SINGLE-STEP SEPARATION OF LACTATE DEHYDROGENASE USING THIOPHILIC CHROMATOGRAPHY

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Lactate dehydrogenase (EC 1.1.1.27) from carp (*Cyprinus carpio*) hepatopancreas was purified by the single-step chromatography on thiophilic sorbent. Hydrophobic negative sorption was used as negative adsorption step for final purification. Final specific activity was 8.88 U/mg protein and the yield 84%. The enzyme is not adsorbed during hydrophobic interaction chromatography, but proteolytic activity is adsorbed completely on hydrophobic sorbent. Thus hydrophobic adsorption can be used as a prepurification step.

Key words: Lactate dehydrogenase; Carp; Thiophilic chromatography; Enzymes; Oxidoreductases; *Cyprinus carpio*.

Thiophilic chromatography[1] is predominantly used for the separation of immunoglobulins (*e.g.* refs[2,3]). A comparative study[4] showed high selectivity of thiophilic sorbents for immunoglobulins. Thiophilic chromatography was only used exceptionally for the isolation of proteins other than immunoglobulins (*e.g.* ref.[5]).

Lactate dehydrogenase is an enzyme of importance both in biochemical analysis and clinical diagnostics (refs[6,7], *etc*.) and in fine organic synthesis[8]. This is the reason for the permanent interest in the possibilities of the production of this enzyme. Mammalian lactate dehydrogenase was separated and purified by different methods including ion-exchange chromatography[9], dye-ligand affinity chromatography[10], immobilized metal-ion affinity chromatography[11,12], and hydrophobic interaction chromatography[12,13]. With the only exception of dye-ligand affinty chromatography, neither from this methods was found to be efficient for purification of lactate dehydrogenase from marine as well as freshwater fish. Fish viscera, on the other hand, seems to be an extremely promising source of enzymes, as this material is not utilized as food and forms usually unpleasant wastes in fish processing.

In connection with the long-term research of enzymes of fish hepatopancreas, particularly in the carp (*Cyprinus carpio*), we also studied the isolation of lactate dehydrogenase from this source. It was shown that lactate dehydrogenase of carp hepatopancreas is sorbed very effectively on a thiophilic sorbent, 2-sulfanylethanol

bound to agarose using divinyl sulfone. The isolation of the enzyme is described in this paper.

EXPERIMENTAL

Carp hepatopancreas was obtained from Rybarstvi s.r.o., Lahovice, Czech Republic. The tissue was removed immediately after the fish was killed, ground and stored at –30 °C until used. The hydrophobic sorbent Iontosorb Phenyl (bead cellulose substituted with phenyl groups), Iontosorb DEAE, Iontosorb Oxin, and Iontosorb Blue were produced by Iontosorb, Usti nad Labem, Czech Republic. The thiophilic sorbent (abbreviated as T-gel), Sepharose 6B with 2-sulfanylethanol bound by divinyl sulfone, was a gift of Prof. Porath, Biochemical Centre, University of Uppsala, Sweden.

Lactate dehydrogenase activity was determined according to Bergmeyer and Berut[14]. One unit is the amount of the enzyme which reduce 1 μmol of lactate to 1 μmol of product during one minute at optimum pH and 30 °C. The concentration of proteins was determined according to Hartree[15].

The determination of purity and molecular weight was carried out using Superose column 12 HR 10/30 (Pharmacia, Uppsala, Sweden). All the chromatography was carried out using an FPLC apparatus, model 250, Pharmacia Uppsala, with the column XK16/20 at 4°C. The UV-VIS photometer Philips, model PU8730, was used for photometric measurements.

The hepatopancreatic extract was prepared by grinding 50 g of semi-defrosted material on a mincer and then extracting it at *ca* 4 °C with 75 ml (1.5-fold sample volume) of 10 mM Tris-HCl (pH 7.5) buffer, which contained 0.5 mM dithiothreitol. The suspension was stirred in an ice bath for 30 min and then centrifuged (30 min, 14 000 rpm, 4 °C). The supernatant was filtered through cotton gauze to remove fat and then stored at 4 °C for 1 h. Both filtration and centrifugation were then repeated under the same conditions. The clear extract obtained was used for further work.

RESULTS AND DISCUSSION

The thiophilic chromatography of the crude extract of carp hepatopancreas was carried out using a slightly modified procedure described by Belew[3]: ammonium sulfate was replaced by potassium sulfate. The clear extract was then mixed with solid potassium sulfate to obtain concentration 0.5 mol/l. The resulting suspension was an extract in 10 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM dithiothreitol and 0.5 M K_2SO_4. This was further divided into 10 ml portions which were applied onto a T-gel column (V_t = 24 ml) previously equilibrated with the same buffer. After the absorbance at 280 nm decreased to a stable low value, the sorbed proteins were eluted first with the same buffer without potassium sulfate, and then with 30% solution of propan-2-ol in the same buffer. The course of chromatography is shown in Fig. 1. The ordinate shows the absorbance measured, the activity was recalculated to present both lines within one scale. A low proportion of lactate dehydrogenase, representing about 14% of total activity, was eluted with the first buffer, but the main part was eluted with buffer lacking potassium sulfate (84% of original activity). No activity of lactate dehydrogenase was eluted with 30% propan-2-ol.

The chromatography of the extract of lactate dehydrogenase on hydrophobic sorbent Iontosorb Phenyl (V_t = 20 ml) was carried out exactly in the same way to make it

possible to compare both methods. The only difference consisted in replacing 0.5 M potassium sulfate with 0.8 M ammonium sulfate, because hydrophobic sorption provides inferior results in 0.5 M potassium sulfate. As shown in Fig. 2, the result is reverse. Only *ca* 10.5% of original activity was sorbed on hydrophobic sorbent, whereas the main fraction, 89%, was eluted under starting conditions.

The isolation efficiency, specific activities and other data are presented in Table I.

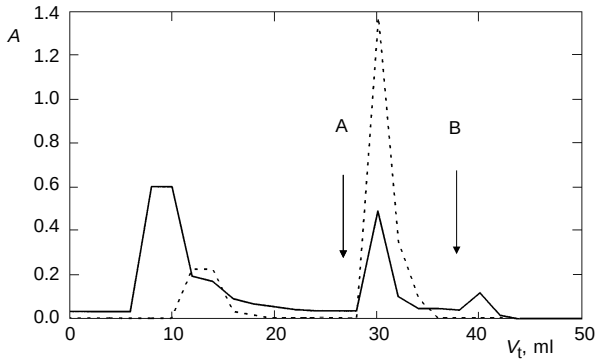

FIG. 1

Thiophilic chromatography of crude extract from carp hepatopancreas on T-gel (2-sulfanylethanol bound to agarose by divinyl sulfone) (V_t = 24 ml). The extract (10 ml portions) was applied to the column in 20 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM dithiothreitol and 0.5 M K_2SO_4. After the elution of non-sorbed proteins, the column was eluted with buffer lacking potassium sulfate (application at point A) and the procedure was completed with the elution using 30% propan-2-ol in the same buffer (application at point B). The solid line represents absorbance at 280 nm, the dashed line shows the activity of lactate dehydrogenase (recalculated to the same scale)

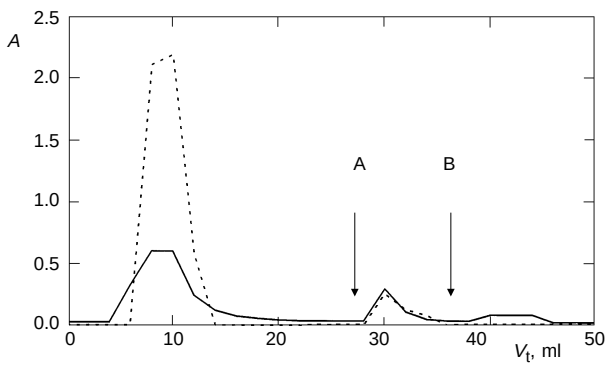

FIG. 2

Hydrophobic chromatography of crude extract from carp hepatopancreas on Iontosorb Phenyl (V_t = 20 ml). All conditions are the same as in Fig. 1

TABLE I
Evaluation of the procedure of lactate dehydrogenase separation from carp hepatopancreas

Step	Volume ml	Protein concentration, mg/ml	Activity EU/ml	Total proteins, mg	Total activity EU	Activity yield, %	Specific activity EU/mg protein	Degree of purification
Crude extract	10	29.2	34.8	292	348	100	1.19	1
Fraction non-adsorbed on Iontosorb Phenyl	108	1.12	2.86	120.9	309	89	3.22	1.19
Fraction adsorbed on Iontosorb Phenyl	31.8	0.83	1.15	264	36.5	10.5	0.96	–
Fraction non-adsorbed on T-gel	101	2	0.48	212	48.5	14	0.25	–
Fraction adsorbed on T-gel	14	2.36	20.95	33	293.3	84	8.88	7.46

The results obtained by the thiophilic chromatography were compared with earlier methods: anion-exchange chromatography, dye-ligand affinity chromatography, oxin-Cu^{2+}-immobilized metal-ion affinity chromatography and hydrophobic interaction chromatography on phenyl substituted bead cellulose. The results shown in Table II indicate that the specific activity of resulted purified enzyme is significantly lower compared with thiophilic chromatography.

The purity of carp lactate dehydrogenase isolated by the method described in present paper was tested using gel chromatography on Superose 12 HR 10/30 (Pharmacia, Uppsala). As shown in Fig. 3, three peaks are present in the chromatogram. The first one (R_t = 26.78 min) is free of lactate dehydrogenase activity and contains 10% of all protein according to peak area and 12.5% according to peak height. The second one (R_t = 28.09 min) contains 1.25% of total protein according to peak area and 4.4% according to peak height. This second peak represents the minor part of lactate dehydrogenase activity. The major part of activity is concentrated in the third peak (R_t = 29.83 min.), which

TABLE II
Isolation of lactate dehydrogenase from carp hepatopancreas by various chromatographic methods

Iontosorb chromatography	Activity yield	Specific activity U/mg protein	Degree of purification
DEAE-	103.7	4.51	3.79
Blue-	106.4	4.28	3.6
Oxin-CU^{2+}-	56.7	2.63	2.21

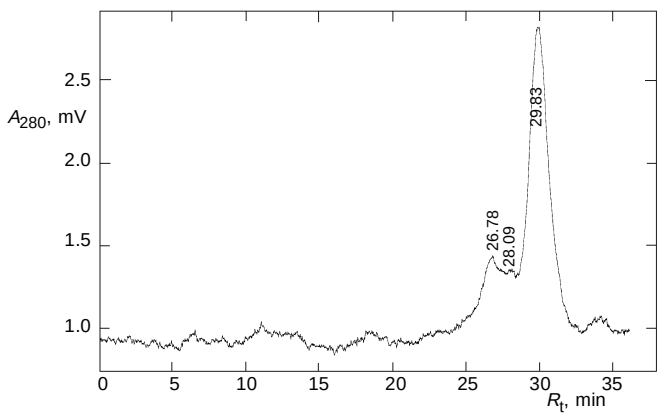

FIG. 3
Gel permeation chromatography of purified carp hepatopancreatic lactate dehydrogenase on the Superose column 12 HR 10/30 (Pharmacia, Uppsala)

contains 88.6% of total proteins according to peak area and 83.1% according to peak height. The enzyme purity is supposed to be more then 83% and less than 88% according to these results.

The molecular weight calculated from gel chromatography is approximately 26 000, which is lower compared with the mammalian enzyme.

The purification methods usually used for purification of mammalian and bird lactate dehydrogenase (see Introduction) give much worse results for carp enzyme (degree of purification as well as resulting specific activity) as shown in Table II.

Thiophilic sorption was originally developed for the isolation of immunoglobins. There is a pronounced difference in sorption of these compounds between hydrophobic and thiophilic sorbents. In spite of this, these gels differ even more markedly in the sorption of the carp hepatopancreas lactate dehydrogenase.

In our opinion thiophilic chromatography of carp lactate dehydrogenase on a thiophilic gel represents a new area of utilization of these sorbents and provides a possibility to use them for isolation of other enzymes as well. On the basis of present results, it can be expected that thiophilic sorption can be used for preparative separation of lactate dehydrogenase from carp hepatopancreas. The separation procedure may be complemented with negative sorption on a hydrophobic sorbent.

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