A NEW PROCEDURE TO PREPARE HIGHLY PURIFIED AND CRYSTALLIZED YEAST PYRUVATE DECARBOXYLASE

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Received 3 May 1974

1. Introduction

Cytoplasmatic yeast pyruvate decarboxylase (EC 4.1.1.1, PDC) has been prepared and characterized in various ways from brewer's yeast [1–3] or baker's yeast [4–6]. Holzer et al. [1] obtained a 10-fold enrichment by acetone and ammonium sulfate precipitation. Ullrich et al. [2, 3] improved this procedure to obtain activities between 20–40 U/mg and a subsequent high purification refinement using Sephadex G-200 yielded preparation of 80 U/mg [7] but no tendency to crystallize has been observed so far.

Specific activities up to 54 U/mg were obtained by Juni and Heym [4] via a combination of heat treatment at 60°C followed by ammonium sulfate precipitation and adsorption on alumina gel C.

Gounaris et al. [6] purified PDC from baker's yeast by rechromatography on DEAE-cellulose; Katsumata et al. [5] by an additional isoelectric focussing. Both obtained preparations showing two peaks of enzymatic activity after separation on DEAE-Sephadex.

2. Materials and methods

2.1. *Chromatography on CM-Sephadex C-50*

After soaking CM-Sephadex 6–8 hr with 0.5 M phosphate buffer pH 5.18 it was carefully equilibrated with 0.05 M disodium hydrogen phosphate adjusted to pH 5.18 by adding concentrated phosphoric acid or better to pH 5.10 by adding of concentrated acetic acid. The chromatographic separations were performed on a 3 × 25 cm column (gel bed 10 cm in height) at 4°C and an elution rate of 25 ml/hr taking the same buffer as used for equilibration.

Crude enzyme preparations of 20–35 U/mg obtained by the usual methods (e.g. [3]) (1.2 g of the enzyme precipitate dissolved in 1 ml elution buffer) with a conductivity not exceeding 200% that of the elution buffer, were added on the column. To achieve proper conductivity separation from ammonium sulfate was performed by a 2-fold centrifugation through a 3 × 3 cm Sephadex G-25 column placed in a swing-out rotor whose velocity was increased very slowly up to 2600 *g* (the Sephadex G-25 used in this separation procedure had been equilibrated with elution buffer and centrifuged to dryness before).

The eluting protein fractions were recorded at 280 nm with a Uvicord (LKB, Sweden) and collected in 4.1 ml fractions which were adjusted to pH 6.2 by a preceding addition of 0.5 ml 0.2 M phosphate buffer pH 6.9 in the case of elution with phosphate buffer pH 5.18 or 2.5 ml in the case of elution with phosphate/acetate buffer pH 5.10 to the collecting tubes.

2.2. *Crystallization of the CM-Sephadex treated preparations*

Only fractions with activities exceeding 75 U/mg were used for the crystallization procedure according to Zeppezauer et al. [8]. The polyacrylamide gel serving as diaphragm contained 1.8 M ammonium sulfate. Dialysis was performed at 4°C against 2.5 M ammonium sulfate. After 8–14 days small fragile crystal druses could be obtained (fig. 1) which showed an extinction quotient at 280 and 260 nm of 1.63

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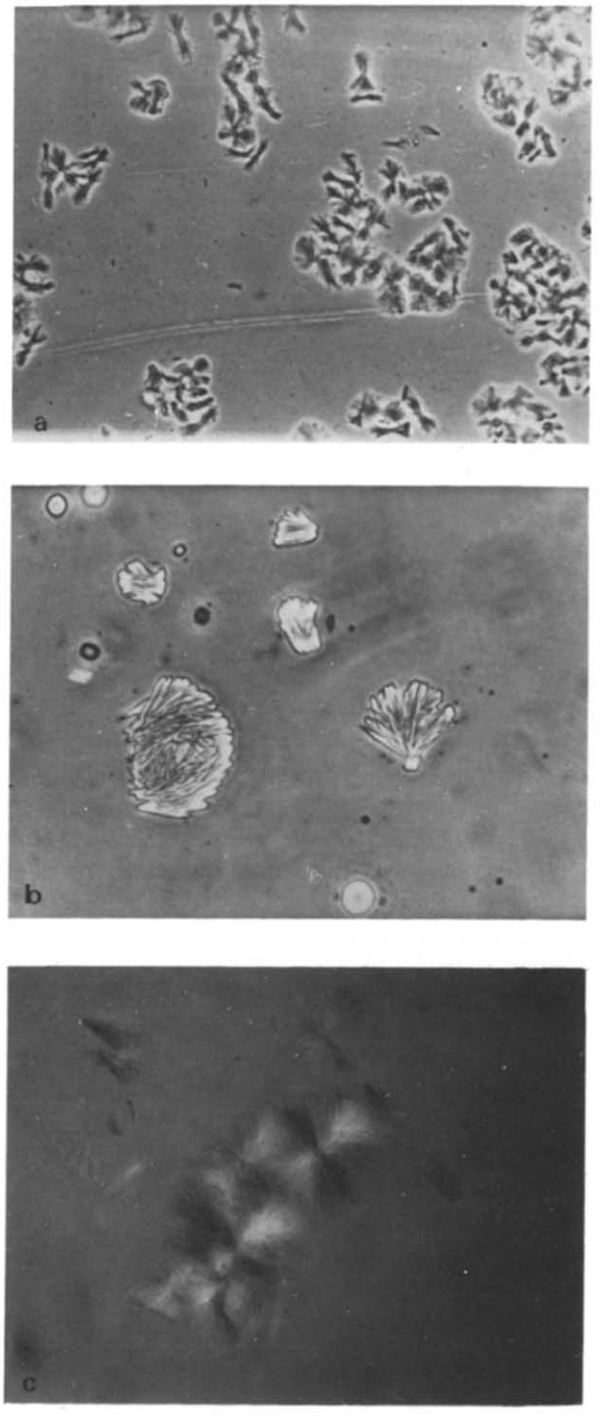

Fig. 1. Microphotographs of the crystallized pyruvate decarboxylase. a) Magnifed 126 X (phase contrast picture), b) Magnified 256 X (phase contrast picture). c) Magnified 256 X (polarized light). Microscope: Amplival (VEB Carl Zeiss, Jena). Film: ORWO NP15.

after centrifugation and resolving in 0.05 M phosphate buffer pH 6.8 on account of the rather long standing of the preparations at 4°C the enzymatic activity was reduced by about 15% in comparison to the initial activity.

Ullrich [7, 9] received a quotient of 1.65 taking non-crystallized enzyme preparations with 85 U/mg which had been purified on Sephadex G-200.

2.3. *Protein estimations*

Protein estimations were performed according to Lowry as modified by Gläser and Kleine [10]. Highly purified PDC preparations were characterized spectrophotometrically at $E\,{}^{1\,cm}_{1\%} = 9.85$ (280 nm).

2.4. *Enzyme assays*

Enzymatic activity was measured by the optical test [11] at 340 nm and 30°C using a Unicam spectrophotometer SP 800 A.

2.5. *Electrophoretic measurements*

Enzyme preparations freed from ammonium sulfate (s.a.) were separated electrophoretically either in 7% polyacrylamide gels (4°C, 150 V, 140 min in 0.025 M Tris–phosphate buffer pH 6.8 after 30 min pre-electrophoresis [12]) or in 1% agarose gel (4°C, 40 V, 0.025 M Tris–maleate buffer pH 4,5–7,0 [13]). Protein bands were stained with Coomassie Brilliant Blue G 250 or Amido Black 10 B.

2.6. *Preparation of apo-pyruvate decarboxylase*

PDC (70 U/mg) was incubated 30 min at 4°C in 0.05 M phosphate buffer pH 8.9 which contained additionally 0.2 M glycine and 1 mM EDTA. A subsequent twofold centrifugation through Sephadex G 25 equilibrated with 0.02 M glycine (gel bed 6 X 15 mm) gave an apo-enzyme suitable for electrophoresis. A specific activity of 50–60 U/mg resulted after recombination with excess thiamine pyrophosphate (TPP) and Mg^{2+}.

3. Results and discussion

The one-step procedure described in this paper using CM-Sephadex performs the preparation of highly purified PDC with an average activity of about 75 U/mg.

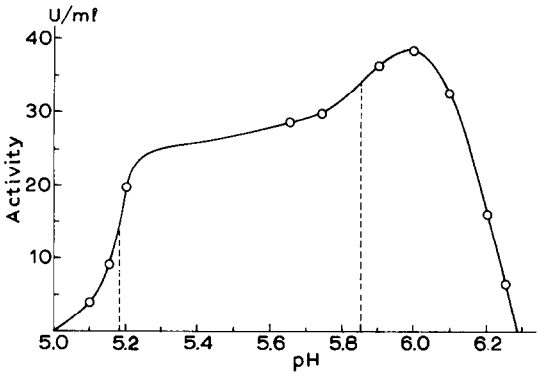

Fig. 2. Elution of pyruvate decarboxylase from CM-Sephadex C-50 in dependence on pH value.

Contrary to Gounaris et al. [6] who carefully avoid pH values below 6.0 in the preparation of PDC from baker's yeast, it could be established that the enzymatic activity of PDC prepared from brewer's yeast at pH 5.0 only diminishes by 9% after standing

Table 1
Chromatographic separation of crude enzymes free of ammonium sulfate via CM-Sephadex C-50 at different pH values

Crude enzyme used (U/mg)	buffer	pH	Maximum specific activity (U/mg)	Quotient of enrichment
35	a	5.35	58	1.66
33	a	5.30	63	1.91
19	a	5.25	35	1.84
32	a	5.18	68	2.13
33	a	5.10	80	2.42
33	a	5.10	79	2.39
7	a	5.10	50	7.14
30*)	b	5.20	85	2.83
22*)	b	5.10	76	3.45

Elution at 4°C and 25 ml/hr. Gel bed 10 X 3 cm.
* 20 X 3 cm. resp.
a – phosphate buffer
b – phosphate/acetate buffer

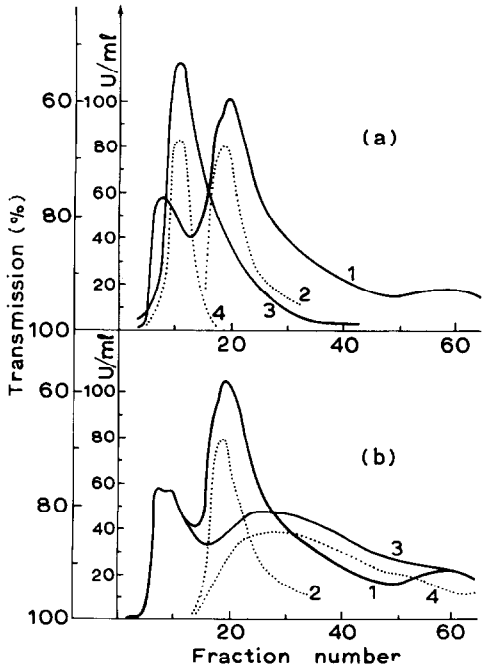

Fig. 3. Chromatography of pyruvate decarboxylase on CM-Sephadex C-50 at different pH values. Volume of the fractions 4.1 ml each. A: 1.2 elution at pH 5.18; 3.4 elution at pH 5.30. B: 1.2 elution at pH 5.18; 3.4 elution at pH 5.10. ... enzymatic activity, —— transmission at 280 nm.

for 5 hr. This permits chromatographic purification at this pH but requires short elution times. The elution profile presented in fig. 2 shows at pH 5.18 as well as at pH 5.87 association–dissociation equilibria with the ion exchanger CM-Sephadex. To provide a good separation effect from contaminating proteins the column was equilibrated and subsequently eluted with phosphate buffer pH 5.15 ± 0.05. In the case of higher pH values (e.g. pH > 5.30) the separating effect between PDC and such proteins eluting at pH 5.15 before PDC is diminished. On the other hand, at lower pH values (e.g. pH < 5.10) the contaminating proteins are also retarded but the PDC is eluted from the column only trailingly (fig. 3). If phosphate/acetate buffer (c.f. Materials and methods) is used in corresponding experiments, all the described effects hold true if the pH values kept lower by 0.1 unit. Table 1 shows a rather small (in comparison to a gradient elution) and therefore critical pH range of only ~ 0.2 pH units which needs a very precise pH adjustment but on the other hand guarantees the very specific separation from the contaminating proteins. PDC fractions purified on CM-Sephadex contain only traces of protein impurities which move faster in polyacrylamide gel electrophoresis than PDC (which can be eliminated by chromatography on

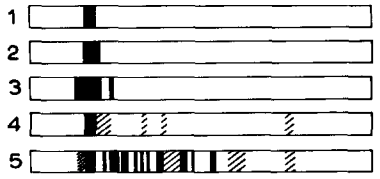

Fig. 4. Electropherograms of pyruvate decarboxylase after different steps of purification. 1. Isoelectric focussing (47 U/mg); 2. preparative electrophoresis (51 U/mg); 3. chromatography on CM Sephadex C-50 and Sepharose 6 B (80 U/mg). To make visible also traces of contaminating proteins a fourfold amount of protein was added as compared to the other pherograms; 4. chromatography via CM Sephadex (73 U/mg); 5. Crude enzyme, prepared according to Ullrich [3] (35 U/mg). ▬ strong; ▨ weak; ▱ very weak staining of the protein fractions.

Sepharose 6 B or Sephadex G-200) and another protein which differs only insignificantly from PDC in this respect (fig. 4). On the other hand the formation of apo-PDC during the electrophoretic procedure by splitting off TPP and Mg^{2+} cannot be excluded. This would explain the changes in the charge distribution (IP) sometimes observed by several authors as well as the existence of several enzymatically active protein peaks. The isoelectric point of PDC from baker's yeast has been determined to be 5.8 by Katsumata et al. [5]. The PDC prepared by the method described in this paper showed an IP of 5.8 whilst the apo-enzyme produced from it showed an IP of 6.0.

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