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Application of high-performance liquid chromatography to the purification, disintegration and molecular mass determination of pyruvate dehydrogenase multi-enzyme complexes from different sources

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ABSTRACT

The pyruvate dehydrogenase complex is associated with the inner mitochondrial membrane. A gentle and rapid purification procedure, especially for the very unstable pyruvate dehydrogenase complex from the extremely thermophilic organism *Thermus aquaticus*, is described. This procedure is based essentially on a combination of hydrophobic interaction and of adsorption chromatography by the rapid fast protein liquid chromatographic technique. Applying the same method, a relative molecular mass of $9.1 \cdot 10^6$ daltons was obtained by gel filtration on Superose 6 HR 10/30 for the pyruvate dehydrogenase complex from *T. aquaticus*. The same column served to resolve the pyruvate dehydrogenase complex into its enzyme components.

INTRODUCTION

The pyruvate dehydrogenase complex is the largest enzyme aggregate of the cell. It consists of three enzyme components, 60 α-chains and 60 β-chains of the pyruvate dehydrogenase (E1, E.C. 1.2.4.1), 60 chains of the dihydrolipoamide acetyltransferase (E2, E.C. 2.3.1.12) and 12 chains of the dihydrolipoamide dehydrogenase (E3, E.C. 1.8.1.4) [1,2]. Whereas in Gram-negative bacteria the enzyme complex is located in the cytoplasm, it is associated with the inner mitochondrial membrane in eukaryotes. Resolution of the enzyme complex from the membrane requires treatment with detergents such as Triton X-100 or digitonin [3,4]. Because of the association of the pyruvate dehydrogenase complex with membrane structures, hydrophobic-interaction chromatography is an appropriate purification method for this enzyme complex. In this paper, we describe a rapid and mild purification procedure based on hydrophobic-interaction and adsorption chromatography with elution gradients controlled by a fast protein liquid chromatographic (FPLC) system. This procedure is

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especially applicable to the isolation of the very unstable pyruvate dehydrogenase complex from the extremely thermophilic organism *Thermus aquaticus*. Gel filtration in the FPLC system was used to determine the molecular mass of the native pyruvate dehydrogenase complex from this organism. The same system was applied to separate the enzyme complex into its components. Because of the instability of the thermophilic enzyme, the bovine pyruvate dehydrogenase complex was taken for this experiment.

EXPERIMENTAL

Materials

Superose 6 HR 10/30, phenyl-Superose HR 5/5 and phenyl-Sepharose CL-4b were obtained from Pharmacia LKB (Freiburg, F.R.G.), the Bio-Gel HPHT hydroxyapatite column from Bio-Rad Labs. (Munich, F.R.G.), bovine serum albumin enzymes, cofactors and substrates for the enzyme tests from Boehringer (Mannheim, F.R.G.) and porcine thyroglobulin, Triton X-100 and the reagents for polyacrylamide gel electrophoresis (PAGE) from Serva (Heidelberg, F.R.G.). All other chemicals were obtained from Merck (Darmstadt, F.R.G.). The *Thermus aquaticus* strain AT62 (DSM No 674) was obtained from the Deutsche Sammlung für Mikroorganismen (Braunschweig, F.R.G.).

The method of Stanley and Perham [5] was used to purify the pyruvate dehydrogenase complex from bovine heart. The enzyme complex from *Bacillus subtilis* was purified according to Maas [6]. Column chromatography was performed with an FPLC system, (Pharmacia LKB) consisting of an LCC 500 controller, two P-500 pumps, a V-7 valve, a FRAC-100 fraction collector and a BT 3030 UV monitor, equipped with an 8-μl flow cell (Biotronic, Maintal, F.R.G.).

Cultivation of T. aquaticus and preparation of crude extracts

The bacteria were cultivated in medium M162 [7], supplemented with 0.4% sodium pyruvate as a carbon source. Fermentation was carried out in the same medium in an Intensor 20b 20-l fermenter (Giovanola Freres, Monthy, Switzerland) at 70°C with 0.2 volumes of air per fermenter unit volume per minute and stirring (1200 rpm) for 15 h. The cells were harvested by centrifugation. For the preparation of the crude extracts, aliquots of 7 g of wet cells were suspended in 14 ml of 50 m*M* potassium phosphate (pH 7.5)–0.1 m*M* EDTA–2 m*M* DTT–3 m*M* $MgCl_2$-5 m*M* β-mercapto-ethanol and sonicated for 4 min at 4°C in a B12 sonicator (Branson Ultrasonics, Danbury, CT, U.S.A.), adjusted to 50 W.

Polyacrylamide gel electrophoresis

PAGE in the presence of 0.1% sodium dodecyl sulphate (SDS) [8] employed a linear gradient of 10–20% (w/v) acrylamide and a ratio of acrylamide to N,N′-methylenebisacrylamide of 100:3.0. Staining of protein bands was performed with 0.2% (w/v) Coomassie Brilliant Blue R-250 in acetic acid–methanol–water (2:9:11, v/v/v) at 60°C for 10 min. For destaining the gels were treated for 30 min at 60°C with acetic–acid–methanol–water (1:3:6, v/v/v).

Enzyme assays and protein determination

The enzymatic activity of the pyruvate dehydrogenase complex was tested

following the reduction of NAD at 340 nm [9] at 37°C for the bovine enzyme and at 70°C for the thermophilic enzyme. The proteins were determined according to Lowry *et al.* [10].

RESULTS AND DISCUSSION

Purification of the pyruvate dehydrogenase complex from T. aquaticus

As the pyruvate dehydrogenase complex from the extremely thermophilic Gram-positive bacterium *T. aquaticus* is very unstable, purification procedures described for the enzyme complex from other sources were not applicable to this organism [11]. Two reasons appeared to be responsible for this instability: (i) dissociation of subunits, especially during chromatography and (ii) destabilization of the native structure of the enzyme complex after detachment from the membrane. A purification procedure was developed based on the hydrophobic nature of the surface of this thermophilic pyruvate dehydrogenase complex in combination with the FPLC technique.

To detach the pyruvate dehydrogenase complex from the membrane fraction, Triton X-100 was added to the crude extract to give a final concentration of 20% (v/v). After stirring for 10 min and centrifuging for 30 min at 100 000 *g*, two layers were obtained. The upper layer, containing the detergent, was discarded. The activity of the pyruvate dehydrogenase complex remained in the lower, clear, yellow layer. This layer, which should be completely free of any traces of the detergent, was applied to a 16 × 2 cm I.D. phenyl-Sepharose CL-4B column, which was equilibrated with 1 *M* potassium phosphate (pH 7.0)–0.1 m*M* dithiothreitol (DTT)–0.1 m*M* EDTA. Under these conditions, the enzyme complex remained bound to the column and, after washing with 100 ml of the equilibration buffer, it was eluted with a 150-ml linear gradient from 1 to 10 m*M* potassium phosphate (pH 7.0)–0.1 m*M* DTT–0.1 m*M* EDTA, followed by a further 100-ml linear gradient from 10 to 1 m*M* potassium phosphate (pH 7.0)–0.1 m*M* DTT–0.1 m*M* EDTA. The gradients were controlled by the FPLC system. The elution profile is shown in Fig. 1. The enzyme activity was eluted as a single peak at the onset of the second gradient, corresponding to a concentration of 9 m*M* potassium phosphate. The active fractions were pooled and carefully layered on a cushion of the same volume of 35% (w/v) sucrose in 50 m*M* potassium phosphate (pH 7.0) in a centrifugate tube. It was centrifuged at 176 000 *g* and 4°C for 4 h. The clear yellow pellet, which contained the enzyme activity, was dissolved in a small volume of 25 m*M* potassium phosphate (pH 7.0).

An HPHT hydroxyapatite column, connected with the FPLC system, served as the final purification step. The column was equilibrated with 25 m*M* potassium phosphate (pH 7.0)–0.2 m*M* DTT. The enzyme solution was applied to the column in portions of 1 ml, each containing 1 mg of protein. Larger amounts of protein were prepared by repeated batchwise chromatography. The pyruvate dehydrogenase complex was eluted with a 10-ml linear gradient from 25 to 500 m*M* potassium phosphate (pH 7.0)–0.2 m*M* DTT. The enzyme complex was completely separated from other proteins (Fig. 2). PAGE of the enzyme preparation after this step established its purity (Fig. 3). The three major protein bands of this preparation observed on the gel (lane 3) represent (from the top) the E2 component and the α- and β-chain, respectively, of the E1 component. The E3 component could not be detected.

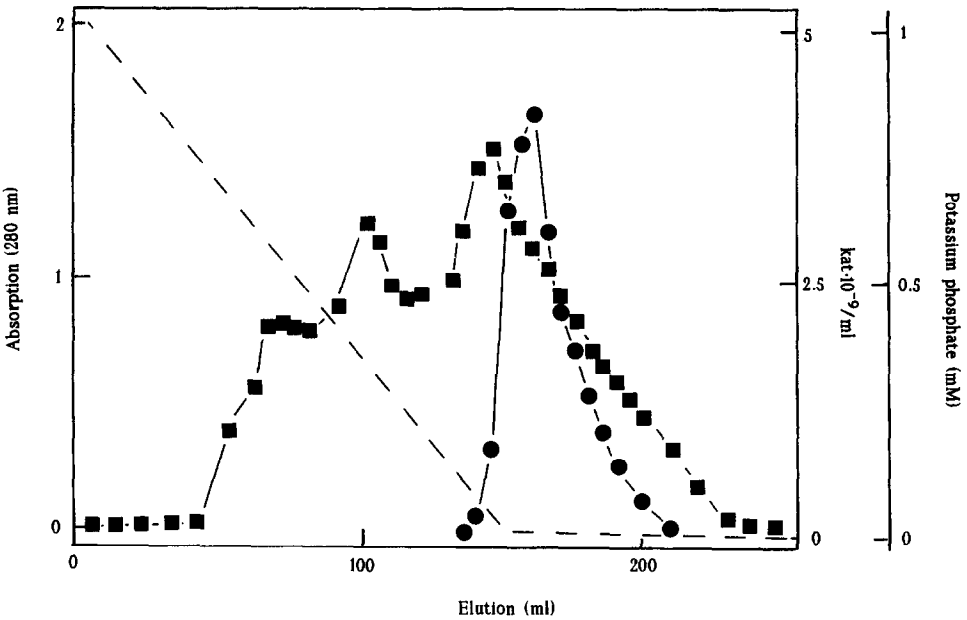

Fig. 1. Elution profile of the pyruvate dehydrogenase complex from *T. aquaticus* after chromatography on phenyl-Sepharose CL-4B. To 12 ml (23 mg of protein) of the Triton X-100 extract the same volume of 2 *M* potassium phosphate was added and it was applied to a 16 × 2 cm I.D. phenyl-Sepharose CL-4B column, equilibrated with 1 *M* potassium phosphate (pH 7.0)–0.1 m*M* DTT–0.1 m*M* EDTA. The column was washed with 100 ml of the equilibration buffer and eluted with a 150-ml linear gradient from 1 to 10 m*M* potassium phosphate (pH 7.0)–0.1 m*M* DTT, followed by a 100-ml linear gradient from 10 to 1 m*M* potassium phosphate (pH 7.0)–0.1 m*M* DTT–0.1 m*M* EDTA. The column was connected to the FPLC system. The flow-rate was 2 ml/min and fractions of 5 ml were collected. ■ = Protein (absorption at 280 nm); ● = activity of the pyruvate dehydrogenase complex; the dashed line indicates the gradient.

There is only a small amount of E3 chains bound to the native enzyme complex [2]. Further, owing to the weak binding of the E3 chains to the pyruvate dehydrogenase complex from *T. aquaticus*, parts of the chains were stripped off during ultracentrifugation [11]. The results of the purification procedure are summarized in Table I. Because of non-linearity, the values for the enzyme activity in the crude extract were underestimated. The yield and purification factor were therefore based on the Triton X-100 extraction.

Molecular mass determination of the pyruvate dehydrogenase complex from T. aquaticus

Special difficulties arise in the molecular mass determination of large enzyme aggregates by methods such as gel filtration and sucrose gradient centrifugation. Comparable large reference proteins with well established molecular masses are rare. Further, the experimental conditions, especially an extended stay in dilute solutions, favour dissociation and lead to underestimation. The structural heterogeneity reported for the pyruvate dehydrogenase complex from *Escherichia coli* [12] may be due to such dissociation processes. The FPLC technique can help to reduce the risk of dissociation.

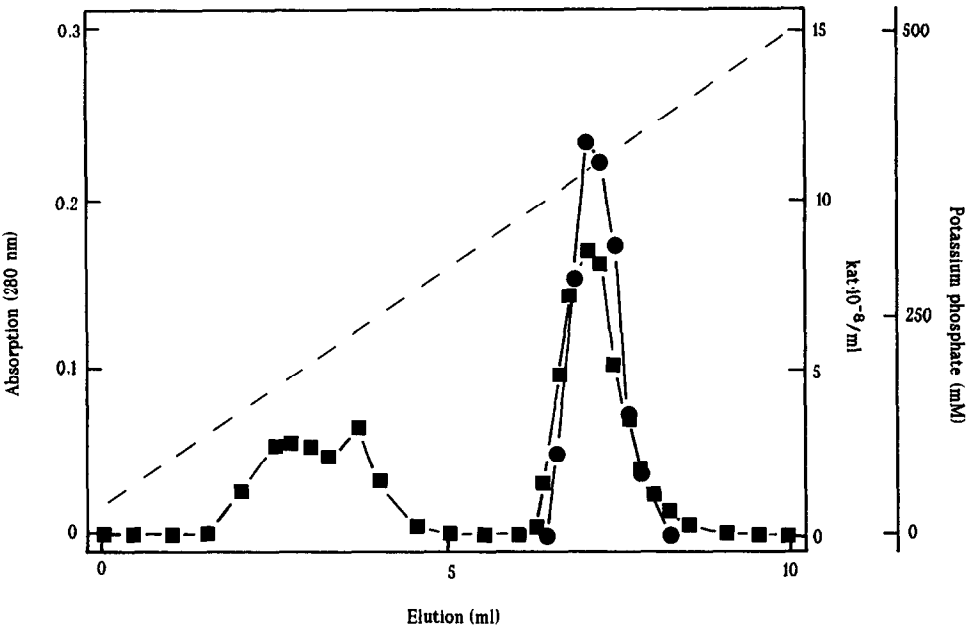

Fig. 2. Chromatogram of the pyruvate dehydrogenase complex from *T. aquaticus* on an HPHT hydroxyapatite column. The column (10 × 0.78 cm I.D.) was equilibrated with 25 m*M* potassium phosphate (pH 7.0)–0.2 m*M* DTT. Solutions of enzyme (1 mg/ml) were applied to the column and eluted with a 10-ml linear gradient from 25 to 100 m*M* potassium phosphate (pH 7.0)–0.2 m*M* DTT. The flow-rate was 0.1 ml per min and fractions of 0.2 ml were collected. ■ = Protein; ● = enzyme activity. The dashed line indicates the gradient.

To determine the molecular mass of the thermophilic pyruvate dehydrogenase complex from *T. aquaticus*, an FPLC Superose 6 HR 10/30 column was calibrated with different reference proteins in the range between $15 \cdot 10^4$ and $67 \cdot 10^4$ dalton and with the pyruvate dehydrogenase complex from bovine heart, having a molecular mass of $8.5 \cdot 10^6$ dalton (Fig. 4). A linear dependence of the retention volunes on the logarithm of the M_r (molecular mass) values was found for all reference proteins up to the very high value of the mammalian enzyme complex. This fact underlines the applicability of the method to large enzyme aggregates. The thermophilic pyruvate dehydrogenase complex was eluted prior to the mammalian complex, corresponding to a relative molecular mass of $9.1 \cdot 10^6$ dalton.

Resolution of the pyruvate dehydrogenase complex from bovine heart into its enzyme components

In the native pyruvate dehydrogenase complex the 60 chain of the E2 component form a stable core, which is surrounded by the α- and β-chains of the E1 component [13]. The contact between both enzyme components is very strong, and partially denaturating conditions, such as high pH and high ionic strength, are necessary to break these bonds [14]. Owing to these denaturating conditions, the reaction time must be kept short and, therefore, quantitative separation of the components cannot be achieved without a severe loss of enzymatic activity. The rate-determining step is the

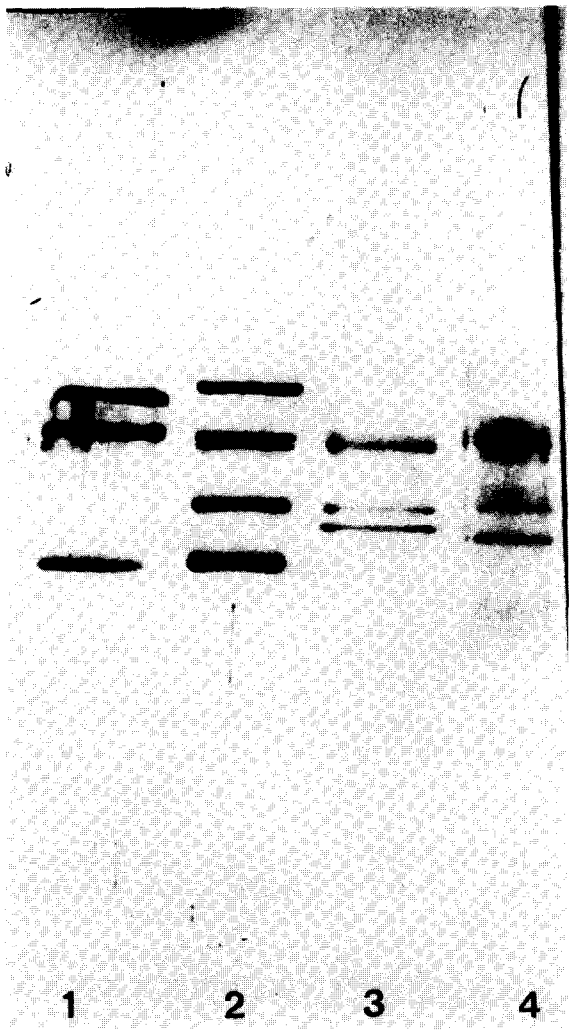

Fig. 3. SDS-PAGE of the pyruvate dehydrogenase complex from *T. aquaticus*. Purified pyruvate dehydrogenase complexes (2 μg of each) from *T. aquaticus* (lane 3), bovine heart (lane 2) and *Bacillus subtilis* lane 4) were applied to the gel. Lane 1 shows the reference proteins (from the top): bovine serum albumin, diaphorase from porcine heart and lactate dehydrogenase from porcine muscle (1 μg of each). The enzyme components of the pyruvate dehydrogenase complexes are as follows (from top to bottom): lane 2 = E2, E3, component X, E1α and E1β; lane 3 = E2, E1α, E1β; lane 4 = E2, E3, E1α, E1β.

chromatographic separation of the subunits. During the whole process, the enzyme components are in contact with the denaturating agents. Thus, a rapid method is required, which permits both the separation of the components and the removal of the denaturating agents in one fast step. Gel filtration in combination with the FPLC technique satisfies these conditions.

To the pyruvate dehydrogenase complex from bovine heart (30 mg/ml) in 50 m*M* potassium phosphate (pH 7.5)–1 m*M* DTT–0.1 m*M* EDTA, solid NaCl, 1 *M* DTT and

TABLE I

PURIFICATION OF THE PYRUVATE DEHYDROGENASE COMPLEX FROM *T. AQUATICUS*

Purification step	Volume (ml)	Protein (mg)	Specific activity (kat × 10^{-9} mg)	Total activity (kat × 10^{-9})	Yield (%)	Purification factor
Crude extract	28	420	0.20	78.3	89	0.62
Triton X-100 extraction	24	276	0.32	88.1	100	1.0
Phenyl-Sepharose Cl-4B	30	122	0.66	80.6	91.5	2.1
Ultracentrifugation	1	2.1	17.5	36.8	41.8	54.9
Hydroxyapatite	1.6	0.34	93.2	31.3	35.3	292

1 *M* glycine–NaOH (pH 9.5) were added to give final concentrations of 1 *M* NaCl, 10 m*M* DTT and 0.1 *M* glycine–NaOH (pH 9.0). After preincubation for 1 h at room temperature, aliquots of 0.1 ml were applied to a Superose 6 HR 10/30 column, equilibrated with 0.1 *M* glycine–NaOH (pH 9.0)–1 *M* NaCl–1 m*M* $MgCl_2$–2 m*M* DTT–0.1 m*M* EDTA. The same buffer was used to elute the enzyme components. Two well separated peaks were eluted (Fig. 5), the first containing the activity of the E2

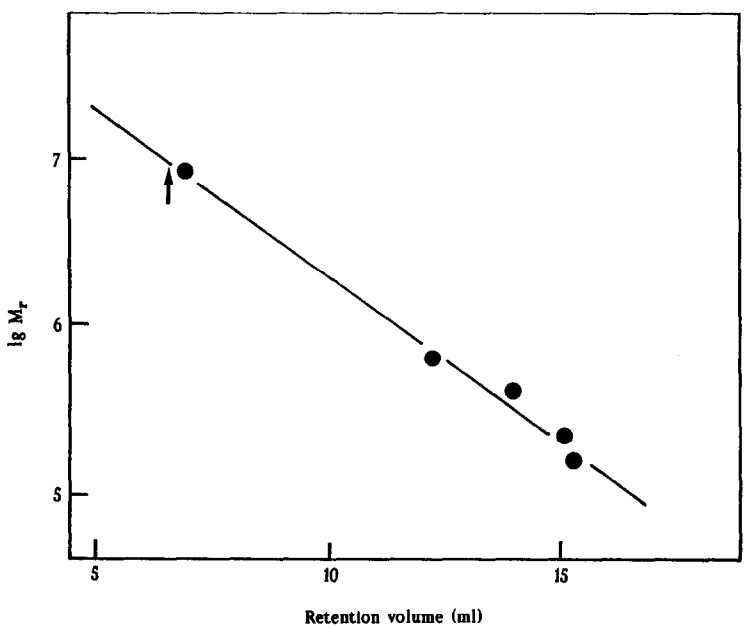

Fig. 4. Molecular mass determination of the pyruvate dehydrogenase complex from *T. aquaticus* on Superose 6. The column was calibrated with the reference proteins (from right to left): aldolase from rabbit muscle ($158 \cdot 10^3$ dalton), catalase from bovine liver ($24 \cdot 10^4$ dalton), ferritin from horse spleen ($45 \cdot 10^4$ dalton), porcine thyroglobulin ($67 \cdot 10^4$ dalton) and pyruvate dehydrogenase complex from bovine heart ($8.5 \cdot 10^6$ dalton). The arrow indicates the position of the pyruvate dehydrogenase complex from *T. aquaticus*. Equilibration and elution were performed with 50 m*M* morpholinopropanesulphonic acid–NaOH (pH 7.0)–150 m*M* NaCl, 0.4 mg of each protein was applied to the column and the flow-rate was 0.5 ml/min. Eluent was monitored at 280 nm and the respective retention volumes were determined.

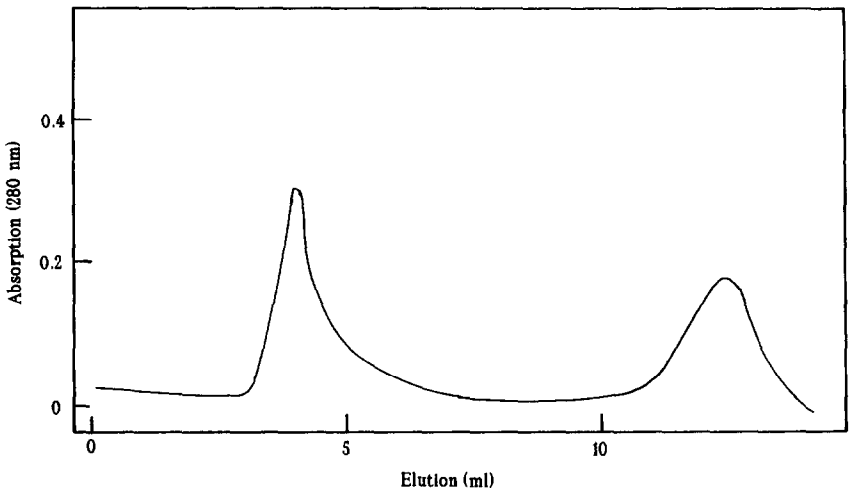

Fig. 5. Separation of the enzyme components of the pyruvate dehydrogenase complex from bovine heart by gel filtration on Superose 6 HR 10/30. The enzyme was pretreated as described in the text. Aliquots of 3 mg of protein in 0.1 ml of buffer were applied to the column, which was equilibrated with 0.1 *M* glycine–NaOH (pH 9.0)–1 *M* NaCl–1 m*M* $MgCl_2$–2 m*M* DTT–0.1 m*M* EDTA. The enzyme components were eluted with the same buffer at a flow-rate of 0.1–0.2 ml/min. The direction of the elution in the diagram is from right to left. The fractions of each peak were pooled and adjusted to pH 7.5 with 1 *M* KH_2PO_4 immediately after elution.

component and the second that of the E1 and E3 components. The pattern of the SDS PAGE, presented in Fig. 6, shows three protein bands for the first Superose peak. One major band corresponds to the E2 chain and a second is at the position of component X. The third band is a proteolytic fragment of the E2 chain, which is often observed in preparations of the mammalian pyruvate dehydrogenase complex [15]. The three protein bands on the gel, found in the second Superose peak, correspond to the E3, E1α and E1β chains, respectively.

CONCLUSION

Various kinds of information can be obtained from the application of the FPLC technique. The elution of the pyruvate dehydrogenase complex at very low ionic strength on phenyl-Sepharose is an indication of hydrophobic regions on the surface of this membrane-associated enzyme complex. This experiment also demonstrates the successful transfer of the conditions from an analytical FPLC phenyl-Superose HR 5/5 column, used for smaller amounts of crude extract (not shown), to the preparative phenyl-Sepharose column with nearly equal resolution.

The higher molecular mass obtained for the bacterial pyruvate dehydrogenase complex in comparison with the mammalian complex on Superose 6 is remarkable, as the mammalian enzyme complex possesses an additional component, X, with a so far unknown function, and two regulatory enzyme components [16,17]. A reason for the high molecular mass of the bacterial enzyme may be the avoidance of dissociation

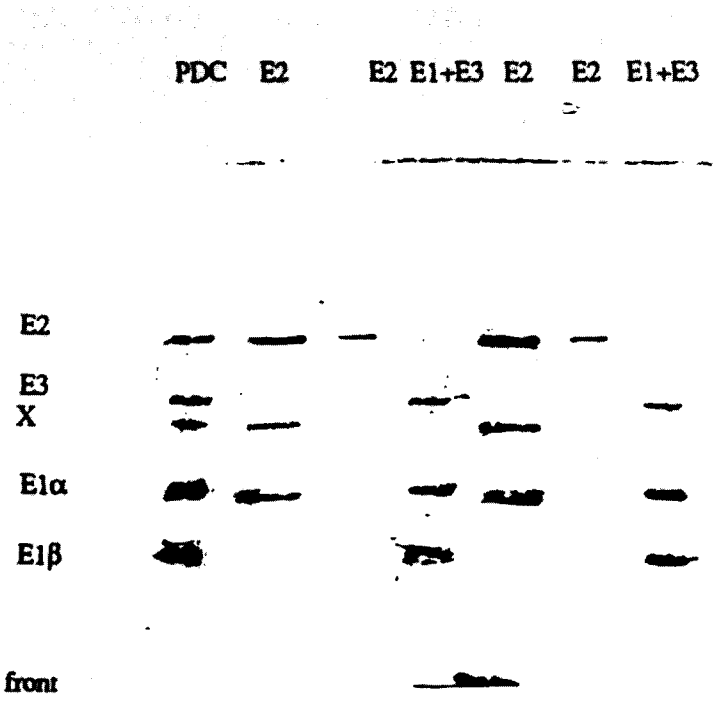

Fig. 6. SDS-PAGE of the enzyme components from the bovine pyruvate dehydrogenase complex. Samples of the purified pyruvate dehydrogenase complex from bovine heart (PDC), and of both of the peaks from the chromatogram shown in Fig. 5, each containing 5 μg of protein, and a further sample of peak 1, containing 0.5 μg of protein (lanes 3 and 6 from the left), were applied to the gel.

processes as a consequence of the fast chromatographic method, although structural differences between the two enzyme complexes cannot be completely excluded.

Separation of the E2 component from both the E1 and the E3 components by gel filtration on Superose 6 at high ionic strength and high pH is an appropriate technique for the dissociation of the native pyruvate dehydrogenase complex. As complex aggregation is mediated via the E2 core, the E1 and E3 components can be easily separated from one another by simple gel filtration without the need for denaturating agents.

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