Separation of isoenzymes of citrate synthase and isocitrate dehydrogenase by fast protein liquid chromatography

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Fast protein liquid chromatography (FPLC) has been shown to be a rapid and effective method of separating isoenzymes of citrate synthase and isocitrate dehydrogenase in extracts of *Pseudomonas aeruginosa* and *Acinetobacter calcoaceticus*. The advantages of FPLC over conventional methods of fractionation are discussed and it is suggested that this may be a valuable and more general technique for isoenzyme resolution.

FPLC *Isoenzyme separation* *Citrate synthase* *Isocitrate dehydrogenase*
(Pseudomonas aeruginosa, Acinetobacter calcoaceticus)

1. INTRODUCTION

A variety of experimental methods may be employed for the analytical separation of isoenzymes, e.g. gel filtration, ion-exchange chromatography, centrifugation and electrophoresis. These methods may also be used for preparative-scale separations but suffer from the disadvantage of being relatively slow procedures. The introduction by Pharmacia of an apparatus system for 'fast protein liquid chromatography' (FPLC) offers the opportunity to perform extremely rapid chromatography on newly developed ion-exchange materials with very high resolution. This technique should, in principle, be advantageously applicable to the resolution of mixtures of isoenzymes.

In the course of our studies on enzymes of the citric acid cycle in diverse bacteria, two isoenzymic forms of citrate synthase (EC 4.1.3.7) have been discovered in various species of *Pseudomonas* [1,2] and two isoenzymes of isocitrate dehydrogenase (EC 1.1.1.42) have been shown to be present in *Acinetobacter calcoaceticus* [3–5]. In both these cases, the isoenzymes differ very substantially in molecular mass, the large forms displaying allosteric regulatory properties not exhibited by the small isoenzymes. A rapid, reliable and high-recovery method for separating these isoenzyme pairs would be a valuable tool in the study both of their occurrence over a wide range of organisms and of any variations in the relative proportions of the isoenzymes under different physiological conditions [1].

We have therefore investigated the use of FPLC for the separation of the isoenzymes of citrate synthase and isocitrate dehydrogenase. Expectations that this method of isoenzyme resolution might be effective, rapid and operable with small amounts of material were clearly fulfilled. The technique may find more general application in both the analytical and preparative separation of isoenzymes.

2. EXPERIMENTAL

2.1. *Organisms and preparation of cell extracts*

Pseudomonas aeruginosa, strain 8602, was kindly provided by Professor P.H. Clarke (University College, London). *A. calcoaceticus*, strain 4B, has been described [4] and was originally isolated from water by P.D.J.W. *Ps. aeruginosa* was grown in

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200 ml of nutrient broth (Oxoid) in a 500-ml flask shaking at 37°C for 16 h. The cells were harvested by centrifugation at 15000 × *g* for 10 min, resuspended in 4 ml of 20 mM Tris-HCl, pH 7.0, containing 1 mM EDTA and 0.1 M KCl, and sonicated (with cooling on ice) for a total of 2 min (4 × 30 s bursts with 30 s cooling between each). After centrifugation at 15000 × *g* for 10 min the supernatant was treated with 2% (w/v) aqueous solution of protamine sulphate (1 mg per 10 mg of extract protein) and stirred on ice for 10 min. The mixture was then centrifuged at 15000 × *g* for 20 min and the supernatant retained (protein content approx. 10 mg/ml).

A. calcoaceticus was grown in the same way as above, but at 32°C. After harvesting, the cells were suspended in 2 ml of 20 mM Tris-HCl, pH 8.0, containing 10 mM $MgCl_2$ and 1 mM EDTA; sonication and treatment with protamine sulphate were carried out as above to give a final supernatant containing protein at 6 mg/ml.

2.2. *Fast protein liquid chromatography*

FPLC was carried out using the equipment supplied by Pharmacia (see [6]). In the case of *Ps. aeruginosa*, the prepared extract was diluted approx. 10-fold with 20 mM Tris-HCl, pH 7.0, containing 1 mM EDTA to give a final protein concentration of 1 mg/ml. This was filtered through a 0.45 μm Millipore filter and 10 ml were applied to a Mono Q (HR 5/5) anion-exchange column (1 ml bed volume) equilibrated with 20 mM 1,3-bis-[tris(hydroxymethyl)methylamino]propane·HCl, pH 7.0. The column was washed with 4 column volumes (4 ml) of this buffer and elution was then continued using a gradient of 0–0.2 M KCl in the same buffer at a flow rate of 1 ml/min. The column effluent was monitored for protein at 280 nm; citrate synthase was assayed as in [7]. In the case of *A. calcoaceticus*, 500 μl of the prepared extract were applied to the Mono Q column equilibrated with 20 mM triethanolamine-HCl, pH 7.7, and the column was washed with 4 ml of the same buffer. Protein elution was then achieved with a gradient of 0–0.35 M NaCl in the same buffer at a flow rate of 2 ml/min. Effluent protein was monitored at 280 nm and isocitrate dehydrogenase was assayed as in [8]. All FPLC runs were conducted at room temperature and the fractions obtained were stored on ice.

3. RESULTS AND DISCUSSION

Two research projects in this laboratory have been concerned with isoenzymes in the citric acid cycle. Earlier studies on citrate synthase from a wide range of organisms (review [9]) showed that this enzyme exists in a 'small' form (M_r ~100000) in Gram-positive bacteria and eukaryotes, and a 'large' form (M_r ~250000) in Gram-negative bacteria. Only the large enzyme displays regulatory sensitivity towards NADH and AMP [9]. Solomon and Weitzman [1] reported the unusual occurrence of both small and large forms of citrate synthase in a mutant of *Ps. aeruginosa* and found that the relative proportions of the two forms varied with the stage of growth of the bacterial culture.

In other work [3,4] we have shown that two isoenzymes of NADP-linked isocitrate dehydrogenase occur in *A. calcoaceticus* and a few other bacteria. One form has an M_r of ~300000 and is sensitive to several allosteric effectors [5,10]; the smaller isoenzyme has an M_r of ~100000 and is not subject to these allosteric controls.

The protein elution profiles obtained by FPLC of extracts of *Ps. aeruginosa* and *A. calcoaceticus* are shown in figs 1 and 2, respectively. The small and large isoenzymes of citrate synthase are particularly well separated. This excellent resolution is the more remarkable if contrasted with the elution pattern on conventional ion-exchange chromatography on DEAE-Sephacel, when only a single peak of citrate synthase was observed. Fractionation by FPLC is therefore superior. Although the separation of *A. calcoaceticus* isocitrate dehydrogenase isoenzymes by FPLC (fig.2) is not as dramatic as that of the citrate synthases, the two isoenzymes are nevertheless clearly resolved. An important feature of both these elution profiles is their reproducibility, the isoenzymes being eluted in identical positions on repeat runs. A further important feature is the high recovery of enzyme activity on FPLC; recoveries in excess of 85% were obtained with both citrate synthase and isocitrate dehydrogenase, whereas recoveries from conventional chromatography on DEAE-Sephacel were in the region of 50–60%.

Previous studies on these two isoenzyme systems have relied on classical methods of separation, particularly those exploiting the differences in molecular size, i.e. gel filtration [4,7] and zonal

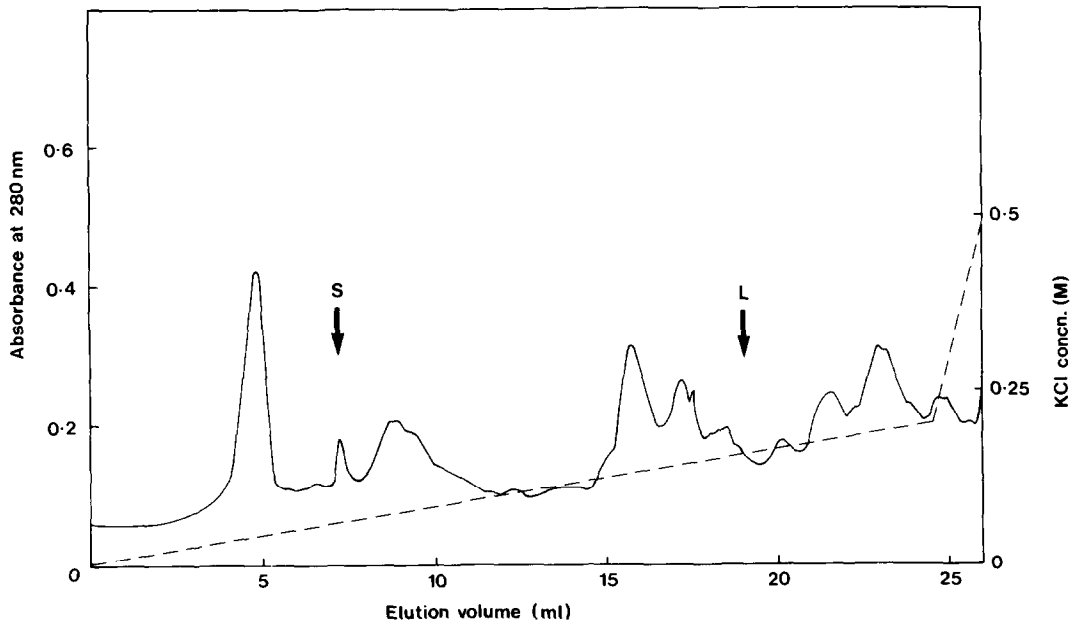

Fig.1. FPLC separation of isoenzymes of citrate synthase from *Ps. aeruginosa*. Experimental conditions are described in the text. S and L refer to the small and large isoenzymes.

centrifugation [3]. These two techniques, as well as conventional ion-exchange chromatography, are relatively time-consuming; FPLC is considerably faster. In the experiments reported here, once the salt gradient had started, elution of the isoenzymes was complete within 10–20 min; the whole FPLC procedure took ~30 min, in contrast to the several hours generally taken by gel filtration or DEAE-Sephacel chromatography. Because of the high speed of FPLC, all experiments could be done at room temperature without significant loss of enzyme activity, thereby avoiding the inconvenience of cold-room operations. The speed of FPLC also reduces the risk of problems arising from the presence of proteases in cell extracts.

Separation by gel filtration generally results in several-fold dilution of eluted enzymes, whereas the FPLC fractionation resulted in the elution of all the isoenzymes in very small volumes. One advantage of this is that relatively smaller amounts of enzyme need to be applied in FPLC in order to measure accurately the eluted activity. Another benefit is that the presence of the eluted enzyme in high concentration and with high recovery permits further examination, e.g. gel electrophoresis, to be undertaken without prior additional steps to concentrate the eluted products.

The results presented here clearly demonstrate the effectiveness of FPLC in achieving rapid and convenient separation of small amounts of isoenzymes in very high yield. We have made further use of these findings in exploring the incidence of citrate synthase isoenzymes in a range of *Pseudomonas* species [2] and in examining the roles of the two isoenzymes of *Acinetobacter* isocitrate dehydrogenase during adaptation of this organism to growth on acetate ([11]; Reeves, H.C. et al., to be published). Although there have been a few other reports of isoenzyme separation by FPLC [12–14] the technique merits greater awareness and attention. The results presented here, together with recent work from this laboratory on the separation of glutamine synthetase isoforms [15], suggest that the technique of FPLC may find wide application to the separation, on both analytical and preparative scales, of a variety of isoenzyme systems.

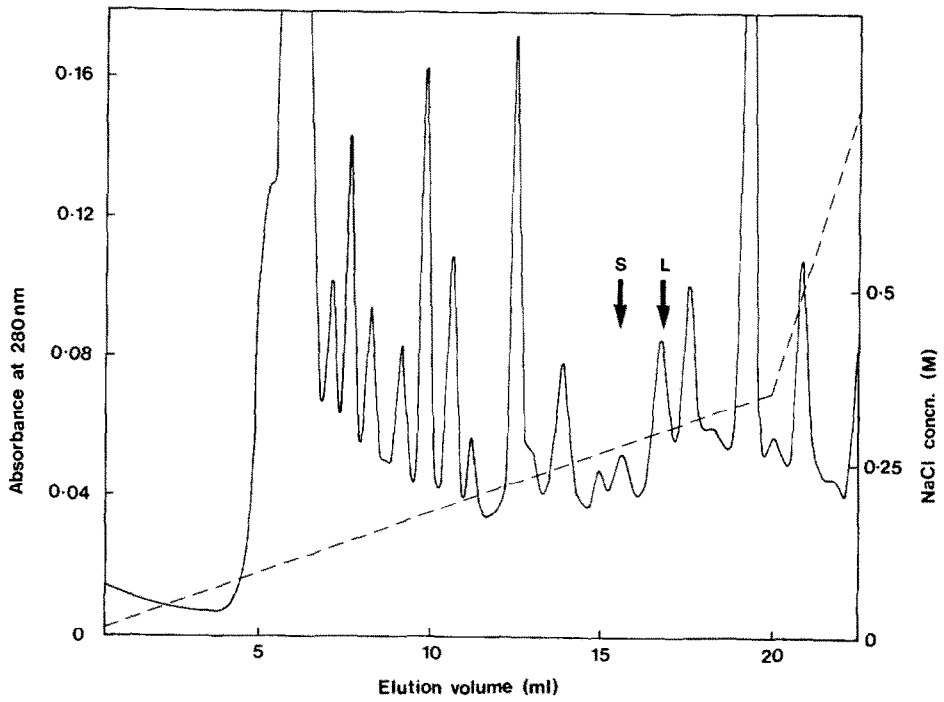

Fig.2. FPLC separation of isoenzymes of isocitrate dehydrogenase from *A. calcoaceticus*. Experimental conditions are described in the text. S and L refer to the small and large isoenzymes.

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