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AFFINITY CHROMATOGRAPHY ON 2',5'-ADP-SEPHAROSE 4B FOR PURIFICATION OF MALIC ENZYME FROM CRUSTACEAN MUSCLE

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SUMMARY

Shrimp abdomenal muscle NADP-dependent malic enzyme (E.C.1.1.1.40) was purified about 1500-fold to a specific activity of 48 units (μmol/min)/mg at 30°C with good quantitative recovery in three chromatographic steps, including affinity chromatography on 2',5'-ADP-Sepharose 4B, a "substrate activation" method using malate substrate plus manganese chloride.

In addition to the malate–manganese chloride substrate pair, succinate or glutamate plus manganese chloride or magnesium chloride could be used in this "substrate activation" method for crustacean NADP-malic enzyme purification on 2',5'-ADP-Sepharose 4B.

Affinity chromatography alone purified malic enzyme almost 43 fold, and the overall method resulted in homogeneous enzyme since polyacrylamide gel electrophoresis of the native purified enzyme revealed only a single band staining for protein and enzyme activity.

INTRODUCTION

During the past decade, affinity chromatography has made great strides as a separation technology in analytical biochemistry. 2',5'-ADP-Sepharose 4B interacts strongly with NADP-dependent dehydrogenases and other enzymes which have affinity for NADP[1]. One of the singular features of the mitochondrial and cytosol NADP-dependent malic enzyme of crustaceans is the lack of binding to 2',5'-ADP-Sepharose 4B[2]. This unique feature is probably related to the structure of the binding site of the enzyme for the nucleotide. The development of substrate activation methods for affinity purification of malic enzyme from crustacean muscle has given a fast and simple purification to homogeneity. The data presented indicate that 2',5'-ADP-Sepharose 4B may be used for purification of NADP-dependent malic

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enzyme from tissues of three crustaceans (shrimp, crayfish, and lobster) by application of malic enzyme specific cofactors for increased binding capacity.

MATERIALS AND METHODS

Animals and preparation of extracts

Shrimp, *Crangon crangon*, were caught in Gdansk Bay, crayfish, *Orconectes limosus*, in the delta of Vistula River, and lobster, *Homarus americanus* were from a local supplier on Canada's eastern Atlantic coast. Abdomenal muscle extracts were prepared as described previously[3].

Enzyme and protein assays

Malic enzyme [L-malate:NADP oxidoreductase (decarboxylating) E.C. 1.1.1.40] activity at all purification steps was followed spectrophotometrically with a Specord or Pye Unicam 1800 UV–VIS spectrophotometer by observing the appearance of NADPH at 340 nm and 30°C. The standard reaction mixture contained: 50 m*M* Tris–HCl, pH 7.5, 0.5 m*M* NADP, 5 m*M* L-malate, 1 m*M* manganese chloride or 1 m*M* magnesium chloride in the case of lobster. Enzyme activities were calculated using $E^{mM}_{340} = 6.22$ for NADPH in a 1-cm light path quartz cell. Protein concentration was determined by the method of Spector[4].

Purification of malic enzyme

All steps of purification were conducted at 5°C.

Ammonium sulfate fractionation. The supernatant fraction (105 000 *g*) was adjusted to pH 7.8 by adding concentrated Tris solution. The solution was then adjusted to 40% saturation with ammonium sulfate by the gradual addition of solid ammonium sulfate. After stirring for 60 min, the suspension was centrifuged at 8000 *g* for 30 min. The resulting supernatant was then brought to 75% saturation with ammonium sulfate and stirred and centrifuged as above. The resulting precipitate was saved and dissolved in a minimum volume of extraction buffer, 10 m*M* Tris–HCl + 2 m*M* EDTA, pH 7.8 (buffer A) and dialyzed against 100 volumes of the same buffer for 24 h with two changes of buffer. Insoluble material was removed by centrifugation at 20 000 *g* for 20 min.

DEAE-Sephacel chromatography. The dialyzed enzyme solution was applied to a column (20 × 2.5 cm) packed with washed DEAE-Sephacel equilibrated with buffer A. After the protein solution had entered the column, washing was started with the buffer A at a rate of 40 ml/h. The washing procedure was continued until a protein peak, devoid of malic enzyme, but containing malate dehydrogenase and lactate dehydrogenase activities, emerged from the column. Malic enzyme activity was then eluted from the column with a linear gradient (0–0.5 *M*) of potassium chloride in buffer A.

2′,5′-ADP-Sepharose chromatography. 2′,5′-ADP-Sepharose 4B or 2′,5′-ADP-agarose was swollen and washed with buffer A, packed into a column (40 × 12 mm), equilibrated by washing with 5 m*M* malate + 5 m*M* manganese chloride in buffer A, and then used for the separation of crustacean NADP-dependent malic enzyme.

Sephacryl S-300 gel filtration. Malic enzyme eluted from the 2′,5′-ADP-Se-

pharose column was concentrated to a small volume using a collodion bag apparatus (Schleicher and Schull, Sartorius, Göttingen, F.R.G.). The enzyme was then applied to a column of Sephacryl S-300 (40 × 2.5 cm) equilibrated with buffer A. Elution was performed at a rate of 20 ml/h. Active fractions from this column were pooled and concentrated as above, and then stored in 60% glycerol in buffer A. The pure enzyme was stable for 6 months when stored −8°C in buffer A and concentrated to > 1 mg of protein/ml; the presence of glycerol was necessary in order to stabilize the enzyme.

Polyacrylamide gel electrophoresis

Polyacrylamide gels [6.3% (w/v) total acrylamide, 3.2% (w/v) of which was bisacrylamide] in a buffer of 135 m*M* tris, 45 m*M* citric acid, pH 7.0 were used to monitor malic enzyme activity under native electrophoresis. Samples were run at 35–40 V and a constant amperage (2.5 mA per tube) in the cold (2°C). Gels were stained for enzyme activity and protein with Coomassie blue. Malic enzyme activity was monitored by incubating gels in the dark at 37°C in a medium consisting of 50 m*M* Tris–HCl, 1 m*M* manganese chloride, 15 m*M* L-malate, 0.5 m*M* NADP, 0.1 mg/ml phenazine-methosulfate and 0.1 mg/ml nitro blue tetrazolium, at the final pH 7.5. Formation of the formazan precipitate corresponding to the location of the enzyme in the gel occurred within 30 min. Yellow gels were destained in distilled water and stored in 7% (v/v) acetic acid.

Reagents

L-Malic, pyruvic, succinic, glutamic, and citric acids, nitro blue tetrazolium, phenazine-methosulfate, 2′,5′-ADP-agarose, asnd bovine serum albumin were purchased from Sigma, St. Louis, MO, U.S.A. NADP, N,N,N′,N′-tetramethylenediamine, acrylamide and N,N′-methylenebisacrylamide were from Reanal, Budapest, Hungary. Tris was from Serva; DEAE-Sephacel, Sephacryl S-300, 2′,5′-ADP-Sepharose 4B, were from Pharmacia. All other chemicals were of the highest reagent quality obtainable.

RESULTS AND DISCUSSION

Table I shows the results of a typical purification of malic enzyme from shrimp abdomenal muscle. An approximately 1500-fold purification was achieved with a final yield of about 75% and a specific activity of 48 units (μmol/min)/mg. This value is close to that reported for the homogeneous NADP-malic enzyme from bovine heart mitochondria[5], rat skeletal muscle[6], *Drosphila*[7], pigeon liver[8], and rat liver[9].

Fig. 1 shows the elution profile of NADP-dependent malic enzyme from the DEAE-Sephacel column. A 20-fold purification of the enzyme with almost quantitative recovery was obtained from this step. Application of 2′,5′-ADP-affinity chromatography under the conditions described earlier[2] for crustacean NADP-malic enzyme was unsuccessful for further purification of the enzyme as the enzyme was not adsorbed on the column. However, crustacean NADP-malic enzyme in highly purified form can be obtained by application of specific cofactors to the buffer to enhance binding capacity of the enzyme for the 2′,5′-ADP-Sepharose column. The pooled NADP-malic enzyme fractions from the DEAE column were made up to 5 m*M*

TABLE I

PURIFICATION OF MALIC ENZYME FROM THE SHRIMP ABDOMEN MUSCLE

Enzyme was isolated from 80 g abdomen muscle. For other conditions see text.

Step	*Protein (mg)*	*Specific activity (μmol/min/mg)*	*Total activity (μmol/min)*	*Yield (%)*	*Purification (fold)*
Homogenate 105000 *g*	1950.6	0.032	62.4	100	1
Ammonium sulfate fractionation (40–75%)	825.6	0.080	66.1	105.8	2.5
DEAE-Sephacel chromatography	93.9	0.656	61.6	98.6	20.5
2′,5′-ADP-Sepharose 4B chromatography	1.98	28.05	55.6	89.1	876.5
Sephacryl S-300 chromatography	0.96	48.39	46.6	74.6	1512.0

malate and 5 m*M* manganese chloride by additions from concentrated stock solutions and then applied directly to a 2′,5′-ADP-Sepharose column previously equilibrated with buffer A also containing 5 m*M* malate and 5 m*M* manganese chloride. After the protein had entered the column, washing was commenced with 100 ml of buffer A containing 5 m*M* malate and 5 m*M* manganese chloride to remove any weakly adsorbed protein. Crustacean NADP-malic enzyme was then eluted by a pulse of buffer A without any additions. Fig. 2 shows the elution behavior of shrimp NADP-malic enzyme from an 2′,5′-ADP-Sepharose column in the presence of 5 m*M* malate and 5 m*M* manganese chloride. In the presence of malate or manganese chloride alone, NADP-malic enzyme passed through the column without being retained (data not shown). However, in the presence of both malate and manganese chloride, the activity was quantitatively adsorbed. Under these conditions, about 90% of the

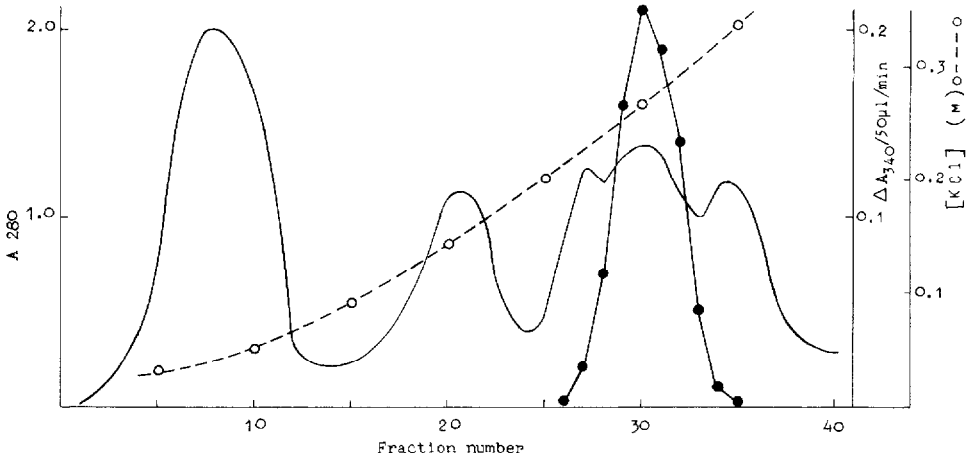

Fig. 1. DEAE-Sephacel chromatography of shrimp NADP-malic enzyme. A linear potassium chloride gradient (○-----○) formed by mixing 300 ml of buffer A with 300 ml 0.5 *M* potassium chloride in buffer A was used to elute malic enzyme activity. Effluent fractions were analyzed for their absorbancy at 280 nm (——) and for malic enzyme activity (●—●) as described under Materials and methods. Fractions of 10 ml were collected.

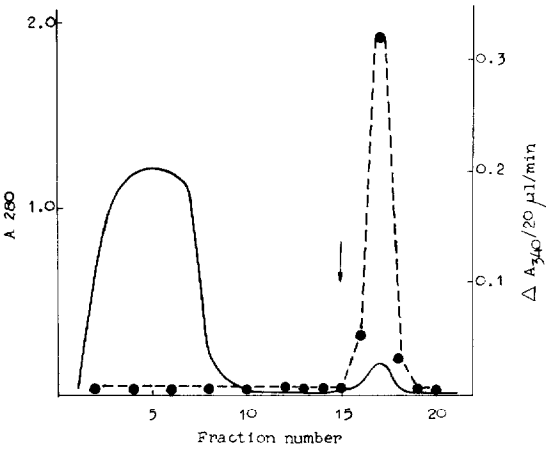

Fig. 2. 2',5'-ADP-Sepharose 4B affinity chromatography of NADP-malic enzyme partially purified on a DEAE-Sephacel column. The pooled peak activity containing 5 m*M* malate and 5 m*M* manganese chloride was applied to a 2',5'-ADP-Sepharose column equilibrated with buffer A + 5 m*M* malate + 5 m*M* manganese chloride. The column was washed with the same buffer until a protein peak was eluted. Buffer A was applied at the arrow. Column fractions (11.5 ml) were analyzed for their absorbancy at 280 nm (——) and for enzyme activity (●——●) as described under Materials and methods.

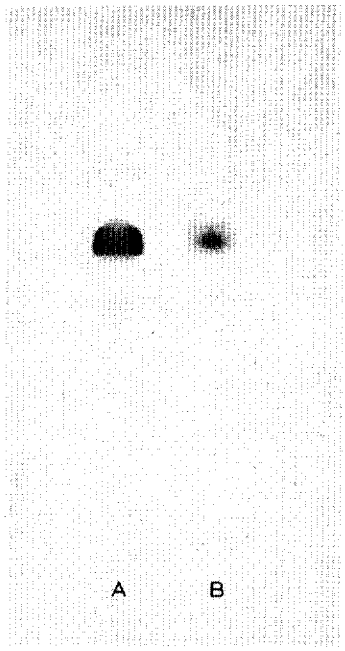

Fig. 3. Polyacrylamide gel electrophoresis of purified shrimp abdomen muscle NADP-malic enzyme. An amount of 5 µg of protein was applied to each gel. Electrophoresis was run for 12 h at 2°C. (A) Stained for enzyme activity with tetrazolium blue; (B) stained for protein. For experimental conditions see Materials and methods.

applied activity could be recovered with a purification of about 870-fold. These observations demonstrated the exceptional utility of an affinity column for binding the enzyme in the presence of its substrate and activator. For final purification, active NADP-malic enzyme fractions were pooled, concentrated as above, and passed over a Sephacryl S-300 column equilibrated with buffer A. Table I summarizes the purification scheme. Polyacrylamide gel electrophoresis of the purified shrimp NADP-malic enzyme under non-denaturating conditions is shown in Fig. 3. A single band of malic enzyme activity and of protein was observed.

In order to understand better the role of crustacean malic enzyme specific cofactors for binding capacity to 2',5'ADP-Sepharose, we tested succinate and glutamate with manganese chloride for elution behavior of shrimp NADP-malic enzyme, malate and pyruvate with manganese chloride for retention of crayfish NADP-malic enzyme and malate with magnesium chloride for lobster NADP-malic enzyme. In the presence of malate, succinate or glutamate in the presence of divalent cation crustacean NADP-malic enzyme was retained. However, in the presence of pyruvate plus manganese chloride crayfish NADP-malic enzyme passed through the column without being retained.

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