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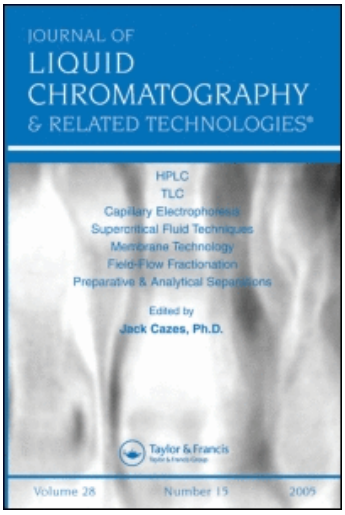

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Rapid Purification of the Unstable Enzyme Carbamoyl Phosphate Synthase by High Pressure Liquid Chromatography

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RAPID PURIFICATION OF THE UNSTABLE ENZYME CARBAMOYL PHOSPHATE SYNTHASE BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

Extracts of soluble proteins obtained from rat liver mitochondria by freeze-thawing and subsequent diafiltration were fractionated by HPLC on a I 250 protein column. The column was eluted either with 0.05 M phosphate buffer pH 6.85 or 0.1 M acetate buffer pH 7.15. Specific fractions obtained by elution with either phosphate or acetate buffer showed a 6.1-fold or 5.5-fold increase in the specific activity of Carbamoyl phosphate synthase when compared with that of crude mitochondrial preparations. The purification and the molecular weight of carbamoyl phosphate synthase were verified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Introduction

Carbamoyl phosphate synthase-I (EC 2.7.2.5), which catalyses the first step in urea synthesis, is located in the matrix of hepatic mitochondria in ureotelic animals (1-5). The enzyme is extremely unstable in its unpurified state, apparently because of its susceptibility to proteolytic degradation (1,4). The rat liver enzyme has been identified with a single polypeptide chain which migrates as a single component with a molecular weight of 165,000 during sodium dodecyl sulphate (SDS)-polyacrylamide electrophoresis, but rapidly degrades, first to a component of molecular weight 155,000 and subsequently to even lower molecular

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weight components during purification by conventional and time consuming methods (4).

During our investigations of this enzyme it became necessary to develop a simple, rapid method for its purification. The method, which is reported in this communication, involves extraction of soluble matrix proteins by freeze-thawing rat liver mitochondria in hypotonic buffer, the concentration of these proteins by diafiltration and fractionation by high pressure liquid chromatography (HPLC) on an I 250 column.

The combined procedures enable purification of carbamoyl phosphate synthase to near-homogeneity with high yield within 2 hours of preparing liver mitochondria. The purified enzyme migrates as a single polypeptide of molecular weight 166,000 during SDS-polyacrylamide gel electrophoresis.

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MATERIALS AND METHODS

Preparation of Mitochondria

Mitochondria were isolated from random-bred Wistar rats weighing between 150 - 200 g essentially as described previously (6); the only modification involved the last two washes, which were carried out in a medium containing 250 mM-sucrose, 1 mM-4-(2-hydroxy-ethyl)-piperazine-ethanesulphonic acid (Hepes), 1 mM-EDTA, pH 7.4.

Preparation of Mitochondrial Matrix

Mitochondrial pellets were suspended in 10 mM Hepes buffer pH 7.4 at a concentration of 20 mg/ml. The suspension was frozen and thawed 3 times by alternate immersion into liquid N_2 and a waterbath at 37 ^{o}C, centrifuged for 10 min. at 10,000 g and the supernatant collected and filtered through a Millex-GS 0.22µm filter unit (Millipore Corporation, Bedford, Ma. U.S.A.) The filtrate which contained the soluble proteins of the mitochondrial matrix was then concentrated 10 times by dia-

filtration using a stirred Model 12 cell (Amicon Corp. Lexington, Mass. U.S.A.) containing a PM 30 (Amicon) membrane.

High Pressure Liquid Chromatography

HPLC was carried out with an ALC/GPC 204 Liquid chromatograph (Waters Associates, Milford, Mass. U.S.A.) using the absorbance detector with a 280 nm filter.

Up to 200 µl of concentrated mitochondrial matrix were injected into an I 250 protein column (Waters Associates) which had been equilibrated either with 0.05 M phosphate buffer pH 6.85 or 0.1 M acetate buffer pH 7.15. The column was eluted with the same buffer with which it had been equilibrated. The flow rate was either 1 ml or 2 ml per min. The elution buffer and the column were maintained at 5 - 6°C by a water jacketed cooling block and the collecting tubes were kept on ice. Fractions were collected manually by following the absorption pattern at 280 nm. For enzyme assays the fractions were used without any further treatment, for SDS-polyacrilamide gel electrophoresis the relevant fractions were concentrated by centrifugation in CF 50A Centiflo ultrafiltration membrane cones (Amicon Corp.)

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Carbamoyl Phosphate Synthase I Assay

Enzyme activity was assayed by a modification of the method described by Clarke (4). Samples varying between 0.05-0.2 ml incubated in a final volume of 0.4 ml with 50 mM-ammonium acetate, 3 mM-ornithine, 10 mM-$MgSO_4$, 6.5 mM-N-acetylglutamate, 20 mM-glycylglycine, 20 mM-$NaHCO_3$, containing 130,000 d.p.m. [^{14}C] $NaHCO_3$, 10 mM-mercaptoethanol, 2.5 mM-ATP, 2.5 mM-phosphoenol-pyruvate, 4 units pyruvate kinase and 0.5 units ornithine transcarbamylase at pH 7.5 for 20 min at 37°C.

The reaction was terminated by the addition of 0.2 ml 30% trichloracetic acid and 0.5 ml portions were pipetted into scintillation vials with 0.1 ml 5 M HCl. The vials were heated for 15 min. with a 150 watt flood-light to remove unreacted bicarbonate.

After cooling, 5 ml Aquasol (New England Nuclear Corp., Boston, Mass. U.S.A.) was added to each scintillation vial. The residual radioactivity due to the conversion of ornithine to citrulline was counted in a model 2211 Packard liquid-scintillation spectrometer for at least 4000 counts and quench corrections were carried out with a quench curve in which acetone was used as a quenching agent.

Assay of Protein Concentration

The protein concentration of samples was determined by the method of Lowry *et al.* (7) with bovine serum albumin (fraction V; Sigma) as standard.

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SDS-Polyacrylamide Gel Electrophoresis

Electrophoresis was carried out on 3 mm thick slab gels measuring 7 cm x 7 cm containing a 6% acrylamide mixture as described by Melnick *et al.* (8) and in a buffer consisting of 0.04 M Tris, 0.02 M sodium acetate, 0.1% SDS, 0.002 M EDTA at pH 7.4. Samples contained between 20 - 80µg protein. At the completion of the run the gels were fixed for 1 hour in a solution containing 57 g trichloracetic acid, 17 g sulphosalicylic acid, 150 ml methanol and 350 ml distilled water and stained with Coomassie blue (8). Destaining was effected in a mixture of Methanol 30: water 63: acetic acid 7. Prior to photographing the gels were swollen in 7% acetic acid. After drying in a GSD-4 Gel slab dryer (Pharmacia, Sweden) the gels were scanned.

For molecular weight determinations the rate of migration of the purified carbamoyl phosphate synthase was compared with that of several standard proteins (myosin, 220,000; heavy meromyosin, 175,000; bovine serum albumin, 68,000).

RESULTS AND DISCUSSION

The HPLC elution profile of the concentrated matrix proteins is shown in Fig. 1A and 1B using phosphate and acetate buffers,

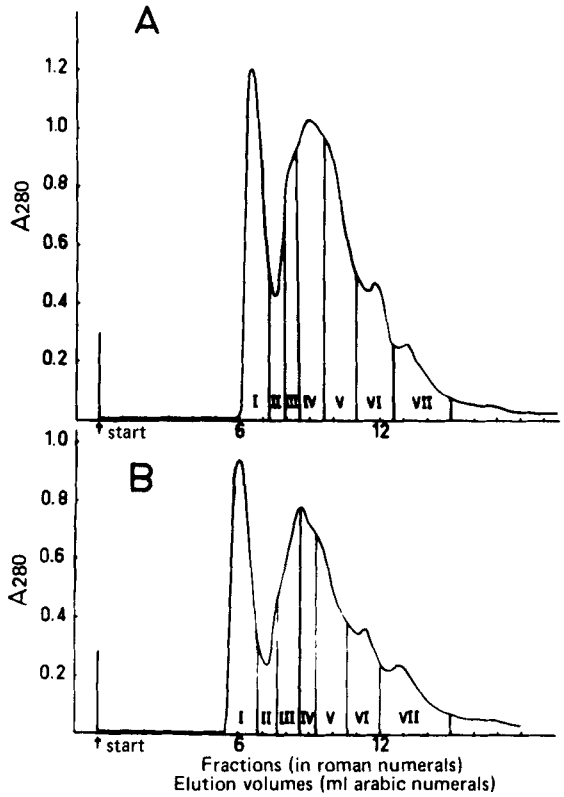

FIGURE 1

HPLC elution profiles. A. eluted with 0.05 M phosphate buffer, pH 6.85. B. eluted with 0.1 M acetate buffer, pH 7.15. Conditions as described in Materials and Methods.

respectively. Only four major peaks were observed as most proteins below a molecular weight of 30,000 had already been removed by diafiltration. Seven fractions were collected in each buffer system and the results are summarised in Table 1. Because of slightly different fraction sizes the enzyme activity was collected in differently numbered fractions in each run. The purification was similar in both buffer systems, except that phosphate clearly inhibited enzyme activity as shown in Table 1.

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TABLE I

Specific Activities of Carbamoyl Phosphate Synthase after Fractionation by HPLC

Fraction	Column eluted with phosphate buffer		Column eluted with acetate buffer	
	Protein	Enzyme activity	Protein	Enzyme activity
	mg/fraction	umol/min./mg protein	mg/fraction	umol/min./mg protein
Mitochondria	----	0.276**	----	0.469
Concentrated mitochondrial matrix	5.28*	0.394**	3.96*	0.602
Fraction I	0.25	0.119	0.10	0.215
Fraction II	0.13	0.968	0.21	2.584
Fraction III	0.51	1.676	0.68	1.186
Fraction IV	1.43	0.545	0.89	0.141
Fraction V	0.94	0.099	0.54	
Fraction VI	0.39	0.045	0.36	0.075
Fraction VII	0.27	0.031	0.33	0.036

* Amount of Protein applied onto Column.

** Assayed in the presence of 0.025 M Phosphate buffer.

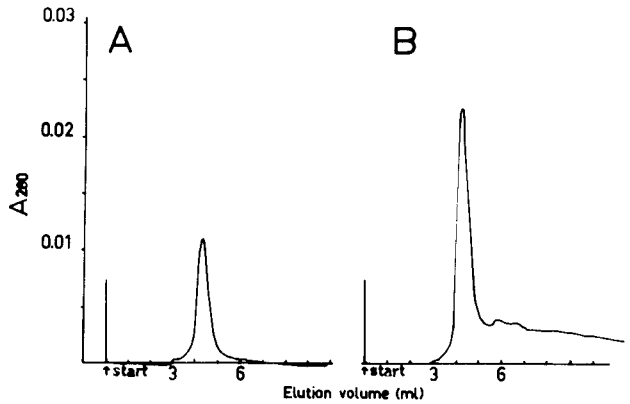

FIGURE 2

Elution profiles of carbamoyl phosphate synthase in the absence (A) and presence (B) of aldolase. Eluant was 0.05 M phosphate buffer, pH 6.85.

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The specific activity of 2.58 units/mg protein for Fraction II (acetate buffer) compared favourably with previously published values of 1.3 units/mg protein for purified rat liver enzyme (4) and 2.44 units/mg protein for crystalline frog liver enzyme (5). From the results presented in Table 1 it may be calculated that on elution with phosphate buffer 74% of the protein and 91% of the carbamoyl phosphate synthase activity were recovered. The corresponding values for the elution with acetate buffer are 78% and 66%.

The fraction with the maximum specific activity rechromatographed with purified aldolase (M.W. 150,000) (Fig. 2). The purification factor of 6.1 and 5.5 from whole mitochondria and 4.8 and 4.2 from mitochondrial matrix suggests that the protein has been purified to near-homogeneity, since carbamoyl phosphate synthase comprises 14 - 18% of total mitochondrial protein and 25% of the matrix proteins (4). This is confirmed in Fig. 3, which compares the protein bands obtained by SDS-polyacrylamide gel electrophoresis of whole mitochondria, matrix proteins, Fraction II (acetate buffer) and Fraction III (phosphate buffer).

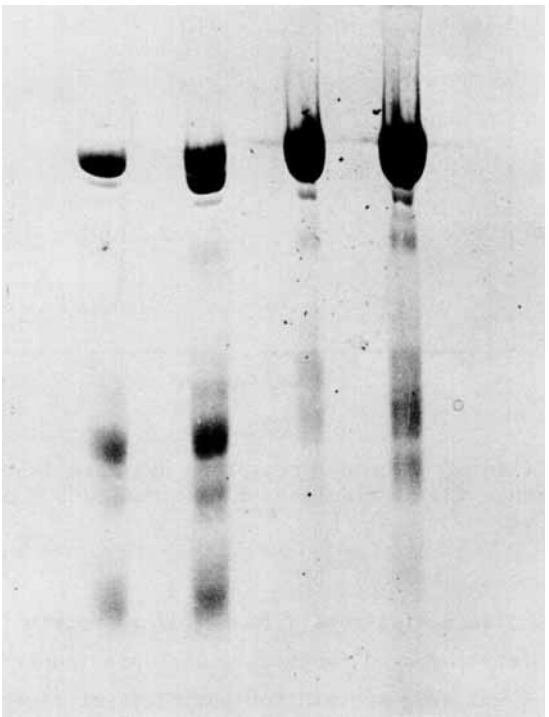

FIGURE 3

Electrophoresis of mitochondrial polypeptides. From left to right. Whole mitochondria, mitochondria matrix, Fraction III after elution with phosphate buffer, Fraction II after elution with acetate buffer.

In Fraction II (acetate buffer) more than 88% of the Coomassie Blue stain is associated with one slow-moving band, as measured by an integrating scanner. This is an underestimate, as it has been shown that Coomassie blue stain intensity increases linearly only to a maximum of 10 μg protein per band (9). This amount was exceeded in order to detect any contaminating proteins.

When compared to suitable marker polypeptides of known molecular weight this slow-moving band had a molecular weight of 166,000, which agrees well with the reported value of 165,000

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also obtained by SDS-polyacrylamide gel electrophoresis (4). It is noteworthy that the undenatured enzyme showed a similar molecular weight during HPLC elution to the SDS-treated enzyme (Fig. 2), in view of suggestions that carbamoyl phosphate synthase may exist as a dimer *in vivo* (2, 10).

The results presented in this communication therefore indicate that the use of an I 250 protein column with HPLC is a suitable technique for the rapid purification of unstable proteins. Such a single step fractionation procedure is particularly appropriate when the protein under investigation comprises a large proportion of the crude protein extract, as in the case of carbamoyl phosphate synthase. However, other work currently being carried out in this laboratory (M.T. Campbell, J.K.Pollak, R. Sutton; unpublished observations) indicates that this purification technique is also applicable to the cytoplasmic precursor of carbamoyl phosphate synthase, which is reported to be 6000 daltons larger than the mature protein (10, 11) and amount to less than 2% of cytoplasmic proteins. This precursor protein is also highly unstable (J.K.Pollak, unpublished observation). Because of the speed and convenience HPLC should be regarded as a powerful tool for protein isolation, separation and purification, especially when used in conjunction with diafiltration.

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