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Chromatographic purification of glucose-6-phosphate dehydrogenase and lactate dehydrogenase from *Leuconostoc mesenteroides*[a]

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SUMMARY

A simple procedure for the simultaneous purification of glucose-6-phosphate dehydrogenase and lactate dehydrogenase from *Leuconostoc mesenteroides* is described. It involves ammonium sulphate precipitation, hydrophobic interaction chromatography and ion-exchange chroamtography. The purity of the final fractions is checked by size-exclusion chromatography and by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. The purified enzymes are suitable for enzymological studies and analytical biochemistry.

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6P-DH, E.C. 1.1.1.49) catalyses the first step in the pentose phosphate pathway, oxidizing glucose-6-phosphate with simultaneous reduction of NADP or NAD[1]. Most glucose-6-phosphate dehydrogenases utilize only NADP as the sole coenzyme, but some *e.g.*, the enzyme from *Leuconostoc mesenteroides*, have the ability to use NAD in addition to NADP[2,3]. The complete absence of cysteine in G6P-DH from *Leuconostoc mesenteroides*, which is unusual in dehydrogenases, is probably responsible for the excellent stability of this enzyme[4]. The resistance to inactivation under a variety of conditions and the dual nucleotide specificity have led to the widespread use of this enzyme in various clinical test kits as an indicator enzyme for the determination of several enzyme activities and for determining the concentrations of numerous metabolites[5].

Several purification procedures for G6P-DH from *Leuconostoc mesenteroides* have been reported. Olive and Levy[3] purified this enzyme by a procedure involving

[a] Dedicated to the memory of Dr. J. Kovář (†1987).

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precipitation with ammonium sulphate, treatment with protamine, ammonium sulphate fractionation, chromatography on hydroxyapatite followed by two crystallizations. Choi *et al.*[6] reported a procedure for the purification of this enzyme by Cibacron Blue F3G-A–Sepharose and hydroxyapatite chromatography. More recently, a purification procedure involving tandem dye–ligand chromatography has been described[7]. Sixty-five immobilized triazine dyes were screened for their ability to purify G6P-DH. However, the best, Matrex Gel Purple A and Matrex Gel Orange B, used in this procedure as "negative" and "positive" adsorbents, respectively, are not commercially available.

In this paper, a rapid semi-preparative procedure for the isolation of G6P-DH from *Leuconostoc mesenteroides* using ammonium sulphate precipitation and two high-performance liquid chromatographic steps is described. In addition, the possibility of the simultaneous purification of NAD-dependent D-lactate dehydrogenase[8,9], which is present in considerable amounts in the sediment after ammonium sulphate precipitation, is discussed. This enzyme has already been purified by Garland[10]. In our procedure, the LDH is a sort of by-product, and a better yield of it could be obtained by easily modifying the procedure. This enzyme can also be used in analytical biochemistry for the determination of several enzyme activities and numerous metabolites[5].

EXPERIMENTAL

Materials

Leuconostoc mesenteroides CCM 2083 (ATCC 12291) was obtained from the Czechoslovak Collection of Microorganisms (Brno, Czechoslovakia) and cultivated as described previously[11]. Glucose-6-phosphate, glucose-1-phosphate, gluconate-6-phosphate, fructose-6-phosphate, sodium pyruvate, NAD and NADH were obtained from Boehringer (Mannheim, F.R.G.). The materials used for electrophoretic separation were mostly from Serva (Heidelberg, F.R.G.). The other chemicals (mostly from Lachema, Brno, Czechoslovakia) were of analytical-reagent grade.

Enzyme preparation

The cells (40 g wet weight) were suspended in 0.1 *M* sodium hydrogencarbonate solution (dilution 1:10) and then sonicated for 15 × 1 min in an ice-bath using a Dynatech (Fremingdale, NY, U.S.A.) sonic dismembrator. Cellular debris was separated by centrifugation at 20 000 *g* for 30 min. The crude cytosol was fractionated by ammonium sulphate precipitation. After removal of the first precipitate (60% saturation), most of G6P-DH and part of the LDH activities were salted out at 90% saturation. These initial procedures were carried out at 4°C and the subsequent chromatographic steps proceeded at room temperature, the eluted fractions being kept in an ice-bath.

The sediment was dissolved in 0.05 *M* sodium phosphate buffer (pH 7.4) containing 1.7 *M* ammonium sulphate and 1 m*M* EDTA and the enzymes were separated on a Phenyl-Superose HR 5/5 column (50 mm × 5 mm I.D.) from Pharmacia–LKB (Uppsala, Sweden) or on an HR 10/10 column (100 mm × 10 mm I.D.) packed with Spheron Micro 300 (12 μm) (Lachema). The columns were attached to a Pharmacia LKB fast protein liquid chromatography (FPLC) system consisting of

two P-500 pumps, an LCC-500 gradient programmer, a UV-1 monitor with an HR-10 cell, a FRAC-100 fraction collector and an REC-482 recorder. As starting and terminating buffers, 0.05 *M* sodium phosphate (pH 7.4) containing 1.7 *M* ammonium sulphate, 1 m*M* EDTA and 0.05 *M* sodium phosphate (pH 7.4) were used at flow-rates of 0.5 ml/min for the Phenyl-Superose column and 2 ml/min for the Spheron Micro 300 column. The samples were injected using a V-7 valve equipped with a 10-ml superloop (Pharmacia–LKB) or sample loops of various volumes. The fractions containing enzyme activities were pooled and concentrated with an Amicon (Danvers, MA, U.S.A.) ultrafiltration cell with a YM-10 membrane. The samples were desalted on an HR 10/10 fast desalting column (Pharmacia–LKB) and applied to a Mono Q HR 5/5 column from Pharmacia–LKB. The columns were attached to the above-mentioned chromatographic system. As starting and terminating buffers, 0.05 *M* sodium phosphate (pH 7.4) and the same buffer with 1 *M* sodium chloride, respectively, were used (flow-rate 1.0 ml/min).

Enzyme analysis

The activity of G6P-DH was determined spectrophotometrically in the presence of NAD and glucose-6-phosphate[3], the activity of LDH in the presence of NADH and pyruvate[8] and the activity of phosphogluconate dehydrogenase in the presence of NADP and gluconate-6-phosphate[12]. The activities of NADH oxidase and NADPH oxidase were determined in the presence of NADH and NADPH, respectively[13]. The activities of phosphoglucomutase[14] and phosphoglucose isomerase[15] were determined by a coupling assay using G6P-DH as the indicator enzyme in the presence of glucose-1-phosphate and fructose-6-phosphate, respectively. The activities of alanine aminotransferase and creatine phosphokinase were detected by means of a Monotest (Boehringer). The temperature for all assays was 25°C. The protein concentration was calculated from the absorbance at 260 and 280 nm[16]. The spectrophotometric measurements were performed using a Cary 118 apparatus (Varian, Palo Alto, CA, U.S.A.).

The purity of the enzymes was checked both by size-exclusion chromatography (SEC) and by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE). SEC was carried out on a Superose 12 column (Pharmacia–LKB) attached to the above-mentioned chromatography system, using 0.05 *M* sodium phosphate (pH 7.4) with 0.15 *M* sodium chloride as the mobile phase (flow-rate 0.7 ml/min). SDS-PAGE was performed as described previously[17] in a Protean Double Slab Cell apparatus (Bio-Rad Labs., Richmond, CA, U.S.A.). The electropherograms were analysed in a VD 620 video densitometer (Bio-Rad Labs.).

RESULTS AND DISCUSSION

Conventional ammonium sulphate fractionation was carried out as the first purification step of the described isolation procedure. It brought about a five-fold purification of G6P-DH and a reduction of the sample volume (Table I). The sediment, after ammonium sulphate fractionation, also contained LDH with a high specific activity (*ca.* 417 nkat/mg). Moreover, this step was compatible with the subsequent hydrophobic interaction chromatography: salted-out homogenate could be used directly without removal of salts.

TABLE I

RAPID CHROMATOGRAPHIC PURIFICATION OF G6P-DH FROM *LEUCONOSTOC MESENTEROIDES*

Details of the procedure as described in the text. All data on LDH are given in parentheses.

Fraction	*G6P-DH (LDH)*				
	Protein (mg)	*Total activity (μkat)*	*Specific activity (nkat/mg)*	*Purification (fold)*	*Recovery (%)*
Crude supernatant	1100	90.9 (235.9)	83 (214)	1	100
Ammonium sulphate fractionation (60–90%)	189	80.9 (78.8)	428 (417)	5.2 (2)	90 (34)
Hydrophobic chromatography	9.6 (31)	56.3 (72.4)	5869 (2336)	71 (11)	63 (31)
Anion-exchange chromatography	1.7 (5.6)	51.8 (65.7)	30 470 (11 770)	367 (55)	58 (28)

Hydrophobic interaction chromatography proved to be a convenient method for further purification of G6P-DH and LDH. The chromatographic conditions were optimized on a Phenyl-Superose HR 5/5 column (loading capacity about 10 mg of protein). A Spheron Micro 300 HR 10/10 column (loading capacity *ca.* 200 mg of protein) was used for preparative chromatography with similar results. Elution with a decreasing salt concentration resulted in a relatively good separation of G6P-DH and

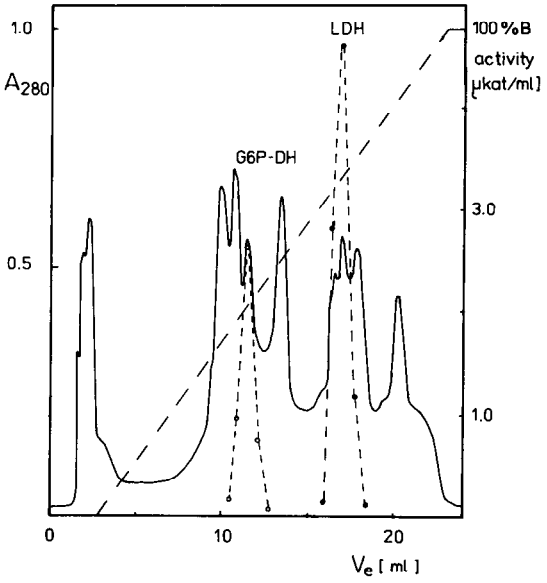

Fig. 1. Chromatography of crude enzyme preparation on a Phenyl-Superose column. Buffers: (A) 0.05 *M* sodium phosphate (pH 7.4) with 1.7 *M* sodium sulphate and 1 m*M* EDTA; (B) 0.05 *M* sodium phosphate (pH 7.4); flow-rate, 0.5 ml/min. V_e, elution volume; solid line, absorbance at 280 nm (A_{280}); dashed lines, gradient; (○) G6P-DH activity; (●) LDH activity. Approximately 10 mg of protein were applied to the column.

LDH from each other and most of the contaminants (Fig. 1). The purification of G6P-DH was *ca.* 14-fold and that of LDH was slightly worse (Table I). The activities of contaminant enzymes (which could interfere in appropriate clinical tests) were measured in the active fractions of G6P-DH and LDH after ammonium sulphate fractionation and hydrophobic interaction chromatography (Table II). It was shown that the enzyme preparations purified only by ammonium sulphate fractionation and hydrophobic interaction chromatography were applicable for use in clinical test kits. They contained only negligible activities of contaminating enzymes.

Chromatography on the strong anion exchanger Mono Q (after desalting) was chosen for the final step in the purification of both G6P-DH and LDH. Highly pure enzymes were eluted with a sodium chloride concentration gradient in both instances (Figs. 2 and 3). The purification of G6P-DH and LDH was *ca.* five-fold and the increase in specific activity achieved by the combination of hydrophobic and ion-exchange chromatographies was nearly 70-fold for G6P-DH and 30-fold for LDH (Table I). SEC and SDS-PAGE showed the homogeneity of G6P-DH (Figs. 4A and 5A). With LDH, if a completely pure enzyme is required subsequent SEC is applied as the final purification step (Figs. 4B and 5B).

The described purification procedure has several advantages over common methods for the purification of G6P-DH from *Leuconostoc mesenteroides*. In the three purification steps we obtained homogeneous G6P-DH with a five-fold higher specific activity than that reported by Hey and Dean[7], who used tandem dye–ligand chromatography in a two-step procedure. Our yield was only slightly worse. The chromatographic materials used here are generally commercially available, in contrast with the dye–ligand matrices used by Hey and Dean[7]. The possibility of the simultaneous purification of G6P-DH and LDH is also an advantage. It is also interesting that even partially purified G6P-DH and LDH (after hydrophobic interaction chromatography) can be used in the appropriate clinical test kits (see Table II).

TABLE II

ACTIVITIES OF THE CONTAMINANT ENZYMES IN THE FRACTIONS AFTER AMMONIUM SULPHATE FRACTIONATION AND HYDROPHOBIC INTERACTION CHROMATOGRAPHY AS % OF G6P-DH AND LDH ACTIVITIES

Enzymes	*Fraction*	
	After ammonium sulphate fractionation	*After hydrophobic interaction chromatography*
G6P-DH	(100)	(100)
Phosphoglucomutase	0.3	–
Phosphoglucose isomerase	8.3	0.4
Creatine phosphokinase	0.03	0.009
6-Phosphogluconate DH	0.3	0.006
NADH oxidase	0.06	0.001
NADPH oxidase	0.008	–
LDH	(100)	(100)
Alanine aminotransferase	0.0003	0.0002

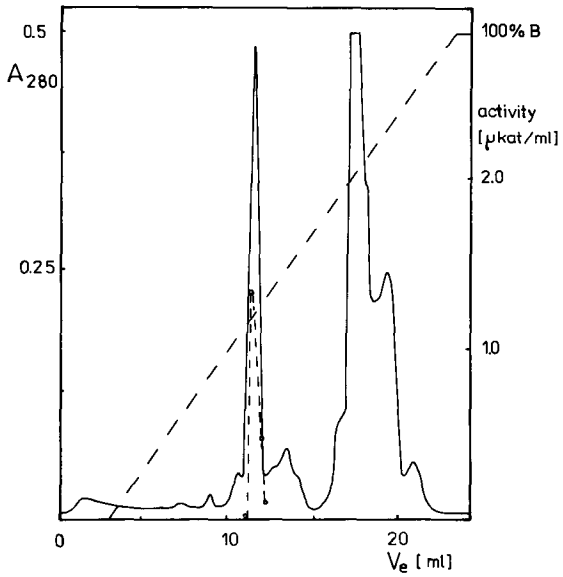

Fig. 2. Chromatography of partially purified G6P-DH on a Mono Q column. Buffers: (A) 0.05 *M* sodium phosphate (pH 7.4); (B) same as A but with 1 *M* sodium chloride; flow-rate, 1 ml/min. Lines symbol as in Fig. 1. Approximately 10 mg of protein were applied to the column.

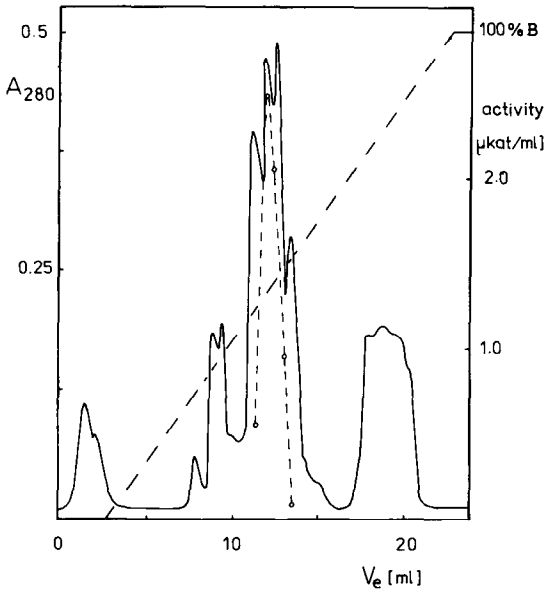

Fig. 3. Chromatography of partially purified LDH on a Mono Q column. Lines as in Fig. 2; (○) LDH activity.

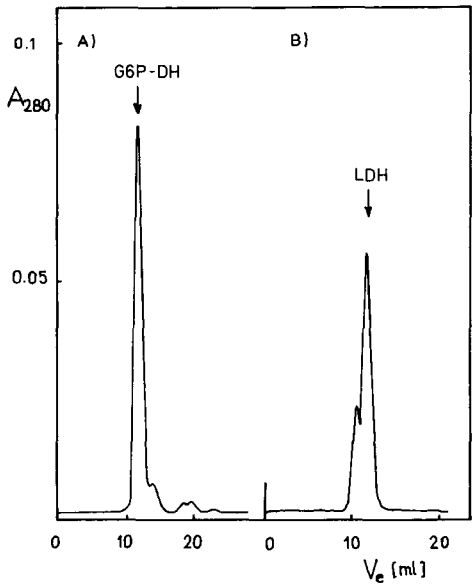

Fig. 4. Chromatography of (A) G6P-DH and (B) LDH on a Superose 12 column after ammonium sulphate fractionation, hydrophobic interaction chromatography and ion-exchange chromatography. Buffer: 0.05 *M* sodium phosphate (pH 7.4) with 0.15 *M* sodium chloride; flow-rate, 0.7 ml/min. Solid line as in Fig. 1.

This rapid chromatographic procedure is suitable for the preparation of several miligrams of G6P-DH and LDH. The chromatographic steps (see Table I) can be carried out in about 3 h, including the concentration with the ultrafiltration cell. Therefore, the whole procedure starting from the cell suspension of *Leuconostoc mesenteroides* can be performed in one working day. The purified G6P-DH and LDH

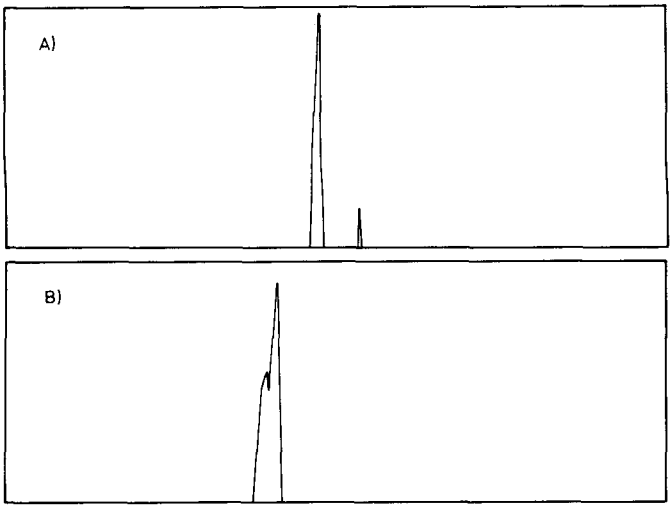

Fig. 5. SDS-PAGE densitograms of (A) G6P-DH and (B) LDH after ammonium sulphate fractionation, hydrophobic interaction chromatography and ion-exchange chromatography.

are suitable for enzymological studies and for analytical purposes. Their amounts are sufficient for *ca.* 1000 assays of glucose or 700 assays of creatine phosphokinase in the case of G6P-DH and for *ca.* 2500 assays of alanine aminotransferase in the case of LDH (under the conditions given in refs. 18–20). The method described can be easily modified for the preparation of both enzymes on a large scale.

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