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Purification and Characterization of Cytosolic NADP Specific Isocitrate Dehydrogenase from *Pisum sativum*[1]

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ABSTRACT

Cytosolic NADP-specific isocitrate dehydrogenase was isolated from leaves of *Pisum sativum*. The purified enzyme was obtained by ammonium sulfate fractionation, ion exchange, affinity, and gel filtration chromatography. The purification procedure yields greater than 50% of the total enzyme activity originally present in the crude extract. The enzyme has a native molecular weight of 90 kilodaltons and is resolved into two catalytically active bands by isoelectric focusing. Purified NADP-isocitrate dehydrogenase exhibited K_m values of 23 micromolar for DL-isocitrate and 10 micromolar for NADP, and displayed optimum activity at pH 8.5 with both Mg^{2+} and Mn^{2+}.

NADP-isocitrate dehydrogenase (*threo*-Ds-isocitrate: NADP oxidoreductase [decarboxylating]; EC 1.1.1.42) has been studied in several different higher plants (3–5, 8, 9). Randall and Givan (9) used protoplasts as a source of organelles and concluded that in pea leaves NADP-IDH[2] was localized primarily in the cytosol (90%), to a lesser degree in the chloroplast, but not at significant levels in peroxisomes or mitochondria. The subcellular localization of NADP-IDH suggests that the two forms of the enzyme may have different functions in the metabolism of the plant. The chloroplast form of NADP-IDH is generally believed to supply 2-oxoglutarate for glutamate synthesis (3) while the cytosolic enzyme is thought to function in the movement of reducing power between the mitochondria and cytosol (7). However, little is known about the metabolic role of cytosolic NADP-IDH and its relationship with the NADP-IDH present in the chloroplast. In this study, we have purified the cytosolic NADP-IDH to homogeneity and characterized the enzyme.

MATERIALS AND METHODS

Purification of NADP-Isocitrate Dehydrogenase. *Pisum sativum* L. cv Little Marvel seedlings were grown on vermiculite in a controlled environment growth chamber (12 h illumination with an intensity of 100 $\mu E/m^2 \cdot s$, at 25°C) for 2 weeks. Leaves were cut from the seedlings and blended for 5 s with (1:3 w/v) ice-cold isolation medium (25 mM Hepes-NaOH [pH 7.6], 0.35 M sucrose, 2 mM EDTA, and 2 mM sodium isoascorbate). The homogenate was strained through eight layers of cheesecloth and centrifuged at 27,000*g* for 15 min at 4°C to remove nuclei, chloroplasts, and other organelles. Crystalline $(NH_4)_2SO_4$ was added to the supernatant with stirring, until a final concentration of 45% was achieved and was left overnight at 4°C. The precipitate was removed by centrifugation at 27,000*g* for 15 min. Additional crystalline $(NH_4)_2SO_4$ was added to the supernatant, to achieve a final concentration of 80% and was mixed overnight at 4°C. The precipitate was collected by centrifugation at 27,000*g* at 4°C and resuspended in affinity column buffer (ACB), pH 7.0 (10 mM K-phosphate, 5 mM citrate, 2 mM $MgCl_2$, 10% glycerol, 1 mM benzamidine, and 0.01% NaN_3).

The protein suspension was desalted with a Bio-Gel P-6DG column (2.5 × 8 cm) at a flow rate of 24 ml/h prior to chromatography on DEAE-Sephadex A-50. This procedure was advantageous in that most of the pigments were removed from the extract. The sample from the desalting column was applied to a DEAE-Sephadex A-50 column (2.5 × 30 cm) equilibrated with ACB. Column effluent was monitored at 280 nm using an ISCO model UA-5 absorbance monitor. The column was washed with ACB at a flow rate of 24 ml/h until the eluant was free from protein. A 400 ml linear gradient of 0 to 0.5 M KCl in ACB was used to elute NADP-IDH. Fractions containing activity were combined and dialyzed against three 1-L changes of ACB buffer.

The desalted enzyme suspension was placed onto an Amicon Dyematrix Procion Red affinity column (1.5 × 8 cm) equilibrated with ACB. The column was washed with 100 ml of ACB at a flow rate of 18 ml/h. NADP-IDH was eluted with a 50 ml linear gradient of 0 to 1 mM NADP and 0 to 25 mM isocitrate in ACB. Fractions containing enzyme activity were pooled, dialyzed at 4°C against ACB, and concentrated to a final volume of 1.0 ml using a Micro-ProDiCon model 10 concentrator. The concentrated sample was applied to a Sepharose-6B column (1.5 × 40 cm) equilibrated with ACB and eluted at a flow rate of 24 ml/h. Fractions containing NADP-IDH activity were pooled, concentrated, and stored at 4°C.

Determination of Subunit Molecular Weight. SDS-PAGE (10% slab gel) was performed in the Bio-Rad Mini Protean II vertical electrophoresis system, according to the procedure of Laemmli (6). Samples and SDS mol wt standards were mixed with an equal volume of sample treatment buffer and heated at 100°C for 10 min. Protein bands were visualized by use of the Bio-Rad silver stain. The subunit mol wt of NADP-IDH was determined from a graph of migration distance *versus* log mol wt of standard proteins.

Isoelectric Point Determination. Isoelectric focusing was performed in a vertical polyacrylamide mini-gel system developed in our laboratory (11). Analysis was performed in the pH range 3 to 10 at 4°C. The sample and standards were mixed with an equal volume of treatment buffer (60% glycerol, 4% ampholyte) and applied to the gel. The gel was stained for protein with Coomassie blue or assayed for IDH activity by the method of Reeves *et al.* (10). Migration distance of standards was plotted *versus* isoelectric point to establish the pI of the enzyme.

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[2] Abbreviations: IDH, isocitrate dehydrogenase; ACB, affinity column buffer.

Native Molecular Weight Determination. The native mol wt of NADP-IDH was determined by gel filtration chromatography with a Sepharose 6B column (2.5 × 40 cm) at a flow rate of 21 ml/h. The column was equilibrated with ACB and precalibrated with 1.2 mg each of thyroglobulin, apoferritin, β-amylase, alcohol dehydrogenase, and BSA. Elution volume of the standards was calculated and plotted *versus* log mol wt to establish a standard curve for determination of IDH native mol wt.

Determination of Michaelis Constants. Michaelis constants (K_m values) were calculated from Lineweaver-Burk double reciprocal (1/V *versus* 1/S) plots. The best fit line was derived from analyzing the data with a linear least squares program.

Enzyme Assays. NADP-IDH was routinely assayed by following the reduction of NADP at 340 nm at 25°C using a Cary model 15 recording spectrophotometer. The reaction mixture contained in 1 ml: 0.15 M Tris-HCl (pH 8.5), 1 mM $MgCl_2$, 0.66 mM NADP, and 2.5 mM DL-Na_3 isocitrate. The reaction was initiated by the addition of enzyme. One unit of activity is defined as that amount of enzyme which catalyzes the reduction of 1 μM of NADP per min, at 25°C. Specific activity is expressed as units per mg of protein.

Protein Assay. Protein concentration was determined spectrophotometrically by the Bradford method (1) at 595 nm, utilizing the Coomassie dye binding reagent of the Pierce Chemical Company. A protein standard curve was established using BSA.

RESULTS

Results of the purification are summarized in Table I. The enzyme was purified 1270-fold and the yield was greater than 50%. The purified enzyme has a specific activity of 40 units per mg protein. Analysis of the purified enzyme by SDS-PAGE and subsequent silver staining (Fig. 1) clearly demonstrates that NADP-IDH has been purified to electrophoretic homogeneity.

The native mol wt of NADP-IDH was determined to be 90 kD by gel filtration chromatography. Subunit mol wt was determined by SDS-PAGE of the purified enzyme (Fig. 1). The subunit mol wt was estimated to be 46 kD, indicating a native protein comprised of two subunits with identical mol wt.

The isoelectric point of the enzyme was determined utilizing a vertical isoelectric focusing mini-gel system, in the pH range from 3.0 to 10.0 (Fig. 2). Lanes A and B were stained for protein with Coomassie blue and lane C was excised and stained for NADP-IDH activity. The two catalytically active bands in lane C correspond to the two protein bands in lane B. Isoelectric points of the two bands were estimated to be 5.6 and 5.8, respectively. Both bands corresponded to the 46 kD band displayed by SDS gel electrophoresis, seen in Figure 1. The presence of two active bands suggest the existence of two forms of NADP-IDH in the cytosol of pea leaves.

The K_m value, calculated from Lineweaver-Burk double reciprocal plots, of NADP-IDH for NADP is 10 μM and 25 μM for DL-isocitrate.

The activity of the enzyme was determined as a function of pH by buffering the reaction mixture with either phosphate or Tris-HCl from pH 6.5 to 10. The results shown in Figure 3 indicate that the optimum pH range was quite broad, from 7.5 to 9.5.

Both Mg^{2+} and Mn^{2+} are generally considered to be cofactors for NADP-IDH. Although both metal ions may function as cofactors, results shown in Figure 3 demonstrate that at a constant enzyme concentration and optimum pH (8.5), the maximum velocity of NADP-IDH with Mg^{2+} is approximately 75% of that attained with Mn^{2+}.

DISCUSSION

The procedure described for the purification of cytosolic NADP-specific IDH from pea leaves resulted in an electrophoretically homogeneous enzyme preparation with a high recovery. The purified enzyme has a specific activity of 40 units per mg protein. A short blending time of the pea leaves was necessary to avoid breakage of the chloroplasts and other organelles. After centrifugation of the crude extract, the supernatant was essentially free of Chl, indicating that the extract had little chloroplast contamination. The use of Amicon Dyematrix affinity chromatography proved superior to other chromatographic media tested, in regards to yield as well as purification. Linear gradients containing both NADP and isocitrate were preferentially used over isocratic methods of elution and resulted in increased purity and reduced volume of active eluant.

The mol wt of 90 kD obtained by gel filtration chromatography is similar to that reported for the NADP-IDH from *E. coli* (10). Also, both enzymes consist of two subunits of identical mol wt (2). To our knowledge, no other study has reported a mol wt for cytosolic NADP-IDH isolated from higher plants.

The existence of isozymes of NADP-IDH has been reported to occur in several different higher plants (4, 5, 12). These isozymes are believed to be encoded by several unlinked loci (4). Kiang *et al.* (5) reported that there were four active loci in the cultivated soybean, none of which code for plastid NADP-IDH. Two of the four loci code for cytosol-associated isozymes of NADP-IDH while the other two code for mitochondrial forms of the enzyme. In this study, two forms of the cytosolic enzyme, with pIs of 5.6 and 5.8 are reported, both of which were found to have the same native and subunit mol wt. The protein with a pI of 5.6 is the major form. We also observed that the chloroplast fraction contained an NADP-IDH activity much lower than that of the cytosol (results not shown), which was in agreement with the results of Randall and Givan (9). The active forms of NADP-IDH present in the chloroplast and cytosolic fractions were analyzed by isoelectric focusing and a gel stain specific for NADP-IDH (10). There were no bands in the chloroplast fraction which corresponded to the bands present in the cytosolic fraction (data not shown). Rabbit antibody, raised against purified cytosolic NADP-IDH, was used to test cross-reactivity with the

Table I. *Purification of NADP-IDH from Pea Leaves*

	Total volume	Total activity	Total protein	Specific activity	Yield	Purification
	ml	*units*	*mg*	*units/mg*	*%*	*-fold*
27,000*g* supernatant	400	134	4320	0.031	100	1
45–80% $(NH_4)_2SO_4$ fractionation	18	156	910	0.17	116	5.5
DEAE-Sephadex A-50 chromatography	25	134.5	105	1.28	100	41
Affi red column	10	113	3	37.67	84	121
Sepharose 4B gel filtration	12	79	2	39.5	59	1270

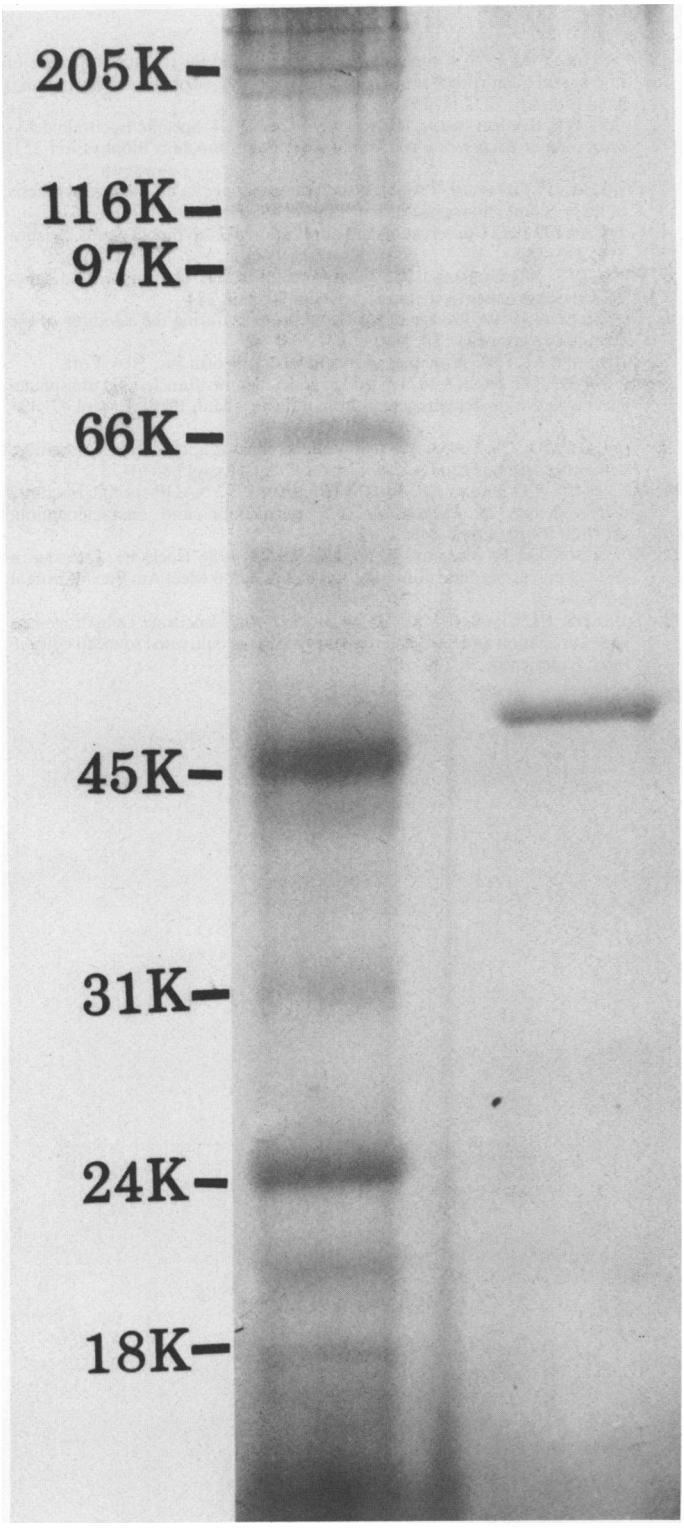

FIG. 1. Enzyme homogeneity and subunit mol wt determination by SDS-PAGE. Left lane contains 10 μg of SDS mol wt standards. Right lane contains 5 μg of purified NADP-IDH. The resolving gel contained 10% acrylamide and was stained with BIO-RAD silver reagent.

chloroplast form. Results from Ouchterlony double immunodiffusion tests indicated that the forms of NADP-IDH present in the cytosol and chloroplast were immunologically distinct and, therefore, might be encoded by different loci.

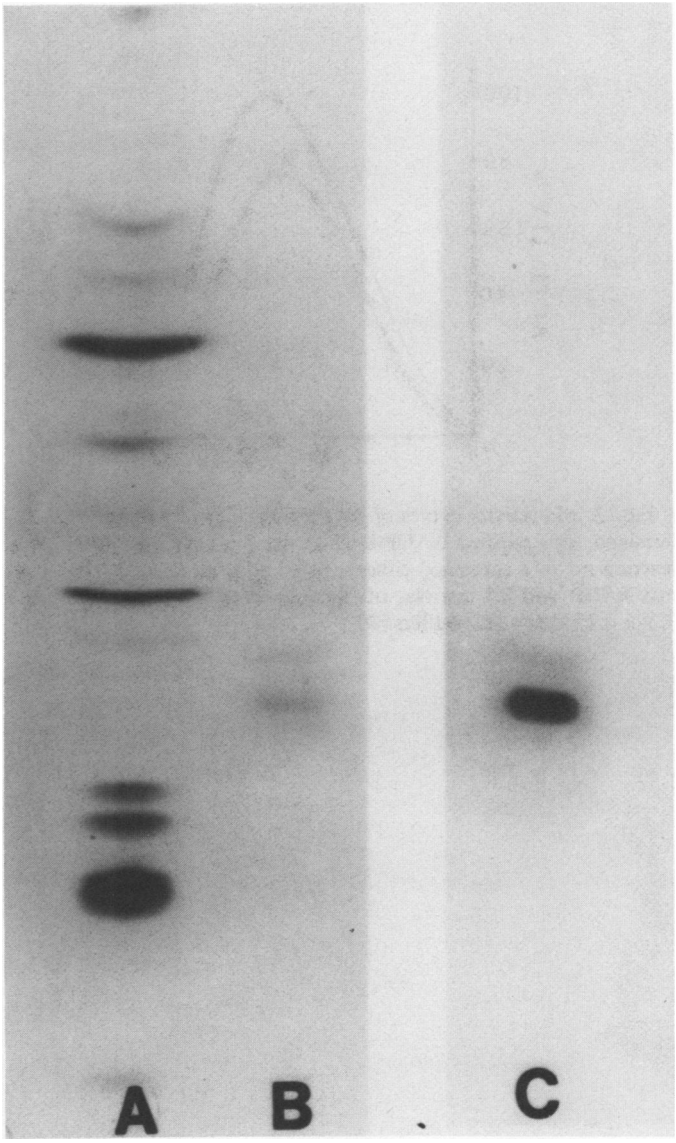

FIG. 2. Isoelectric point determination of NADP-IDH. Isoelectric focusing was performed in a vertical polyacrylamide mini-gel system. Electrophoresis was performed at 4°C in a 5% gel, at 200 V for 2 h, followed by 2 h at 400 V. Lane A contains 15 μg IEF protein standards (Serva Protein Test Mix 9). Lanes B and C contain 10 μg purified NADP-IDH; lane B was stained with Coomassie blue, and lane C was stained for NADP-IDH activity.

The K_m values of cytosolic NADP-IDH (10 μM for NADP and 25 μM for DL-isocitrate) are similar to the values reported for chloroplast NADP-IDH (11 μM for NADP and 35 μM for DL-isocitrate) (9). The cytosolic NADP-IDH has an absolute requirement for a divalent metal ion. Although both Mg^{2+} and Mn^{2+} will fulfill this requirement, the latter is a more effective cofactor *in vitro* than the former at similar concentrations. This is in agreement with reports concerning several forms of NADP-IDH from bacterial and plant sources (8–10).

In this report, cytosolic NADP-IDH was purified and characterized. We are currently conducting studies on the chloroplast form of the enzyme in order to elucidate the relationships between the two forms of NADP-IDH.

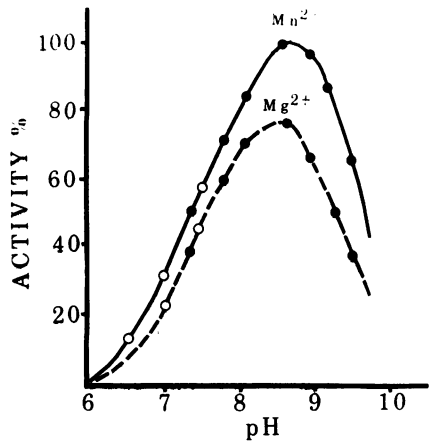

FIG. 3. pH activity curves of NADP-IDH. Enzyme activity was determined with purified NADP-IDH at the pH values indicated. The reaction mixture contained either 1 mM $MgCl_2$ or 1 mM $MnCl_2$, 0.66 mM NADP, and 2.5 mM Na_3 DL-isocitrate in either 0.25 M phosphate (○) or 0.25 M Tris-HCl buffers (●).

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