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NADP$^+$-SPECIFIC ISOCITRATE DEHYDROGENASE OF *ESCHERICHIA COLI*

III. TWO-STEP PURIFICATION EMPLOYING AFFINITY CHROMATOGRAPHY

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Summary

The NADP$^+$-specific isocitrate dehydrogenase (*threo*-D$_S$-isocitrate:NADP$^+$ oxidoreductase (decarboxylating), EC 1.1.1.42) of *Escherichia coli* has been purified to electrophoretic homogeneity by a two-step purification procedure employing affinity chromatography. The overall yield of enzyme was 30% with specific activity 125 μmol/min per ng protein. Electrophoretic homogeneity of the isocitrate dehydrogenase was determined in analytical polyacrylamide gels in a Tris/acetate/EDTA buffer system at pH 7.5 and in a citrate/phosphate buffer system at pH 6.0.

During the course of studies designed to investigate the in vivo regulation of NADP$^+$-isocitrate dehydrogenase (*threo*-D$_S$-isocitrate:NADP$^+$ oxidoreductase (decarboxylating), EC 1.1.1.42) in *Escherichia coli*, it became necessary to isolate electrophoretically homogeneous enzyme from milligram quantities of whole cells. The use of an NADP$^+$-affinity column has led to development of a simple, two-step procedure to isolate highly purified NADP$^+$-isocitrate dehydrogenase from crude extracts of *E. coli*. The purification procedure will be presented in this report.

Experimental Procedure

Materials. D$_S$-isocitrate (trisodium salt, *allo*-free) was purchased from Calbiochem. Agarose-hexane-nicotinamide adenine dinucleotide phosphate (5 μmol NADP$^+$ per ml of agarose), nicotine adenine dinucleotide phosphate (NADP$^+$), Tris (hydroxy methyl) aminomethane, nitroblue tetrazolium and phenazine methosulfate were obtained from Sigma Chemical Co. *N,N,N'*-tetramethyl-

ethylene-diamine (TEMED), acrylamide and *N,N'*-methylene bisacrylamide were purchased from Eastman Organic Chemicals. The dehydrated minimal salts medium, R-100, was purchased from Grand Island Biological Co.

All other inorganic and organic reagents used in this study were of Analytical Reagent grade. Distilled, deionized water (Hydro Service and Supplies, Inc.) was used to prepare all aqueous solutions.

Enzyme source. E. coli, strain K-12, was grown to late log phase in 250-ml Klett flasks containing 150 ml of minimal salts medium containing 0.5% (w/v) glucose as the carbon source. The flasks were incubated at 37° C with shaking at 250 rev./min for 8—10 h. The cells were harvested by centrifugation at 4° C at 10 000 × *g* for 10 min using a Sorvall RC2-B centrifuge. The pellet was stored at −20° C.

Preparation of crude cell-free extract. For each purification, 200 mg of frozen cells were suspended in 1.5 ml of 10 mM citrate/phosphate buffer, pH 5.0, containing 2 mM $MgCl_2$ and 10% (v/v) glycerol (hereafter referred to as, "affinity column buffer"). The suspension was sonicated in a 15-ml Corex glass tube for 5 min employing a Sonifier Cell Disruptor Model W1YOD (Heat systems - Ultrasonic, Inc.) equipped with a micro-tip. The samples were immersed in an ice-bath during sonication. Cell debris was removed by centrifugation at 27 000 × *g* at 4° C for 20 min.

Enzyme assay. Enzyme assays were conducted at 25° C in quartz cuvettes with a 1-cm light path using a Cary Model 15 recording spectrophotometer.

The activity of $NADP^+$-specific isocitrate dehydrogenase was assayed at 340 nm as described earlier [1]. One unit is that amount of enzyme which catalyzes the reduction of 1 μmol of $NADP^+$ per min.

Protein was determined by the method of Waddell [2] using crystalline bovine serum albumin as the primary protein standard.

Equilibration of the affinity column. 5 ml of agarose-hexane nicotinamide adenine dinucleotide phosphate (in 0.5 M NaCl) was diluted with 5 ml of affinity column buffer. A 1 × 15-cm column was packed with the slurry, at a flow rate of 12 ml/h with a peristaltic pump (LKB Producter AB, Sweden) and then washed with four void volumes of the buffer. Throughout the experiment, the column was maintained at a temperature of 1.5° C in a constant-temperature bath.

First passage through the agarose-hexane-$NADP^+$ column. The crude extract was applied to the column at a flow rate of 12 ml/h. The effluent was monitored for protein at 280 nm with an ultraviolet absorbance monitor and 1.2 ml fractions were collected. Non-adsorbed protein was eluted with approximately 2 void volumes of affinity column buffer. The eluted fractions were assayed for isocitrate dehydrogenase activity and the active fractions combined.

Regeneration of agarose-hexane-$NADP^+$. The column was stripped of all bound protein by washing with approximately 15 ml of 2 M KCl at a flow rate of 12 ml/h and was then re-equilibrated with 4 void volumes of affinity column buffer.

Second passage through the agarose-hexane-$NADP^+$ column. The combined, active fractions from the first passage were re-applied to the column at a flow rate of 12 ml/h and any non-adsorbed protein was eluted with affinity column buffer. When the protein concentration in the effluent returned to zero, a linear

gradient of $NADP^+$ from 0 to 2 mM was applied.

The gradient was prepared as follows: 10 ml of affinity column buffer were placed in the mixing chamber and 10 ml of 2 mM $NADP^+$ in affinity column buffer were placed in the gradient chamber of a gradient mixer. The gradient was applied at a flow rate of 12 ml/h. Isocitrate dehydrogenase was specifically eluted under these conditions.

Analytical polyacrylamide disc gel electrophoresis. Polyacrylamide disc gel electrophoresis was carried out with 7% gels polymerized using 0.085% ammonium persulfate and 0.1% TEMED as the catalysts. The buffer systems used to determine enzyme homogeneity were the citrate phosphate (pH 6) system of Reeves et al. [3] and the Tris/acetate/EDTA (pH 7.5) system reported by Caton and Goldstein [4].

Electrophoresis was carried out at room temperature at a constant current of 4 mA per gel using Bromophenol Blue as the tracking dye.

The gels were stained for protein in a 0.5% solution of Coomassie Blue in 7% acetic acid and subsequently destained in 7% acetic acid.

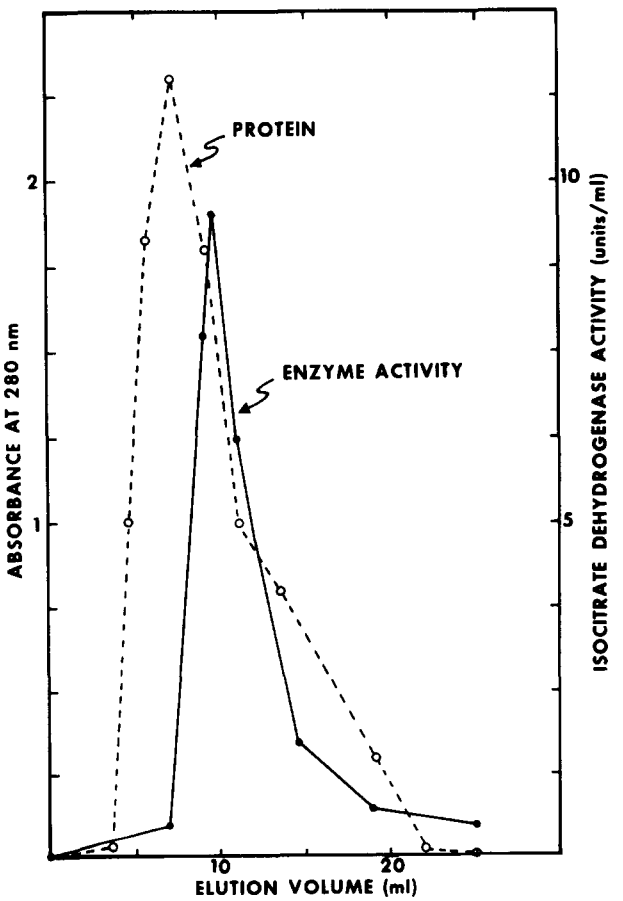

Fig. 1. Elution profile of *E. coli* crude extract from the agarose-hexane-$NADP^+$ column. Details are given in Experimental Procedure.

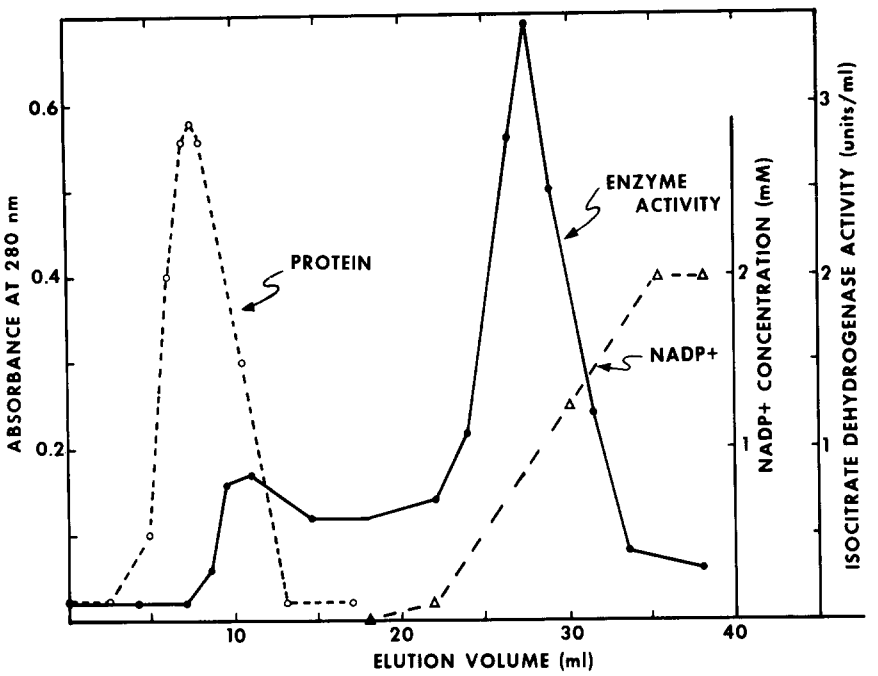

Fig. 2. Elution profile of partly purified enzyme from the agarose-hexane-NADP$^+$ column. A linear gradient of NADP$^+$ was started after 18 ml had eluted from the column. Details are given in Experimental Procedure.

Gels were stained for NADP$^+$-isocitrate dehydrogenase employing the specific enzyme stain previously described by Reeves et al. [3].

Results

When 1.5 ml of a crude sonic extract of *E. coli* K-12 containing 66 mg protein was applied to the column, the isocitrate dehydrogenase activity corresponded with the last half of the eluted protein peak (Fig. 1). 60% of the initial activity was recovered in a volume of 4 ml (Table I) and the specific activity of isocitrate dehydrogenase in the combined active fractions was 1.45.

When the active fractions from this step were applied to the regenerated affinity column under the same conditions as in the first pass, all of the isocitrate dehydrogenase activity was bound (Fig. 2) while contaminating proteins eluted

TABLE I

SUMMARY OF PURIFICATION

Step	Total protein (mg)	Total units	Specific activity	Recovery (%)	Purification (-fold)
Sonic extract	18.60	19.1	1.02	100	—
First passage	9.80	14.2	1.45	74	1.4
Second passage	0.05	6.4	125.00	34	123.0

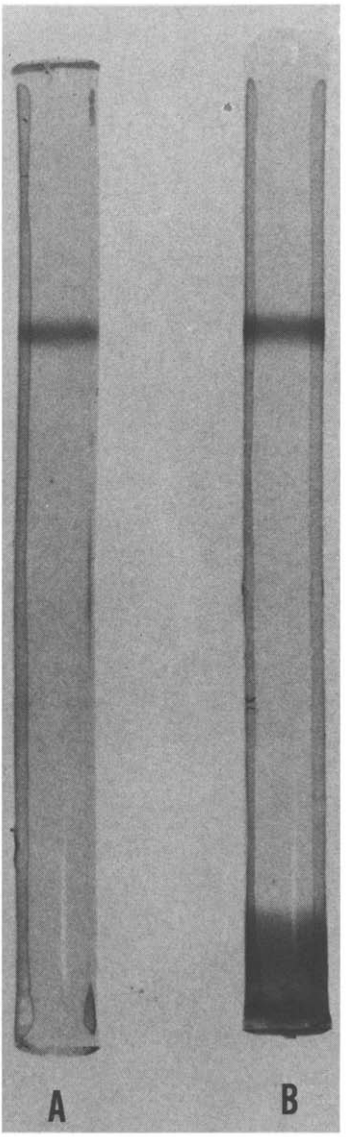

Fig. 3. Polyacrylamide gel electrophoresis of *E. coli* isocitrate dehydrogenase in the Tris/acetate buffer system. The sample applied had a specific activity of 125 units/mg protein. Gel A was stained for protein; gel B was stained for enzyme activity. Electrophoresis was towards the anode. Details are given in Experimental Procedure.

in a sharp peak. The isocitrate dehydrogenase was specifically eluted from the column employing a linear gradient of $NADP^+$. The enzyme eluted at a concentration of $NADP^+$ corresponding to 1 mM (Fig. 2). The total recovery of the purified isocitrate dehydrogenase was 30% with a specific activity of 125 (Table I).

Homogeneity of the purified enzyme was established by electrophoresis in

7% polyacrylamide gels in a Tris/acetate/EDTA system at pH 7.5 and in a citrate/phosphate system at pH 6.0. In both systems, a single protein band was obtained corresponding in position to the enzyme activity region in a duplicate gel (Fig. 3).

Discussion

Although $NADP^+$-isocitrate dehydrogenase has previously been purified in our laboratory [3], the procedure involves six distinct fractionation steps and requires several days. Further, as in most classical enzyme purifications relatively large amounts of starting material are required in order to achieve a homogeneous enzyme preparation in high yield.

The isolation of electrophoretically homogeneous enzyme from crude extracts obtained from milligram quantities of whole cells became important in other studies in our laboratory concerning the in vivo regulation of $NADP^+$-isocitrate dehydrogenase activity. Although anti-isocitrate dehydrogenase rabbit serum had previously been prepared, and the appropriate antigen-antibody complex could be readily isolated from crude sonic extracts of *E. coli* employing quantitative precipitation techniques, it was not possible subsequently to dissociate this complex and recover the pure, catalytically active enzyme. Antigen-antibody complexes have been reported to be difficult to dissociate by several workers [6—8], and often involve the use of rigorous procedures which may lead to the loss of catalytic activity.

The studies reported in this communication describe a simple, two-step procedure for the isolation of electrophoretically homogeneous $NADP^+$-isocitrate dehydrogenase. Minimal amounts of starting material are required, and the purification can easily be completed in one day. The further development of similar procedures employing affinity chromatographic techniques for the isolation of homogeneous enzyme from limited amounts of starting material will undoubtedly become increasingly significant.

Acknowledgement

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