Purification by Immunoabsorption and Immunocchemical Properties of NADP-Dependent Malic Enzymes from Leaves of C<sub>3</sub>, C<sub>4</sub>, and Crassulacean Acid Metabolism Plants

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ABSTRACT

NADP:malic enzyme from corn (Zea mays L.) leaves was purified by conventional techniques to apparent homogeneity as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Antibodies raised against this protein in rabbits were purified, coupled covalently to protein A-Sepharose CL-4B, and used as an immunoaffinity resin to purify the NADP:malic enzymes of the C<sub>3</sub> plants spinach (Spinacia oleracea L.) and wheat (Triticum aestivum L.), of the Crassulacean acid metabolism (CAM) plant Bryophyllum daigremontianum R. Hamed et Perr. de la Bathie and the C<sub>4</sub> plants corn, sugarcane (Saccharum officinarum L.), and Portulaca grandiflora L. Such procedures yielded homogeneous protein preparations with a single protein band, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, except for P. grandiflora L. with two bands. The specific activities of the purified proteins ranged between 56 and 91 units (milligrams per protein). NADP:malic enzyme represented up to 1% of the total soluble protein in C<sub>3</sub> plants, 0.5% in the CAM plant, and less than 0.01% in C<sub>4</sub> plants. In immunotitration tests involving immunoprecipitation and immunoinhibition of activity by an anti-serum against the corn leaf enzyme, the NADP:malic enzymes of corn and sugarcane showed virtually full identity of epitopes, while the NADP:malic enzymes of the C<sub>3</sub> and CAM plants exhibited a cross-reaction of one-twentieth and one-fourth by these tests, respectively. The NADP:malic enzyme of P. grandiflora exhibited characteristics more closely related to the enzymes of C<sub>3</sub> and CAM plants than to those of C<sub>4</sub> plants.

The decarboxylation of malate via malic enzyme may be accomplished by NADP or NAD as cofactor. In plants at least two different enzymes are known. The NADP:malic enzyme (EC 1.1.1.40) reacts primarily with NADP, has only little activity with NAD, and needs Mg<sup>2+</sup> or Mn<sup>2+</sup> as cofactor (13, 23). The NAD:malic enzyme (EC 1.1.1.39), which is known to be mitochondrial, reacts predominantly with NAD, to about 30% of this activity with NADP, and requires also Mg<sup>2+</sup> or Mn<sup>2+</sup> as cofactor (28). Another type of NAD:malic enzyme was found in a particular subgroup of C<sub>4</sub> plants (NAD:malic enzyme subgroup). This enzyme reacts primarily with NAD, to about 5 to 30% of this activity with NADP, and operates with Mn<sup>2+</sup>, which cannot be replaced by Mg<sup>2+</sup> (14). In this paper we will refer to the NADP:malic enzyme (EC 1.1.1.40) in green leaves.

NADP:malic enzyme has an essential function in the metabolism of photosynthetic leaf tissue, although the role is considered to vary in plants with different modes of photosynthesis. Most importantly, this activity is needed in leaves of some C<sub>3</sub> plants to split malate into pyruvate and CO<sub>2</sub>. C<sub>4</sub> plants have been subgrouped, depending on which enzyme degrades C<sub>4</sub> acids, into a NADP:malic enzyme, a NAD:malic enzyme, and a phosphoenolpyruvate carboxykinase type (12). NADP:malic enzyme is present in high activity in the NADP:malic enzyme subgroup of C<sub>4</sub> plants and only in low activity in the other two subgroups.

CAM plants fix CO<sub>2</sub> during the night, mainly into C<sub>4</sub> acids, which in turn become degraded into CO<sub>2</sub> and a C<sub>3</sub> compound during the subsequent day period before CO<sub>2</sub> is refixed in the Calvin cycle. Either NADP:malic enzyme or phosphoenolpyruvate carboxykinase may accomplish this reaction (21). Again, the activity of one enzyme is high in one group of plants and low in the other, while the activity of the other enzyme is present in the opposite pattern. Finally, C<sub>3</sub> plants, which do not require NADP:malic enzyme directly for photosynthetic CO<sub>2</sub> fixation, exhibit NADP:malic enzyme activity, too, although in likewise low amounts.

NADP:malic enzymes of several plant species and tissues have been shown to exhibit large differences in kinetic properties. These differences have been discussed by Nishikido and Wada (20) and Pupillo and Bossi (23) in that one type of NADP:malic enzyme exists in C<sub>3</sub> and CAM plants and in nongreen tissues of C<sub>4</sub> plants and another type in green leaves of C<sub>4</sub> plants only. Differences may be correlated with the fact that the NADP:malic enzymes of the two types appear to be compartmented in different subcellular locations. In this respect NADP:malic enzyme was found in the chloroplasts of C<sub>4</sub> plants (15). For CAM plants, however, reports vary. In phylloclades of Opuntia ficus-indica, one isoenzyme each was reported in the cytosol, the chloroplasts, and the mitochondria (19). In Bryophyllum calycinum and Crassula lycopodioides (24) and in Mesembryanthemum crystallinum (30), it was reported mostly or totally in the cytosol. In C<sub>3</sub> plants NADP:malic enzyme was restricted to the cytosol (8, 27).

A comparative study on NADP:malic enzymes was therefore initiated to elucidate differences of these enzymes in plant species differing in their C<sub>3</sub>, C<sub>4</sub>, and CAM type of photosynthesis. It was our goal to purify the NADP:malic

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enzymes from several C₃, C₄, and CAM plants and to compare their structural and kinetic properties. Their subunit structure and immunochemical properties will be analyzed in this paper. In a subsequent study the kinetic differences of these enzymes were evaluated (M Fathi, C Schnarrenberger, unpublished results).

**MATERIALS AND METHODS**

**Plant Material**

Plants of corn (*Zea mays* L., type Badischer Landmais), sugarcane (*Saccharum officinarum* L.), and *Bryophyllum daigremontianum* R. Hamed et Perr. de la Bâthie were raised in a greenhouse. Spinach (*Spinacia oleracea* L., type Norveto) was grown in the open. *Portulaca oleracea* L., *Portulaca grandiflora* L., and wheat (*Triticum aestivum* L., type Colibri) were kept in growth chambers with daily cycles of 10 h in 5.5 kLux white light (Powerstar Lamps, Osram, HQI, 400 W) at 22°C and of 14 h in the dark at 16°C.

**Enzyme Assays**

All enzyme assays were performed at 25°C in a 1 mL reaction mixture and recorded with an Eppendorf 1101 m (Netherer and Hinz, Hamburg, FRG) at 340 nm or with a Carl Zeiss (Oberkochen, FRG) photometer at 334 nm. NADP:malic enzyme (l-malate:NADP oxidoreductase [oxaloacetate-decarboxylating], EC 1.1.1.40) from C₄ plants was assayed according to Hatch and Mau (13) in 1 mL of 50 mm Tricine/KOH (pH 8.3), 0.1 mm EDTA, 2 mm MgCl₂, 0.3 mm NADP, and 5 mm l-malate. For the assay of NADP:malic enzyme from C₃ and CAM plants, we used an assay mixture consisting of 50 mm Heps/KOH (pH 7.2), 0.1 mm EDTA, 0.5 mm MnCl₂, 0.3 mm NADP, and 5 mm l-malate (10). NAD:malic enzyme (l-malate:NAD⁺ oxidoreductase [oxaloacetate-decarboxylating], EC 1.1.1.39) was assayed in 25 mm Heps/KOH (pH 7.5), 5 mm dithioerythritol, 0.1 mm EDTA, 0.05 mm coenzyme A, 2 mm MnCl₂, 2.5 mm NAD, and 5 mm l-malate (6). One unit of activity corresponds to the reduction of 1 µmol of NAD per min. Protein was determined with the Coomassie brilliant blue G-250 reagent according to Bradford (4). If samples contained (NH₄)₂SO₄ or polyvinylpyrrolidone, proteins were treated with 2% sodium deoxycholate and precipitated with 24% TCA (2) and determined by the method of Lowry et al. (17). Salt concentrations were determined by conductivity.

**Conventional Enzyme Purification Procedures**

For purification of malic enzyme from corn the procedure by Asami et al. (1) was followed with some modifications. All handling of enzymes was performed at 0 to 4°C and, for homogenization, a buffer was used consisting of 200 mm potassium phosphate (pH 8.0), 20 mm 2-mercaptoethanol, and 0.5% (w/v) polyvinylpyrrolidone. For preparing an extract of corn and sugarcane leaves, portions of 100 g of leaf tissue without midribs were cut with scissors into 1 cm broad strips and homogenized with a five-fold excess of buffer for 1 min in a Waring Blendor (Waring Products Division, New Hartford, CT) and subsequently for 2 min in an Ultra-Turrax (Janke-Kunkel, Staufen, FRG), each at maximum speed. The crude homogenate was squeezed through cheesecloth and clarified by centrifugation for 45 min at 20,000 g in a GSA rotor of Sorvall RC 2B centrifuge (Du Pont de Nemours, Bad Nauheim, FRG). For plants other than corn and sugarcane, a twofold excess of buffer was sufficient and the homogenization with the Ultra-Turrax was omitted.

For (NH₄)₂SO₄ gradient solubilization, 400 g Celite 545 (Serva, Heidelberg, FRG) and solid (NH₄)₂SO₄ up to 70% final relative saturation were added to 4 L of crude extract. The pH was maintained at 7.0 to 8.0 with 0.5 M KOH. The suspension was settled in a 9 cm wide glass column and proteins were solubilized and eluted by a 2-L gradient of 70 to 0% (NH₄)₂SO₄ in 0.1 M potassium phosphate (pH 8.0). Fractions with more than 30% of the maximum NADP:malic enzyme activity were pooled, precipitated by 70% (NH₄)₂SO₄, resuspended, and dialyzed in 10 mM potassium phosphate and 10 mM 2-mercaptoethanol (pH 8.0).

Subsequently, the enzyme was bound to a column (3 x 50 cm) of DEAE-cellulose (DE32, Whatman, Maidstone, England) equilibrated with dialysis buffer (see above) and eluted by a linear gradient of 1200 mL of 0 to 0.5 M KCl in the same buffer. Fractions with malic enzyme activity were combined, precipitated by 70% (NH₄)₂SO₄, and dialyzed overnight. The scales of this and the previous step were changed according to the amounts of crude extracts used.

The next purification step was performed on a 3 x 14 cm column of hydroxylapatite (Bio-Rad, München, FRG) equilibrated with 10 mM potassium phosphate and 10 mM 2-mercaptoethanol (pH 8.0). After loading the proteins, NADP:malic enzyme was eluted with a 350 mL gradient of 0 to 0.4 M potassium phosphate in 10 mM 2-mercaptoethanol at pH 7.5.

The last step of purification involved chromatography on Blue-Sepharose 4B. The coupling of 2 g Cibacon Blue F3G-A (Serva, Heidelberg, FRG) onto 10 g Sepharose 4B (Pharmacia, Freiburg, FRG) was done according to the procedure by Böhme et al. (3). The resin was sedimented into a glass column of 2 x 10 cm, equilibrated with buffer (10 mM potassium phosphate and 10 mM 2-mercaptoethanol [pH 8.0]). The enzyme solution was diluted with 10 mM 2-mercaptoethanol (pH 8.0), up to a conductivity of less than 5 x 10⁻³ S and loaded onto the column. The column was washed with one volume of column buffer. Elution of NADP:malic enzyme was achieved with a linear gradient of 0 to 0.7 M KCl in column buffer. Fractions with NADP:malic enzyme activity were combined, concentrated with an Amicon ultrafiltration cell (model 203, Amicon, Lexington, MA) and Diaflo ultrafilter PM 10, and dialyzed. The purified NADP:malic enzyme was stored as a solution of 1 mg protein per mL in 10% (v/v) glycerol at -20°C.

**Immunization and Purification of Antibodies**

Prior to immunization, the purified NADP:malic enzyme was subjected to preparative SDS-PAGE in order to remove minor impurities. Two hundred µg of the purified NADP:malic enzyme with either Freund's complete or incomplete adjuvant were mixed and used for immunization of...
two rabbits by standard procedures. After collection of the antisera (400 mL) antibodies were prepurified by precipitating twice with 0 to 50% (NH₄)₂SO₄ at pH 7.5 and, after dialysis, by adsorption of other proteins to a DEAE-cellulose column equilibrated with 30 mm potassium phosphate (pH 7.5). Proteins in the flow-through fractions were concentrated in an Amicon ultrafiltration cell (PM 10 membrane) and stored in batches at −20°C.

Highly specific anti-NADP:malic enzyme IgGs² were obtained by immunoabsorption chromatography on a NADP:malic enzyme-Affigel 10 column. About 2.5 mL of wet packed Affigel 10 was coupled with 4.2 mg of purified NADP:malic enzyme in 5 mL of 0.1 M Hepes/KOH (pH 8.0), for 4 h at 4°C and further processed according to the product information manual of Bio-Rad. Then, NADP:malic enzyme-Affigel 10 was equilibrated with 40 mM Hepes/KOH (pH 8.0), and agitated gently with 200 mg of prepurified anti-malic enzyme-IgGs in 12.5 mL of 40 mM Hepes/KOH (pH 8.0), for 4 h. The material was sedimented into a glass column (1 x 6.5 cm) and washed with 50 mL of 0.4 M NaCl in 40 mM Hepes/KOH (pH 8.0), to remove unbound IgGs. Specific anti-NADP:malic enzyme IgGs were eluted with 4 mM MgCl₂ in 100 mM Hepes/KOH (pH 8.0), at a flow rate of 30 mL h⁻¹ and dialyzed immediately against 2 L of 20 mM Hepes/KOH (pH 8.0), for 2 h and against 2 x 2 L PBS overnight. The IgGs were concentrated to 1 mg mL⁻¹ with an Amicon ultrafiltration cell (PM 10 membrane) and stored at −20°C.

### Purification of NADP:Malic Enzyme by Immunoabsorption

For the preparation of a resin, 10.6 mg of specific anti-NADP:malic enzyme IgG in 20 mL PBS was coupled covalently to 3 mL of wet packed protein A-Sepharose CL-4B (Pharmacia/Freiburg) according to Gersten and Marchalonsis (11). For removal of free binding sites, the gel was incubated overnight in 0.1% (w/v) BSA in PBS. Excess proteins were removed by washing the gel in a glass column with 30 mL 3 mM MgCl₂ in 100 mM Hepes/KOH (pH 8.0), and with 150 mL 20 mM Hepes/KOH (pH 8.0). Aliquots (0.4 mL) of the gel material were stored at 4°C in 20 mM Hepes/KOH (pH 8.0), and 0.02% mercaptoethanol. The latter was removed by washing with 40 mM Hepes/KOH (pH 8.0) prior to use.

NADP:malic enzyme (enriched by (NH₄)₂SO₄ gradient elution and chromatography on DEAE-cellulose) was applied to a column (0.4 mL) of anti-NADP:malic enzyme-IgG-protein A-Sepharose CL 4B equilibrated with 40 mM Hepes/KOH (pH 8.0). Unbound NADP:malic enzyme was reapplied to the column once again. The protein concentration was limited to 4 mg mL⁻¹ maximum to prevent plugging. The flow rate was 50 mL h⁻¹. The column was washed with 20 mL of 0.4 M NaCl in 40 mM Hepes/KOH (pH 8.0), and NADP:malic enzyme was eluted with 10 mL of 4 mM MgCl₂ in 100 mM Hepes/KOH (pH 8.0). The eluate was dialyzed immediately against 20 mM Hepes/KOH and 10 mM 2-mercaptoethanol (pH 8.0). The dialysate was concentrated with an Amicon ultrafiltration cell (PM 10 membrane) and stored in 10% (v/v) glycerol at −20°C.

² Abbreviation: IgG, immunoglobulin G.

### Immunochemical Assays

For immunoprecipitation, 50 μL of NADP:malic enzyme, 50 μL enriched antibodies, and 20 μL immunoprecipitin (12% wet packed, formalin fixed Staphylococcus aureus Cowan I cells [Bethesda Research Laboratories, Neu Isenburg, FRG]) were mixed in a 1.5 mL Eppendorf reaction vessel and incubated for 10 min at room temperature. After centrifugation for 1 min at 5400g, an aliquot of 25 μL supernatant was used to determine the activity of NADP:malic enzyme. For immuno-inhibition studies the activity of NADP:malic enzyme was assayed in the presence of enriched antibodies (or serial dilutions of it).

### Electrophoresis

Disc-polyacrylamide-gel electrophoresis under non-denaturating conditions was performed according to Davis (9) in slab gels with a 14 x 8 x 0.1 cm separation gel and a 3 x 8 x 0.1 cm collection gel on top at 80 to 120 V for 2 to 3 h. SDS-PAGE was done using the system developed by Laemmli (16). Samples were treated with 10 mM 2-mercaptoethanol, 1% SDS for 2 min in a boiling water bath prior to electrophoresis. Proteins were visualized by treatment with 0.07% (w/v) Coomassie brilliant blue R-250 (Serva, Heidelberg) in 50% methanol and 10% acetic acid for 45 min and destaining with 5% methanol and 10% acetic acid.

### Molecular Masses

Molecular masses of native enzymes were estimated by velocity sedimentation centrifugation in 5 mL gradients of 5 to 20% (w/w) sucrose in 20 mM potassium phosphate and 1 mM dithiothreitol (pH 7.5), according to Martin and Ames (18). Markers were catalase (240 kD), glucose-6-phosphate dehydrogenase (116 kD), and hexokinase (51 kD). Centrifugation was performed for 13 h at 270,000 g in a SW 41 Ti rotor of a Beckman L2/65 ultracentrifuge. Subunit molecular masses were determined by SDS-PAGE (16) with phosphorylase a (95 kD), human serum albumin (69 kD), carbonhydrase (31 kD), and Cyt c (12.5 kD) as markers.

### RESULTS

To purify NADP:malic enzyme from a variety of plants, we used a specific immunoabsorbent method as an essential step in the procedure. We therefore purified the NADP:malic enzyme of corn leaves according to a modified conventional procedure described by Asami et al. (1). A typical purification protocol is given in Table I. This procedure resulted in an almost homogeneous protein preparation as visualized by SDS-PAGE (Fig. 1).

Such a preparation was further purified by preparative SDS-PAGE and used for immunization of rabbits. In an Ouchterlony double diffusion test, the respective antiserum showed a single and confluent precipitation line with either purified NADP:malic enzyme or with an enzyme preparation enriched 15-fold by chromatography on DEAE-cellulose. During immonoelectrophoresis, only a single precipitation line was observed with either probe (data not shown). Specific anti-
bodies were purified by conventional methods and by
immunoabsorption onto purified NADP:malic enzyme im-
obilized to Affi-Gel 10. During the latter step, the amount of
protein was reduced by 97.9% while the antibodies became
highly enriched (see Table II).

The highly purified antibodies against corn-leaf
NADP:malic enzyme were linked covalently to a protein A-
Sepharose CL 4B and used as an immunoabsorbent to purify
NADP:malic enzyme from corn and several other plants. To
eliminate other interfering materials (polyphenols, membrane
particles, etc.), it was necessary to prepurify the NADP:malic
enzymes by (NH₄)₂SO₄ gradient solubilization and anion-
exchange chromatography on DEAE-cellulose. NADP:malic
enzymes of such preparations could be bound to the immu-
noadsorbent with an efficiency of 28 to 62%. The remaining
activity was recovered in the eluate. The bound enzyme could
be eluted by 4 mM MgCl₂ with a recovery of 27 to 45% relative
to the amount adsorbed to the column. Table III contains the
protocols for the purification of the enzymes from the C₃
plants spinach and wheat, from the CAM plant Bryophyllum
daiquirimontianum, and from the C₄ plants corn, sugarcane,
and Portulaca grandiflora. From Figure 2 it can be seen that
such enzyme preparations were homogeneous, showing a
single peptide, except for P. grandiflora which had two pep-
tides in a ratio of about one to one.

The recovery of only 27 to 45% of the bound activity from
the immunoabsorbent was largely due to the fact that elution
was only achieved by high salt concentrations (4 mM MgCl₂)
which, in part, inactivated the enzyme. Two mM MgCl₂ eluted
only half the activity of what 4 mM MgCl₂ did. It was therefore
necessary to use 4 mM MgCl₂ for elution and to remove MgCl₂
from the eluate immediately. Elution by other means was
ineffective, i.e. by aqueous bidestillata, by pH 4.0, pH 10.0, or 3
m sodium rhodanide, pH 7.4. It is remarkable to note that
the NADP:malic enzyme from P. oleracea was not bound to the
immunoabsorbent at all (see Table III bottom).

The specific activities of the purified NADP:malic enzymes
of the six species ranged from 56 to 91 units (mg protein)⁻¹
with the trend that the specific activities of the enzymes from
C₃ and CAM plants were somewhat lower than the specific
activities of the enzymes from C₄ plants.

To determine the immunochemical cross-reaction of the
NADP:malic enzymes from species of C₃, C₄, and CAM
plants, we used enriched antibodies against the corn leaf
NADP:malic enzyme for immunoinhibition in a specific immu-
noabsorption procedure with a constant amount of pu-
rified NADP:malic enzyme (Fig. 3). In another immuno-
inhibition test, the inhibition of activity was determined in the
presence of antibodies (Fig. 4). In both sets of experiments,
the titers for 50% residual activity or activity inhibition were
almost equally high for the enzymes of the C₄ plants corn and
sugarcane (Table IV). However, the titers were only 3 to 6% in
the immunoprecipitation assay and 17 to 33% in the immuno-
inhibition assay, respectively, with the NADP:malic

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**Table I. Purification Protocol of NADP:Malic Enzyme from Green Corn Leaves**

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific Activity</th>
<th>Purification</th>
<th>Recovery</th>
</tr>
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<tr>
<td></td>
<td>mg</td>
<td>units</td>
<td>units mg⁻¹</td>
<td>-fold</td>
<td>%</td>
</tr>
<tr>
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<td>1.2</td>
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<td>100</td>
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<tr>
<td>(NH₄)₂SO₄ gradient solubilization</td>
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<td>7.3</td>
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<td>62</td>
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<tr>
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<td>1.4</td>
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<td>Blue-Sepharose</td>
<td>5</td>
<td>0.45</td>
<td>93.7</td>
<td>77</td>
<td>9</td>
</tr>
</tbody>
</table>

**Table II. Enrichment of Anti-Malic Enzyme-IgGs**

The presence of anti-malic enzyme-antibodies was assayed by
immunoprecipitation of 0.1 unit corn-leaf-NADP:malic enzyme (15-
fold enriched by chromatography on DEAE-cellulose) with 15 μg
protein of antibody preparation and by determining subsequently the
malic enzyme activity which was not precipitated. Each value repre-
sents the average of doubelets. Controls with preimmune serum or
without S. aureus Cowan I cells showed no precipitation of malic
enzyme (data not shown).

<table>
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<th>Purification Step</th>
<th>Residual Activity</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units mL⁻¹</td>
<td>mg</td>
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<tr>
<td>Control without antisera</td>
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<tr>
<td>Crude antisera</td>
<td>0.80</td>
<td>16 400</td>
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<tr>
<td>(NH₄)₂SO₄ precipitation</td>
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<td>2 600</td>
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<tr>
<td>DEAE-cellulose</td>
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<tr>
<td>Malic enzyme-Affi-gel 10</td>
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</table>
enzymes of the C₃ plants spinach and wheat and of the CAM plant B. daigremontianum as compared with the titer of the NADP:malic enzyme from corn leaves. The enzyme of the C₄ plant P. grandiflora behaved rather like the enzymes of the C₃ and CAM plants.

The molecular masses of the native NADP:malic enzymes were determined for the enzymes of corn, spinach, and B. daigremontianum as representatives for C₄, C₃, and CAM plants by velocity sedimentation centrifugation in a sucrose gradient (Fig. 5). These analyses indicated molecular masses of 220 kDa for the enzymes from corn and B. daigremontianum and of 250 kDa for the enzyme from spinach. The molecular masses of the subunits of all purified NADP:malic enzymes were estimated to be 64 to 68 kDa in size except the enzyme of P. grandiflora which showed two peptides of 67 or 69 kDa. From this one may conclude that all NADP:malic enzymes are homotetrameric proteins under native conditions except the enzyme of P. grandiflora which could also be a heterotetrameric protein.

**DISCUSSION**

The purification of NADP:malic enzyme by immunoaffinity, as applied in the course of this paper, proved highly efficient and reliable with the enzymes of several C₄, CAM, and C₃ plants. Five out of six enzyme preparations were obtained with a single protein band as judged by SDS-PAGE. Only the enzyme of the C₄ plant Portulaca grandiflora showed two bands. This was observed in two independent experiments. One reason for this finding could be that one of the two bands is an impurity. Alternatively, the NADP:malic enzyme in this species could consist of two different subunits or there could be two NADP:malic enzymes. Nevertheless, all these NADP:malic enzymes appear to be of tetrameric structure when compared with the mass of the native proteins as determined for corn, spinach and B. daigremontianum.

Purification of NADP:malic enzymes from plants by non-conventional methods have already been described when enzymes of grapes (26) and corn (7) were bound to ADP-Sepharose and eluted with NADP/NaCl or NADP. Similarly, the enzyme of rat liver could be bound to Procion Red-Agarose and be eluted with NADP/NaCl (31). Attempts in our laboratory to purify the corn leaf NADP:malic enzyme by affinity chromatography on ADP-Sepharose or Blue-Sepharose, failed since it was not possible to elute bound enzyme with NADP/NaCl or NADP.

The antibodies against the corn leaf NADP:malic enzyme
crossreacted sufficiently with the enzymes of the other five plants to act as immunoadsorbent. It is remarkable that the NAD:malic enzyme from the NAD:malic enzyme type of C₄ plant, *P. oleracea*, failed to do so. Therefore, this enzyme must be immunochemically very different from the NAD:malic enzyme.

The specific activities of the purified malic enzymes from leaves of the six different C₃, C₄, and CAM plants are on the order of 56 to 91 units (mg protein)⁻¹. These values are in the same order as the highest reported values, e.g. for the purified enzymes of corn leaves (1, 22) and *B. tubiflorum* (5). It is not clear to what extent, if at all, the values in our preparations were affected by MgCl₂ inactivation during elution from the immunoadsorbent resin.

It is interesting to note from our data that the degree of purification over crude extracts correlates closely with the fact that the activity of NAD:malic enzyme is considerably lower in C₃ plants than in C₄ and CAM plants known to have such high activities (12, 21). Thus, NAD:malic enzyme represents

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**Table IV. Percent Cross-Reaction of NADP:Malic Enzyme from Sugarcane, P. grandiflora, Wheat, Spinach, and B. daigremontianum with the Corn Leaf Enzyme as Determined by Immunoprecipitation in an Immunoprecipitation Assay or by Inhibition of Enzyme Activity by Enriched IgGs against the Corn Leaf NADP:Malic Enzyme**

<table>
<thead>
<tr>
<th>Source of Enzyme</th>
<th>Percent Cross-Reaction</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td></td>
<td>Inhibition of activity</td>
</tr>
<tr>
<td>Corn</td>
<td>100</td>
</tr>
<tr>
<td>Sugarcane</td>
<td>79</td>
</tr>
<tr>
<td><em>P. grandiflora</em></td>
<td>6</td>
</tr>
<tr>
<td>Wheat</td>
<td>6</td>
</tr>
<tr>
<td>Spinach</td>
<td>3</td>
</tr>
<tr>
<td><em>B. daigremontianum</em></td>
<td>4</td>
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</tbody>
</table>
intracellular compartmentation of NADP:malic enzymes in differing plant tissues and species appear to be contradictory. On a simplified basis one may suppose a localization of NADP:malic enzyme in the chloroplasts of C₄ plants and in the cytosol of C₃ and CAM plants (for references see introduction). Comparing this with other cell compartment specific isoenzyme systems like the cytosol and plastid isoenzymes of sugar phosphate metabolism (25), one would expect very low cross-reactivity of NADP:malic enzymes even if compartmented differently in different plant species. The immunological differences between the NADP:malic enzymes of the C₄ plants corn and sugarcane and the NADP:malic enzymes of the C₃ and CAM plants seem to be in accord with such a view. The NADP:malic enzyme of the C₄ plant P. grandiflora does not fit this view. This enzyme displayed an immunological cross-reaction more like the enzymes of the C₃ and CAM plants than that of the C₄ plants. This finding, however, could be erroneous since protein was used as a basis for both immunotitration tests. This may not have been appropriate when one of the two protein bands in the SDS gels were not immunologically reactive. If this were the case, the enzyme of P. grandiflora would be immunologically between C₃ and CAM plants and the two other C₄ plants. It is therefore hard to judge whether the NADP:malic enzymes of C₄ plants form a group of immunologically closely defined enzymes or not.

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LITERATURE CITED


Figure 5. Velocity sedimentation of NADP:malic enzymes (dashed lines) from corn (top), spinach (middle), and B. daigremontianum (bottom). Markers used were (a), catalase; (x), glucose-6-phosphate dehydrogenase; and (O), hexokinase.