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Note

Rapid chromatographic purification of the mitochondrial isoenzyme of beef heart malate dehydrogenase

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Malate dehydrogenase (MDH, E.C. 1.1.1.37) occurs in virtually all eukaryotic cells in at least two forms (isoenzymes) identified as mitochondrial (m-MDH) and cytosolic (c-MDH) according to their location[1]. It plays important metabolic rôles, being a necessary component of the tricarboxylic acid cycle and of the malate shuttle[1], and is used in analytical biochemistry as an indicator enzyme for the determination of the activity of aspartate aminotransferase (AST, GOT), ATP citrate lyase and citrate synthase[2–5]. MDH is also applied for the determination of oxaloacetate and malate and of other substances, *e.g.*, acetate, acetyl-coenzyme A (CoA) and citrate[6].

The purification of beef or pig heart MDH involves mostly ammonium sulphate fractionation and several chromatographic steps[7–9] (using cation and anion exchangers, molecular sieves or hydroxyapatite). The ion-exchange methods depend on the fact that c-MDH has an acidic isoelectric point, whereas m-MDH has a basic one[1].

The aim of the present paper is to describe a quick semi-preparative purification using two high-performance procedures (hydrophobic chromatography and cation-exchange chromatography). A combination of these methods yields homogeneous m-MDH.

EXPERIMENTAL

Materials

Beef hearts were obtained from a slaughter-house and were stored at −60°C. NADH and sodium pyruvate were obtained from Reanal (Budapest, Hungary) and Lachema (Brno, Czechoslovakia), respectively. The materials used for electrophoretic separations were mostly from Serva (Heidelberg, F.R.G.).

Enzyme preparation

Beef heart tissue (50 g) was homogenized with 100 ml of 20 m*M* sodium phosphate buffer (pH 7) and centrifuged at 6000 *g* for 30 min. The crude homogenate was precipitated with ammonium sulphate, most of the MDH activity being salted out between 40 and 75% saturation. These initial procedures were carried out at 4°C; the subsequent chromatographic steps proceeded at room temperature, the eluted fractions being kept in an ice-bath.

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The supernatant containing MDH activity (15 ml) was separated in two stages on a glass column (120 mm × 12 mm I.D.) packed with Spheron-Micro 300 (12 μm, Lachema). The column was attached to two pumps (P-500) and a gradient programmer (GP-250) from Pharmacia (Uppsala, Sweden). As starting and terminating buffers, 0.1 *M* sodium phosphate (pH 7) 30% saturated with ammonium sulphate and 0.1 *M* sodium phosphate (pH 7) were used (flow-rate 4 ml/min). The samples were injected with a V-7 valve equipped with a 10-ml superloop (Pharmacia). The separations were evaluated by an UV-1 monitor (λ = 280 nm) and a FRAC-100 collector (Pharmacia). The fractions containing m-MDH were diafiltrated and concentrated with an ultrafiltration cell (Amicon, Danvers, MA, U.S.A.) with a YM-10 membrane. The sample was divided into two portions and applied to a Mono S HR 5/5 column (50 mm × 5 mm I.D.) from Pharmacia or to a column of the same dimensions packed with Spheron-Micro SB 300 (12 μm, Lachema). The columns were attached to the above-mentioned chromatographic system. As starting and terminating buffers, 20 m*M* sodium phosphate (pH 7) and the same buffer with 0.3 *M* sodium chloride were used (flow-rate 1.5 ml/min).

Enzyme analysis

The activity of malate dehydrogenase was assayed spectrophotometrically in the presence of NADH and pyruvate at 25°C[10]; the protein concentration was calculated from the absorbance at 280 nm. The activity of aspartate aminotransferase (AST, GOT) was determined using a GOT opt. Monotest (Boehringer, Mannheim, F.R.G.). The spectrophotometric measurements were performed in a Cary 118 apparatus (Varian, Palo Alto, CA, U.S.A.). Gel permeation chromatography was carried out on a Superose 12 column (Pharmacia) attached to the above-mentioned chromatographic system, using 0.1 *M* sodium phosphate (pH 7) with 0.15 *M* sodium chloride as the mobile phase (flow-rate 0.7 ml/min). Electrophoretic analysis of MDH was carried out in 15% starch gels using the separation and staining conditions described[11]; polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed as described previously[12].

RESULTS AND DISCUSSION

The conventional ammonium sulphate fractionation was carried out as the first purification step in the described isolation procedure. It brought about a nearly four-fold purification of MDH and a reduction of the sample volume (Table I). Moreover, it was compatible with the subsequent hydrophobic chromatography (the salted-out homogenate could be used directly without diafiltration).

The chromatography on a Spheron-Micro column was a convenient method for further purification of MDH. The unsubstituted Spherons show moderate hydrophobicity and can be used for hydrophobic chromatography of several proteins[13]. The elution with a decreasing salt concentration resulted in a relatively good separation of the main MDH isoenzymes from each other and from most of the contaminants (Fig. 1). The isoenzymes were identified by means of starch electrophoresis; c-MDH (the prevailing form in the first peak with MDH activity in Fig. 1) migrated to the anode at the pH value used[11], whereas m-MDH (the most important constituent of the second peak with MDH activity in Fig. 1) moved in the opposite direction.

TABLE I

PURIFICATION OF THE MITOCHONDRIAL ISOENZYME OF MALATE DEHYDROGENASE FROM BEEF HEART

Details of the procedure as described in the text. The activity of m-MDH is given (it corresponds to *ca.* 60% of the total MDH activity in the initial steps); m-MDH and c-MDH were separated nearly completely on a Spheron-Micro column (Fig. 1).

Fraction	*Protein (mg)*	*Total activity (U)*	*Specific activity (U/ml)*	*Purification*	*Recovery (%)*
Crude supernatant	2650	4000	1.5	(1)	(100)
Ammonium sulphate fractionation (40–75%)	540	2900	5.4	3.6	73
Hydrophobic chromatography (Spheron-Micro)*	53	2800	53	35	70
Cation-exchange chromatography (Mono S)*	2.7	2450	915	610	62

* The separations were carried out in two stages.

The purification of m-MDH was approximately ten-fold (Table I), that of c-MDH was slightly worse.

Chromatography on columns containing strong cation exchangers (with sulphonyl groups) was chosen for the final step in the purification of m-MDH. Both materials tested (Mono S and Spheron-Micro SB) gave comparable results. Homogeneous m-MDH was eluted with a sodium chloride concentration gradient (Fig.

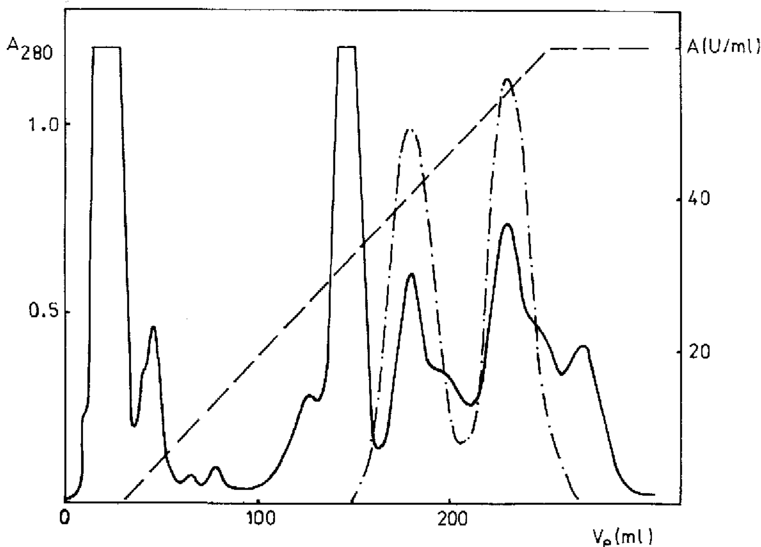

Fig. 1. Chromatography of crude malate dehydrogenase on a Spheron-Micro column. Buffers: A, 0.1 *M* sodium phosphate (pH 7) 30% saturated with ammonium sulphate; B, the same but without ammonium sulphate. V_e = Elution volume; ——, absorbance at 280 nm (A_{280}); -----, gradient; –·–·–, MDH activity (U/ml). Approximately 270 mg of protein were applied to the column.

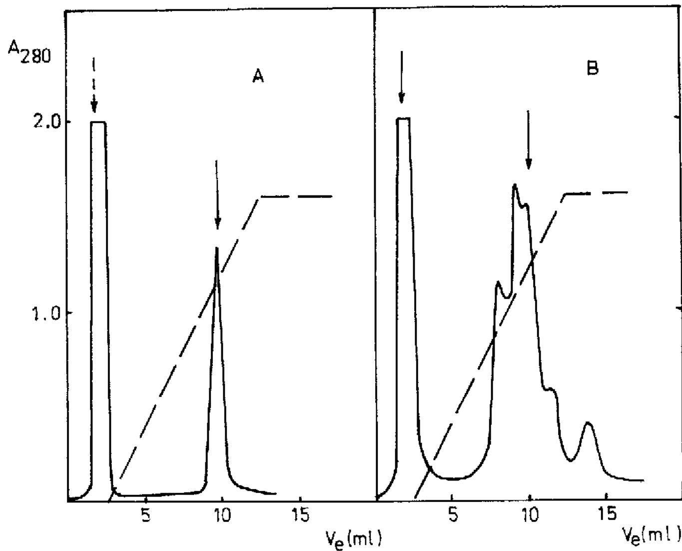

Fig. 2. Chromatography of malate dehydrogenase on a Mono S column. Buffers: A, 20 m*M* sodium phosphate (pH 7); B, the same but with 0.3 *M* sodium chloride. Full and broken arrows correspond to high and low MDH activities, respectively; other symbols as in Fig. 1. (A) Separation of m-MDH purified by hydrophobic chromatography (see Fig. 1 and Table I); *ca.* 25 mg of protein with *ca.* 1400 units of m-MDH were injected. (B) Separation of crude MDH (salted-out with ammonium sulphate); a comparable MDH activity to that in (A) was injected.

2A). The purification of m-MDH was nearly twenty-fold in this step (see Table I). The chromatography on cation exchangers was also carried out with the crude homogenate (after ammonium sulphate fractionation and diafiltration), *cf.*, Fig. 2B. The increase in specific activity was nearly 100-fold in this case, however, the m-MDH obtained was not homogeneous. This means that the hydrophobic chromatography removed some impurities which could not be separated on the cation exchangers (compare Fig. 2A and B). The increase in specific activity achieved by the combination of hydrophobic and ion-exchange chromatography was nearly 200-fold (Table I). The homogeneity of the final product (Table I) was shown by re-chromatography on a Mono S column (after diafiltration), by chromatography on a Superose 12 column and by SDS-PAGE.

The rapid chromatographic procedure described is suitable for the preparation of several milligrams of the homogeneous mitochondrial isoenzyme of malate dehydrogenase. Larger columns for high-performance hydrophobic and cation-exchange chromatography can be used for the preparation of m-MDH on a preparative scale. The purified m-MDH can be used for exact enzymatic studies as well as for analytical purposes (it does not contain detectable activities of aspartate aminotransferase or of other enzymes). The amount of m-MDH prepared on the semi-preparative scale (Table I) was sufficient for more than 1000 assays of aspartate aminotransferase in biological materials (under the conditions given in ref. 2). On the other hand, non-negligible AST activities were found in the partially purified MDH preparations. The MDH/AST activity ratio in the m-MDH fraction obtained in the first chromatographic step, *i.e.*, after hydrophobic chromatography, was approximately

400. However, this partially purified enzyme can also be used for AST activity assay in most biological samples, giving the blank AST activity of 1–2 U/l (which is one order of magnitude lower than the upper limit for the normal values of AST in human blood serum[14] and is comparable with the standard error of the AST assay[2]).

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