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Short Report

Purification of NADP-Malic Enzyme with the High Yield from Leaves of *Mesembryanthemum crystallinum* Exhibiting Crassulacean Acid Metabolism*

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CAM 型 *Mesembryanthemum crystallinum* の葉身の NADP-リンゴ酸酵素の高回収率精製法：斎藤和幸・縣　和一・朝倉政江・窪田文武（九州大学農学部）
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NADP-malic enzyme (L-malate : $NADP^+$ oxidoreductase [oxaloacetate-decarboxylating], EC 1.1.1.40) catalyzes the following reaction in the presence of a divalent metal ion :

$$\text{malate} + NADP^+ \rightleftarrows \text{Pyruvate} + CO_2 + NADPH.$$

NADP-malic enzymes from several plant species and tissues have been shown to exhibit large differences in kinetic and immunochemical properties. These differences have been discussed by Nishikido and Wada[8], Pupillo and Bossi[9] and Fathi and Schnarrenberger[4], and it appears that one type of NADP-malic enzyme exists in C_3, Crassulacean acid metabolism (CAM) plants and in nongreen tissues of C_4 plants and a second type exists only in green leaves of C_4 plants. The differences in properties are thought to be correlated with the fact that the NADP-malic enzymes of the two types appear to be compartmentalized in different subcellular locations.

In the inducible CAM plant *Mesembryanthemum crystallinum*[12], the significance of NADP-malic enzyme in the decarboxylation of malic acid is indicated by the considerable increases in extractable activities during the induction of CAM[5,11]. However, there are few reports on purified NADP-malic enzymes from CAM plants.

Nevertheless, we have recently purified NADP-malic enzyme from leaves of *M. crystallinum*[10], the yield was low. The present report describes a method which allows purification of NADP-malic enzyme with a high yield.

Materials and Methods

M. crystallinum plants were grown from seed as previously described[10]. CAM was induced under the water culture conditions added 400 mM NaCl for two weeks. The fully developed leaves were harvested during the late daylight period and stored at −55°C until use.

The initial steps in the purification procedure were as previously described[10], and involved preparation of crude extracts, fractionation with 50-70% ammonium sulfate and DE 52 (Whatman, Maidstone, England) chromatography. Enzymatically active fractions from DE 52 chromatography were pooled and applied to a column of hydroxylapatite (Bio-Rad Co., Richmond, California, U.S.A. ; 5 cm i.d. ×8.6 cm) that had previously been with buffer A, which contained 50 mM Tris-HCl, pH 7.3, 5 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetate (EDTA), 10% (w/v) glycerol, and 10 mM 2-mercaptoethanol. The enzyme was eluted with a linear 500-mL gradient of potassium phosphate (0-200 mM) in buffer A. The fractions with maximum

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Table 1. Purification of NADP-malic enzyme from leaves of *M.crystallinum* exhibiting CAM.

Purification step	Total volume (ml)	Total protein (mg)	Total activity (units)	Yield (%)	Specific activity (units mg)	Purification (fold)
Crude extract	1,145.0	4,022.67	241.36	100.0	0.06	1.0
Ammonium sulfate, 50~70% fraction	35.0	973.50	233.64	96.8	0.24	4.0
DE 52	62.5	116.41	168.71	69.9	0.69	11.5
Hydroxylapatite	115.5	18.46	167.12	69.2	9.05	150.8
Sepharose 6B	43.5	8.10	144.73	60.0	17.87	297.8
Blue Sepharose CL-6B	56.5	1.77	125.17	51.9	70.72	1,178.7

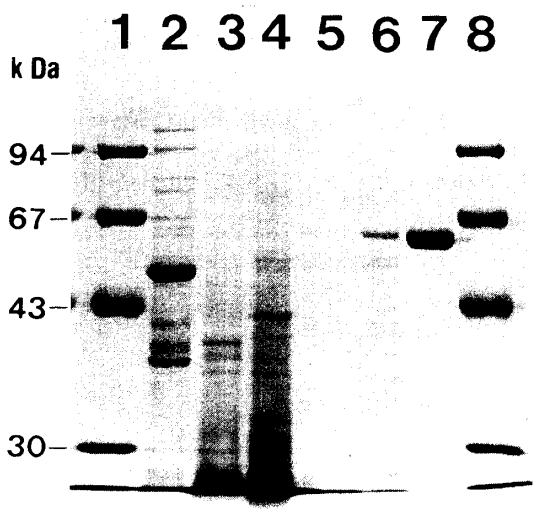

Fig. 1. Results of SDS PAGE of NADP-malic enzyme at different stages of purification from *M. crystallinum* exhibiting CAM. Lanes 1 and 8, standard proteins; lane 2, crude extract; lane 3, ammonium sulfate fraction; lane 4, after DEAE-gel ion-exchange chromatography; lane 5, after hydroxylapatite chromatography; lane 6, after gel-filtration; lane 7, after affinity chromatography.

enzymatic activity were pooled and precipitated with 75% ammonium sulfate. The precipitate dissolved in a small volume of buffer A. The concentrated solution was chromatographed on a column of Sepharose 6B (Pharmacia LKB, Uppsala, Sweden; 1.6 cm i.d. ×86 cm) which had previously been equilibrated with buffer A. The peak fractions with enzymatic activity were pooled and applied to a column of Blue Sepharose CL-6B (Pharmacia LKB; 2.6 cm i.d. ×11.6 cm) that had previously been equilibrated with buffer A. The enzyme was eluted with a linear 320-mL gradient of NADPH (0-0.65 mM) in buffer B, which contained 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.1 mM EDTA, 10% (w/v) glycerol, and 10 mM 2-mercaptoethanol. The most active fractions were pooled and stored at 4°C.

The enzyme was assayed at 30°C as previously described[10]. One unit of activity is defined as the amount of enzyme that catalyzed the reduction of 1 μmol of $NADP^+ \cdot min^{-1}$ under the conditions of the reaction.

The system developed by Laemmli[7] for SDS polyacrylamide gel electrophoresis (SDS-PAGE) was used. Analytical polyacrylamide gels containing SDS were stained with Coomassie brilliant blue R-250.

Protein concentrations were determined spectrophotometrically at 595 nm by the method of Bradford[2], with Coomassie brilliant blue G-250 (Bio-Rad Co.). A standard curve was established using bovine serum albumin.

Results and Discussion

The Blue Sepharose CL-6B chromatography was a very efficient method for purifying the enzyme from leaves of *Mesembryanthemum crystallinum* exhibiting CAM[10]. However, the large amounts of NADP-malic enzyme could not be applied to the column of Blue Sepharose CL-6B, since the large amounts of contaminating proteins were adsorbed onto the column. The efficient purification was achieved by chromatography on hydroxylapatite. The specific activity was 13-fold greater than after chromatography on DE 52. This efficient separation was due to the elution of enzymatic activity at approximately 85 mM potassium phosphate, behind most of the

other proteins. NADP-malic enzyme obtained from the column of hydroxylapatite was further purified by chromatography on Sepharose 6B. The subsequent affinity chromatography on Blue Sepharose CL-6B was also efficient. The overall purification of the enzyme from *M. crystallinum* exhibiting CAM is shown in Table 1. The enzyme was purified about 1, 180-fold to a specific activity of 71 units (mg protein)$^{-1}$ with a yield of 52%. This yield was much higher than the value reported previously[10]. The specific activity of the final preparation is of the same order as the highest reported values for the purified enzymes from C_3, C_4 and CAM plants[1,3,4,6,10]. SDS-PAGE of the final preparation obtained by affinity chromatography on Blue Sepharose CL-6B revealed a single band without any contaminating bands (Fig. 1). Futher studies are in progress to compare immunochemical properties of NADP-malic enzymes from C_3, C_4 and CAM plants using an antibody against the purified enzyme.

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