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Competitive elution of lactate dehydrogenase from Cibacron Blue–bead cellulose with Cibacron Blue–dextrans

DANICA MISLOVIČOVÁ*, PETER GEMEINER and EVA STRATILOVÁ
Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, 842 38 Bratislava (Czechoslovakia)
and
MARTA HORVÁTHOVÁ[a]
Laboratory of Biochemistry, Centre of Physiological Sciences, Slovak Academy of Sciences, Vlárska 3, 833 06 Bratislava (Czechoslovakia)

ABSTRACT

The efficiencies of elution of lactate dehydrogenase (LDH) from Cibacron Blue (CB)–bead cellulose with eluents ensuring competitive (Cibacron Blue–dextran), biomimetic (NADH) and displacing (KCl) mechanisms were compared. Competitive elution with CB–dextran T 10 was shown to be the most effective providing a 38 fold purified enzyme in 83% yield. As shown by fast protein liquid chromatography and polyacrylamide gel electrophoresis, this LDH preparation was free from protein contaminants but contained CB–dextran. CB–dextran was then removed by ion-exchange chromatography and the yield of LDH decreased to 62%. When using a longer colum, the enzyme was resolved partially in two fractions. The isoelectric point of the main fraction was 7.3.

INTRODUCTION

The use of dye-ligand chromatography has expanded in several directions, *e.g.*, with the rational development of new dyes[1,2] and their derivatives[3] and the search for a universal strategy for their testing[4,5]. In a search for the most effective elution strategy, attention was focused on the utilization of competition between immobilized and mobile dyes for the same binding site of the enzyme[6,7]. The efficiency of competitive elution was evaluated from parameters determined by analytical zonal chromatography[6,7] and batchwise adsorption[6].

This paper describes a study of competitive elution of the enzyme under the conditions of preparative dye-ligand chromatography. For this study, as in previous work[6,7], Cibacron Blue 3G-A (C. I. Reactive Blue 2) was chosen as the effective component of both the immobilized dye-ligand and the competitive eluent. Cibacron Blue (CB) was used in the conjugated form, namely with porous bead cellulose

[a] Present address: Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Vlárska 3, 833 06 Bratislava, Czechoslovakia.

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(immobilized dye-ligand) and with dextrans of the T series (competitive eluent). Lactate dehydrogenase (LDH) from beef muscle was the enzyme that provided biomimetic interaction with dye-ligands. All three basic components of the model system, *i.e.*, reactive dye (CB), dye-ligand matrix (bead cellulose), and enzyme (LDH) from various sources have been used previously in systematic studies of dye-ligand chromatography[6–9].

EXPERIMENTAL

Materials

Bead celluloses Ostsorb B [porosity 90%, dry weight 12.5% (w/w), particle diameter in the range 100–400 μm] and Ostsorb DEAE [ion-exchange capacity 1.1 mmol g^{-1}, dry weight 25% (w/w)] were obtained from Spolchemie (Ústí n/L, Czechoslovakia), dextran T 10 from Pharmacia (Uppsala, Sweden) and low-molecular-weight dextran substance (molecular mass 4000) and dextran 70 Spofa pulvis (molecular mass *ca.* 60 000) from Biotika (Slovenská Ľupča, Czechoslovakia). Cibacron Blue 3G-A (C.I. Reactive Blue 2) was kindly provided by Ciba Geigy (Basle, Switzerland). Coomassie Brilliant Blue G-250 was purchased from Serva (Heidelberg, F.R.G.).

Ostsorb B and the dextrans were derivatized (under base catalysis) with Cibacron Blue 3G-A at 80°C[6,9]. The degree of substitution (DS) was determined spectrophotometrically either in aqueous solution (CB–dextrans) or in cadoxene [cadimium tris-(ethylenediamine)-hydroxide] solution (CB–bead cellulose) at 610 nm[9] and/or 630 nm[6]. An extract from beef flank muscle[6], after filtration and lyophilization, contained *ca.* 4.4 units of LDH (L-lactate:NAD oxidoreductase, E.C. 1.1.1.27) per milligram of solid material. This material was used throughout this work.

Methods

The activity of LDH was established spectrophotometrically[6,10]. The protein concentration was established by the method of Bradford[11]. Zonal chromatography was performed[6] at 25 ± 0.5°C using an immobilized dye column (29 × 1.1 cm I.D.) which was equilibrated and developed with a fixed concentration of NADH or dye–dextran T 10 conjugate in 20 m*M* phosphate buffer (pH 8.5) at a flow-rate of 15 ml h^{-1}. Chromatographic analyses were started by application of 200 μl (42 mg of lyophilizate) of LDH (*ca.* 200 U). The results obtained in zonal chromatography were analysed using the equation for monovalent interactions[6,12].

Elution experiments

Dye-affinity chromatography of LDH on CB–bead cellulose (DS = 1.155 mmol l^{-1}) was performed at ambient temperature using a column (12 × 1.1 cm I.D.) equilibrated with 20 m*M* phosphate buffer (pH 8.5). Chromatography was started by application of 5 ml of a solution of crude LDH (500 mg of lyophilizate). The unbound proteins were washed out with equilibration buffer (*ca.* 300 ml). The elution of LDH was performed using three different eluent solutions of KCl (3 *M*), NADH (1 m*M*) or CB-dextran T 10 (50 μM) in equilibration buffer. The flow-rate was 35 ml h^{-1} and 5-ml fractions were collected. The fractions with maximum activity of LDH were pooled and subjected to repeated ultrafiltration on Ultracell Amicon YM 10 immediately

after elution (KCl, NADH) or after removing CB–dextran on a DEAE-bead cellulose column (5 × 1.1 cm I.D.). The products were stored at −18°C in 50% glycerine.

Ion-exchange chromatography

Chromatography was performed on a DEAE-bead cellulose column (11.5 × 1.5 cm I.D.) at ambient temperature. The column was equilibrated with 20 m*M* phosphate buffer (pH 8.5) and loaded with 10 ml of a solution of LDH (purified on CB–bead cellulose by elution with CB–dextran T 10). LDH was then eluted with a linear gradient of 0–0.1 *M* ammonium sulphate in equilibration buffer. The flow-rate was 42 ml h^{-1} and 10-ml fractions were collected. For stabilization of the enzyme during ultrafiltration and chromatography on DEAE-cellulose it was advantagenous to add L-cysteine (5 m*M*) and EDTA (1 m*M*) to the LDH solution.

Fast protein liquid chromatography (FPLC)

The purity of LDH was checked by size-exclusion chromatography. Pharmacia FPLC equipment with a standard prepacked column (30.0 × 1.0 cm I.D.) of Superose 12 HR 10/30 was used. The column was preconditioned as follows: eluting buffer 50 m*M* phosphate (pH 7), flow-rate 30 ml h^{-1}, UV absorbance monitored at 280 nm, ambient temperature. Fractions of 0.5 ml were collected in order to measure the catalytical activity of LDH.

Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed according to the method of Laemmli[13] using a gel which contained 12.5% of acrylamide. Proteins were stained with Coomassie Brilliant Blue G-250. Ultrathin-layer isoelectrofocusing in polyacrylamide gels on polyester films was performed according to Radola[14]. Proteins were stained with Coomassie Brilliant Blue G-250.

RESULTS AND DISCUSSION

The results of zonal analytical chromatography of LDH on CB–bead cellulose are summarized in Table I. From these data it follows that, in the system CB–cellulose–LDH-mobile ligand, there are significant differences in the $K_{M\text{-}L}$ values, representing the interaction of LDH with CB–dextran T 10 and of LDH with NADH. The values presented suggest that CB–dextran should be a more effective competitive eluent than NADH. However, the elution profiles of LDH are dependent on the concentration of the dye immobilized on bead cellulose. At a low concentration of CB (137.6 μmol l^{-1}) the elution with CB–dextran T 10 is characterized by a single LDH peak. When the used bead CB–cellulose was substituted to a greater extent (1155 μmol l^{-1}), double peaks occurred in the elution profiles of LDH in competitive elution with CB–dextran T 10 (not shown). They were ascribed[7] to partial separation of LDH isoenzymes.

LDH was eluted from CB–cellulose (1155 μmol l^{-1}) by CB–dextrans differing in average molecular mass (4000, 10 000 and 60 000; Fig. 1). The differences in DS values of these three CB–dextrans (see Experimental) were compensated by uniform concentration of the dye (50 μM) in the eluent. The best elution profile and almost total

TABLE I

DISSOCIATION CONSTANTS $K_{M\text{-}L}$ AND $K_{I\text{-}L}$

$K_{M\text{-}L}$ represents the dissociation constant of the LDH–immobilized dye complex and $K_{I\text{-}L}$ the dissociation constant of the LDH–mobile dye complex.

CB–cellulose: concentration of immobilized dye (μmol l^{-1})	*Mobile ligand*	$K_{M\text{-}L}$ *(μM)*	$K_{I\text{-}L}$ *(μM)*
137.6	CB–dextran T 10[a]	9.1	1.6
	NADH	200.0	1.8
1155.0	CB–dextran T 10[a]	20.0[b]	3.15[b]
		8.0	1.0

[a] The CB–dextran T 10 as the mobile dye was 130 μmol g^{-1}.
[b] Dissociation constants calculated from double peak in the elution profiles.

recovery (93%) of LDH was achieved after elution with CB–dextran T 10 (Fig. 1).

The desorption effect of CB–dextran T 10 was compared with that of KCl and NADH. The elution profiles of LDH were similar (Fig. 2) despite the fact that the eluents were applied in very different concentrations, *i.e.*, 50 μ*M* CB–dextran T 10, 1 m*M* NADH and 3 *M* KCl. However, the yields of LDH were different (Table II). They decreased in the same order as the concentrations of the eluents increased. The separation effect achieved with all eluents was sufficiently strong to provide an almost equally purified (38-fold) LDH (Table II). This was also shown by chromatograms of the purified LDH obtained by size-exclusion FPLC. The chromatograms were almost identical in all three instances. They revealed only one peak at the same position corresponding to active LDH, as is demonstrated by the chromatogram obtained after elution with 50 μ*M* CB–dextran T 10 (Fig. 3). Purification of LDH was confirmed also by the SDS-PAGE method (Fig. 4).

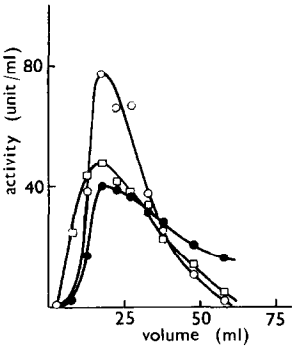

Fig. 1. Elution of LDH from CB–bead cellulose by using CB–dextrans as eluting agent. The elution of LDH was performed after washing out the unbound proteins from a 10-ml column of CB–bead cellulose with equilibration buffer; 500 mg of crude enzyme (2100–2200 U) was loaded onto this column. For elution of bound LDH solutions of CB–dextrans of various molecular mass were used: (○) CB–dextran T 10; (□) CB–dextran 60 000; (●) CB–dextran 4000.

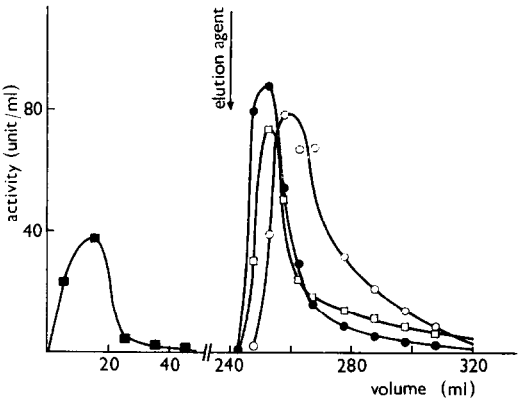

Fig. 2. Elution profiles of beef muscle extract from CB–bead cellulose. Eluting agents: (□) 3 *M* KCl; (●) 1 m*M* NADH; (○) 50 μM CB–dextran T 10; (■) equilibration buffer.

The most effective means of separating of LDH from CB–dextran was shown to be ion-exchange chromatography on DEAE-bead cellulose. Here CB–dextran is adsorbed on the ion-exchanging cellulose. It was found further that by using a sufficiently long column and gradient elution with ammonium sulphate, it is possible to remove CB–dextran and, at the same time, to resolve LDH into two fractions. Fig. 5 shows the results of the gradient elution of LDH by ammonium sulphate (0–0.1 *M*) from the DEAE-bead cellulose column (20 ml). Both LDH fractions are free from protein contaminants, as indicated by their FPLC traces (not shown). However, from the elution profile in Fig. 5 it follows that the separation of these two fractions was not

TABLE II

COMPARISON OF COMPETITIVE, BIOMIMETIC AND DISPLACING ELUTION OF LDH FROM CB–BEAD CELLULOSE

The solution of 500 mg of crude LDH in 5 ml of buffer (2100–2200 units, specific activity 4.4 units/mg solid) was loaded on an equilibrated CB–bead cellulose column (10 ml, concentration of immobilized dye = 1155 μmol l^{-1}) and the unbound proteins were washed out with equilibration buffer. The bound LDH was eluted with one of the solutions of KCl, NADH or CB–dextran T 10 each in equilibration buffer. Separation of CB–dextran T 10 from LDH was performed on a DEAE-bead cellulose column (4 ml) with equilibration buffer.

Eluent	*Volume (ml)*	*Activity (units ml^{-1})*	*Total activity (units)*	*Specific activity (units mg^{-1})*	*Yield (%)*	*Purification factor*
Equilibration buffer	250	1.2	300	1.2	13.6	0.14
3 *M* KCl	70	20.2	1417	329.5	66.2	38.8
1 m*M* NADH	70	23.5	1644	328.8	79.8	38.7
50 μM CB–dextran T 10	50	34.8	1740	—[b]	82.85	—[b]
$(NH_4)_2SO_4$ gradient[a]	70	18.5	1294	306.0	61.6	36.0

[a] Performed by linear gradient elution (0–0.1 *M*).
[b] Not determined.

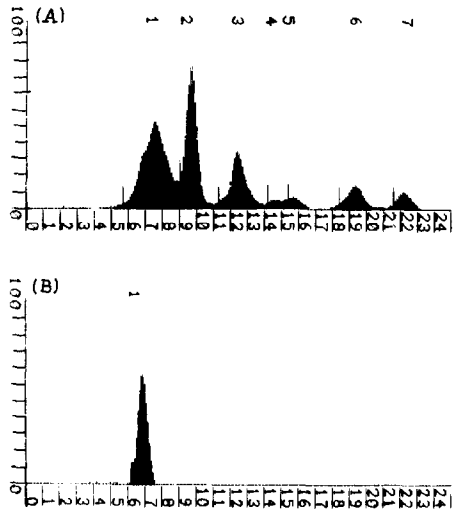

Fig. 3. Elution profiles of (A) crude and (B) purified LDH recorded with the aid of FPLC equipment. The absorbance of the effluent was monitored at 280 nm (ordinate); the numbers on the abscissa signify fraction numbers. The column (Superose 12 HR 10/30) was loaded with 20 U of LDH contained in 0.2 ml.

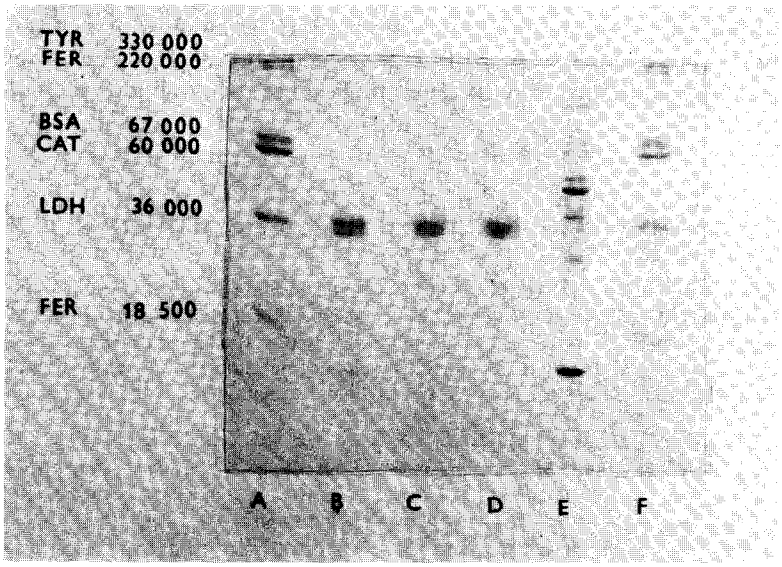

Fig. 4. SDS-PAGE of LDH. The gel consisted of 12.5% acrylamide in Tris–glycine buffer. Gels were stained with 0.2% (w/v) Coomasie Brilliant Blue G-250 in methanol–acetic acid–water (50:10:40) for 6 h, then destained in the same solvent for the same time. Samples A and F, Pharmacia electrophoresis calibration kit containing TYR = hog thyroid thyroglobulin, FER = horse spleen ferritin, CAT = beef liver catalase, LDH = beef heart lactate dehydrogenase and BSA = bovine serum albumin. Sample B, product from NADH elution. Sample C, product from KCl elution. Sample D, product from CB–dextran elution. Sample E, crude LDH from beef muscle.

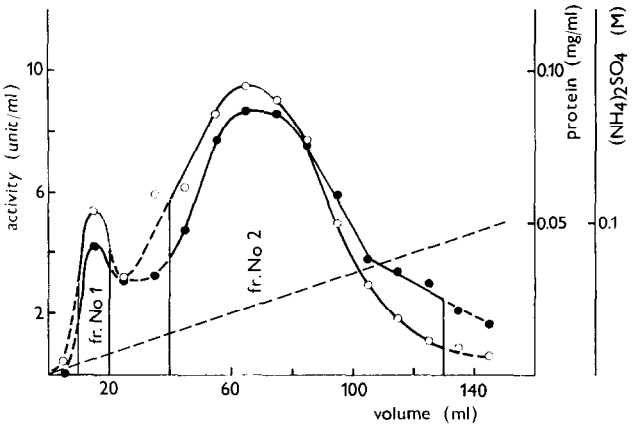

Fig. 5. Elution profile of LDH on DEAE-bead cellulose column. Ion-exchange chromatography was performed on a 20-ml DEAE-bead cellulose column by linear gradient elution with 0–0.1 M $(NH_4)_2SO_4$ in phosphate equilibration buffer; 10-ml fractions were collected and analysed for (○) LDH activity and (●) protein. The two main fractions (1, 10 ml; 2, 90 ml) were pooled.

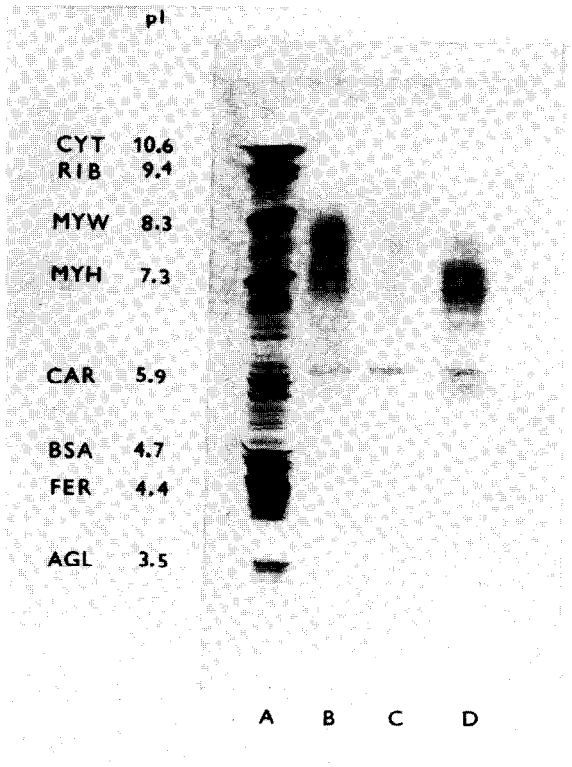

Fig. 6. Ultrathin-layer isoelectrofocusing of LDH in 50 μM polyacrylamide developed gel on silanized polyester film. Gel: 5% T, 3% C, Servalyt pH 3–10 carrier ampholyte 3%. Protein staining with Coomassie Brilliant Blue G-250. Sample A, mixture of marker proteins: CYT = cytochrome c; RIB = ribonuclease; MYW = sperm whale myoglobin; MYH = horse myoglobin; CAR = carbonic anhydrase; BSA = bovine serum albumin; FER = horse spleen ferritin; AGL = amyloglucosidase. Sample B, product from CB-dextran elution. Sample C, fraction No. 1 and sample D, fraction No. 2 from ion-exchange chromatography of LDH.

satisfactory. Therefore, only those pooled parts of both fractions which would least interfere with each other were subjected to isoelectric focusing. After this procedure, only fraction 2 (Fig. 6) could be analysed satisfactorily by isoelectric focusing. This fraction was enriched by an isoenzyme of LDH with a lower isoelectric point (p*I* 7.3).

Removal of the competitive eluent (CB–dextran T 10) and simultaneous resolution of LDH into two fractions by ion-exchange chromatography was accompanied by loss of material and a decrease in specific activity. These losses were minimized (20% and 7%, respectively) when CB–dextran was removed on a short column (4 ml) of DEAE-bead cellulose (Table II).

It can be concluded that competitive elution of LDH from CB–bead cellulose with CB–dextran T 10 is more effective than biomimetic (NADH) and displacing (KCl) elutions. Removal of CB–dextran after elution is accompanied by a loss of material and a decrease in specific activity. Despite these negative aspects, competitive elution may be utilized in dye-ligand chromatography mainly in those instances when difficulties are encountered in trying to elute a protein from too tight a binding to the immobilized dye.

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