A SIMPLE PURIFICATION OF ACETOACETATE–SUCCINATE CoA-TRANSFERASE USING SUBSTRATE ELUTION CHROMATOGRAPHY

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1. Introduction

Succinyl-CoA: 3-oxoacid CoA-transferase (EC 2.8.3.5) from pig heart can be easily purified using specific substrate elution chromatography; it appears to be a dimer of similar subunits.

The use of highly purified CoA-transferase in a sensitive fluorometric assay for succinyl-CoA has recently been reported [1]. Published purification procedures for this enzyme [1, 2] are not entirely satisfactory for this purpose in several respects: i) they are lengthy with low overall yields; ii) the final product is only 80% pure and contains contaminating activities; iii) they have proved difficult to reproduce.

Incubation of CoA-transferase with acetoacetyl-CoA (or succinyl-CoA) results in the formation of an enzyme-CoA covalent complex with a half life of 42 min at 25°C and pH 6.5 [3, 4], and a consequent increase of negative changes on the enzyme molecule. This suggested the use of substrate elution chromatography [5] as a possible method of purification. We describe a simple procedure based on the specific elution of the enzyme by acetoacetyl-CoA from a cation exchange column of cellulose phosphate.

2. Materials and methods

2.1. Acetoacetyl-CoA was prepared from diketene and coenzyme A as described by Wieland and Rueff [6].

2.2. Protein concentrations were determined spectrophotometrically by the method of Kalckar [7] from the absorbancies at 260 nm and 280 nm, measured against an appropriate blank.

2.3. Activity of CoA-transferase was measured spectrophotometrically in the direction of acetoacetyl-CoA breakdown by a modification of the method of Williamson et al. [8]. The decrease in absorbance at 303 nm was followed in a 2 ml assay mixture at 30°C containing 100 mM Tris–sulphate (pH 8.1), 25 mM $MgSO_4$, 10 mM sodium succinate, 50 μM acetoacetyl-CoA and a suitable amount of the enzyme. The buffer anion used was sulphate rather than chloride as monovalent anions inhibit the enzyme [2]. The extinction coefficient of acetoacetyl-CoA under these conditions is 16.9×10^3 litre·mol^{-1} [9].

2.4. All other enzyme assays were performed by standard spectrophotometric methods. Marker proteins for molecular weight determinations were purchased from Boehringer, Mannheim, while those for subunit molecular weights were obtained as follows: lysozyme, zyme, – S. Behr & Mathew (Sales) Ltd. London, England; carboxymethyl-aldolase – a gift from Dr. I. Gibbons; pepsin – Calbiochem, Los Angles, U.S.A.; crystallised bovine serum albumin – BDH Chemicals Ltd., Poole, England.

2.5. SDS-gel electrophoresis was performed by the method of Shapiro et al. [10], as modified by Weber and Osborn [11]. Protein samples were prepared according to Pringle [12].

2.6. Molecular weights were determined by gel filtration on a column (2 cm × 32 cm) of Sephadex G-200 M (Pharmacia) equilibrated at 5°C with 100 mM potassium phosphate (pH 7.0), containing 1 mM EDTA. The positions of protein peaks were estimated by assaying for enzyme activities.

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2.7. Whatman cellulose phosphate P11 (W. & R. Balston Ltd., Maidstone, England) was pre-cycled using the standard procedure for carboxymethyl-cellulose, except that 0.1 M acids and bases were used, and thoroughly defined. The pre-cycled material from 40 g of dry ion exchanger was equilibrated in 10 mM potassium phosphate (pH 6.5) containing 0.1 mM EDTA ('column buffer'), and poured in a thick slurry to give a column 5 cm × 9 cm. The diameter: length ratio should not be greater than this as the transferase is eluted slowly by the 'column buffer'.

3. Results and discussion

3.1. *Purification*

Fresh pig hearts were cut into small pieces and frozen. The tissue may be stored at −20°C for at least 2 months without loss of activity.

3.1.1. Extraction (step 1)

Two hundred grams of frozen heart was homogenised in a blender for 2 min in 600 ml of 0.4 M KCl/20% ethanol (v/v), pre-cooled to 0°C. The homogenate was centrifuged at 10 000 *g* for 20 min and the supernatant dialysed for 2 × 6 hr against 5 litres of 10 mM potassium phosphate (pH 7.0), containing 0.1 mM EDTA, at 5°C.

3.1.2. Ammonium sulphate fractionation (step 2)

Ignoring any precipitate formed during the dialysis, the dialysate was brought to 50% saturation with $(NH_4)_2SO_4$ by addition of 312 g per litre and stirred for 30 min. The solution was centrifuged at 10 000 *g* for 20 min and the precipitate discarded. The supernatant was brought to 65% saturation by addition of 100 *g* $(NH_4)_2SO_4$ per litre and stirred and centrifuged as before. The precipitate was dissolved in a minimal volume of 'column buffer', and ammonium sulphate removed by gel filtration on a column of Sephadex G-25C (5 cm × 43 cm) equilibrated with 'column buffer'. It is very important to ensure complete removal of the ammonium sulphate for the success of the chromatographic step.

3.1.3. Cellulose phosphate chromatography (step 3)

The desalted enzyme solution was diluted to 200 ml

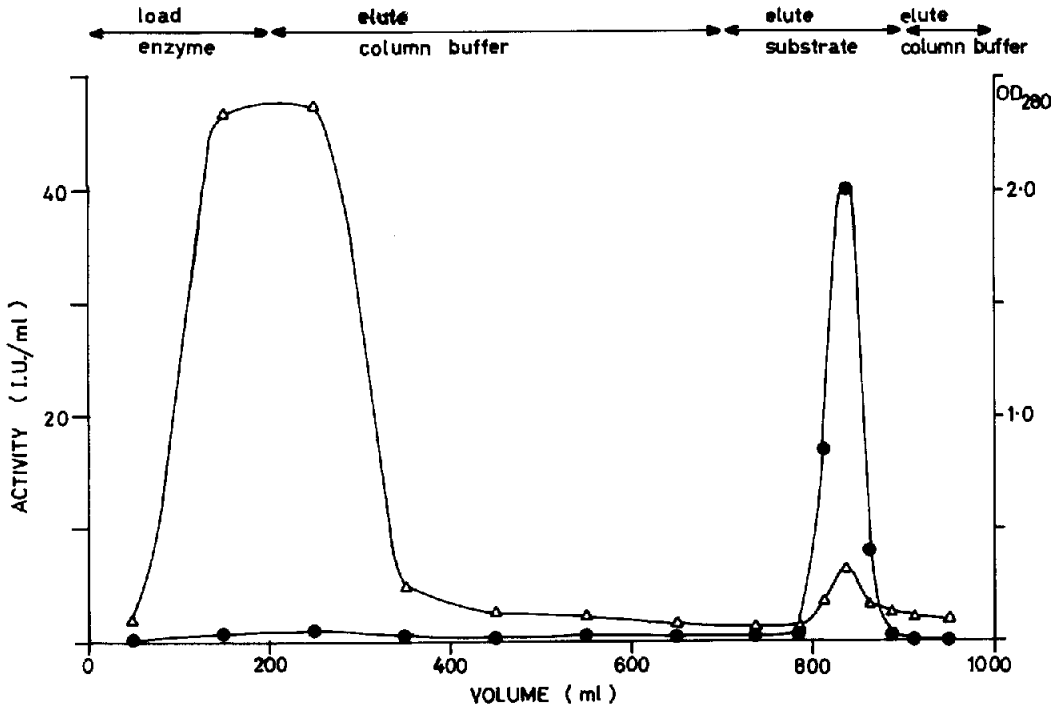

Fig. 1. Elution diagram from a cellulose phosphate column. CoA-transferase activity (●——●——●) and A_{280} (△——△——△), measured against a column buffer blank, are plotted against the eluent volume. For details see text.

Table 1
Summary of purification of CoA-transferase.

Fraction	Volume (ml)	Activity (I.U./ml)	Total units	Protein (mg/ml)	Specific activity	Purification	Yield (%)
Extract	650	3.8	2470	21	0.18	1	100
50–65% $(NH_4)_2SO_4$	45	52.5	2360	32	1.64	9	95
Substrate elution	78	22.5	1755	0.155	145	805	71

with 'column buffer' and loaded onto the column. The column was washed with 'column buffer' until the A_{280} of the effluent decreased to less than 0.1 absorbance units as measured against a 'column buffer' blank. At this stage the column was eluted with 200 ml 'column buffer' containing 10 μM acetoacetyl-CoA. Fractions (25 ml) were collected and those containing the peak of enzyme activity, usually 3 fractions, were pooled and freeze-dried. The results of the chromatography are shown in fig. 1, and the purification is summarised in table 1.

3.2. *Purity and contamination*

The enzyme at this stage has a specific activity of about 100–150 I.U./mg protein, and is free of lactate

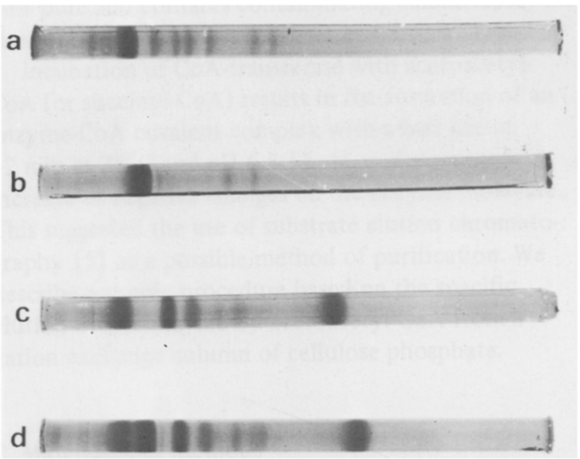

Fig. 2. SDS gel electrophoresis of CoA-transferase and marker proteins: (a) Pooled fractions from a cellulose phosphate column, specific activity, 145 I.U./mg; (b) after further purification on alumina gel to give a specific activity of 155 I.U./mg; (c) marker proteins, from left to right, serum albumin, carboxymethylaldolase, pepsin, lysozyme; (d) alumina-treated CoA-transferase plus marker proteins.

and 3-hydroxyacyl-CoA dehydrogenases, acetoacetyl-CoA thiolase and hydrolase, citrate synthase, carnitine acetyltransferase and NADH oxidase. Very small amounts (less than 0.5% on an activity basis) of malate dehydrogenase may be present, and the freeze-dried transferase, after redissolving in a small volume (4 ml) of distilled water, is slightly yellow coloured.

If homogeneous transferase is required, this may be obtained by an alumina gel step as described by Hersh and Jencks [2] on the pooled fractions from the column step. The transferase is adsorbed onto the gel from the 'column buffer', washed with 100 mM sodium phosphate (pH 7.0), and finally eluted with 200 mM sodium phosphate (pH 8.0) containing 55 g $(NH_4)_2SO_4$ per litre.

3.3. *SDS gels and molecular weight*

Ten percent-SDS gels were loaded with 10–20 μg samples of CoA-transferase preparations. As shown in fig. 2, the pooled fractions from the column step (specific activity 145 I.U./mg) showed seven minor bands and one major band comprising about 90% of the total protein. After an alumina step the transferase was essentially homogenous (specific activity 155 I.U./mg). SDS gel electrophoresis with marker proteins (bovine serum albumin, subunit mol. wt 68 000 [13]; carboxymethyl-aldolase, 44 000 (Dr. I. Gibbons, personal communication); pepsin, 35 000 [14]; and lysozyme, 14 300 [11]) indicated a subunit molecular weight for CoA-transferase of about 55 000 (see fig. 2).

A sample of native transferase together with marker proteins (10–70 I.U. of each) in a total volume of 0.1 ml was diluted to 0.5 ml with 100 mM potassium phosphate (pH 7.0) containing 1 mM EDTA and chromatographed on the Sephadex G-200 column. The markers were pyruvate kinase, mol. wt 237 000 [15]; lactate dehydrogenase, 140 000 [16]; citrate synthase, 87 000 [17]; malate dehydrogenase, 65 000 [18]; and

carnitine acetyltransferase, 58 000 [19]. The CoA-transferase eluted between lactate dehydrogenase and citrate synthase and the molecular weight was estimated by the method of Andrews [18], to be about 113 000 (in contrast to a figure of 78 000 given by Hersh and Jencks [20], based on Sephadex G-100 chromatography).

Comparison of the molecular weigjts obtained by SDS gel electrophoresis and Sephadex G-200 gel filtration strongly suggests that the CoA-transferase molecule is a dimer of similar subunits.

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