Purification of NAD-Dependent Mannitol Dehydrogenase from Celery Suspension Cultures

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Mannitol dehydrogenase, a mannitol:mannose 1-oxidoreductase, constitutes the first enzymatic step in the catabolism of mannitol in nonphotosynthetic tissues of celery (Apium graveolens L.). Endogenous regulation of the enzyme activity in response to environmental cues is critical in modulating tissue concentration of mannitol, which, importantly, contributes to stress tolerance of celery. The enzyme was purified to homogeneity from celery suspension cultures grown on D-mannitol as the carbon source. Mannitol dehydrogenase was purified 589-fold to a specific activity of 365 pmol h⁻¹ mg⁻¹ protein with a 37% yield of enzyme activity present in the crude extract. A highly efficient and simple purification protocol was developed involving polyethylene glycol fractionation, diethylaminoethyl-anion-exchange chromatography, and NAD-agarose affinity chromatography using NAD gradient elution. Sodium dodecyl sulfate gel electrophoresis of the final preparation revealed a single 40-kD protein. The molecular mass of the native protein was determined to be approximately 43 kD, indicating that the enzyme is a monomer. Polyclonal antibodies raised against the enzyme inhibited enzymatic activity of purified mannitol dehydrogenase. Immunoblots of crude protein extracts from mannitol-grown celery cells and sink tissues of celery, celeriac, and parsley subjected to sodium dodecyl sulfate gel electrophoresis showed a single major immunoreactive 40-kD protein.

Mannitol occurs in more than 70 higher plant families (Zimmermann and Ziegler, 1975; Lewis, 1984; Thompson et al., 1986; Bieleski, 1982) and yet relatively little is known about the synthesis or catabolism of this six-carbon acyclic alcohol. In celery (Apium graveolens L. var dulce [Mill.] Pers.) and privet (Ligustrum vulgare [Mill.]), the biosynthesis of mannitol occurs in the cytosol of leaf photosynthetic mesophyll cells through the NADPH-dependent reduction of Man-6-P mediated by the enzyme M6PR (Rumpho et al., 1983; Loescher et al., 1992). Mannitol functions as a phloem-translocated photosynthetic source of energy and in sink tissues, is catabolized for entry into central metabolism by MTD, which is an NAD-dependent mannitol:Man 1-oxidoreductase (Stoop and Pharr, 1992). This enzyme is unique among known mannitol dehydrogenases in that it catalyzes the oxidation of mannitol to Man, whereas all previously known mannitol dehydrogenases from lower organisms are 2-oxidoreductases that oxidize mannitol or mannitol-1-P to Fru or Fru-6-P, respectively.

Recent evidence unequivocally demonstrates that mannitol plays an important role in alleviating osmotic and salinity-induced stress in plants (Tarczynski et al., 1993; Everard et al., 1994; Stoop and Pharr, 1994a, 1994b). This is particularly evident from studies of transgenic tobacco plants engineered to produce mannitol (Tarczynski et al., 1992). This species does not normally contain mannitol. Plants transformed to produce mannitol (Tarczynski et al., 1992) acquire tolerance to salinity (Tarczynski et al., 1993). Furthermore, it has been shown that mannitol catabolism in sink tissues of celery, a plant normally producing and translocating mannitol, is strongly down-regulated in response to osmotic and salinity stress (Stoop and Pharr, 1994b), whereas the capacity of leaves to photosynthetically incorporate CO₂ into mannitol is unaffected by salinity stress (Everard et al., 1994). The reduced catabolism of mannitol is due specifically to reduced MTD activity in growing sinks throughout the plant (Stoop and Pharr, 1994b). Under environmental stress, constant mannitol production (Everard et al., 1994) and reduced mannitol consumption (Stoop and Pharr, 1994) result in accumulation of mannitol throughout the plant. Thus, MTD is a critical enzyme in regulating tissue mannitol concentration during periods of environmental stress.

During periods when celery plants are not subjected to stress, mannitol, along with phloem-translocated Suc, is used as a carbon source for growth and energy in sink tissues. Recent evidence demonstrated that celery cells growing in culture utilized mannitol more effectively than Suc for dry weight gain (Pharr et al., 1995). All of these facts provide compelling reasons to engineer plants to produce and utilize mannitol to determine whether these apparent advantages can be incorporated into other species of plants. As a step in that direction, this work was undertaken to purify MTD to homogeneity and to obtain poly-

Abbreviations: ADH, alcohol dehydrogenase; FPLC, fast protein liquid chromatography; MALDI, matrix-assisted laser desorption ionization MS; M6PR, Man-6-phosphate reductase; MTD, mannitol dehydrogenase, also mannitol 1-oxidoreductase; PMI, phosphomannose isomerase.

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clonal antibodies directed against the enzyme for use in cloning of the Mtd gene.

**MATERIALS AND METHODS**

**Chemicals and Plant Material**

Biochemicals, PEG, NAD-agarose resin N-1008, and Triton X-100 were purchased from Sigma. Bisacrylamide, precasted SDS-PAGE molecular mass standards, and protein assay reagent were obtained from Bio-Rad. Unstained low and high molecular mass standards were purchased from GIBCO-BRL. Nitrocellulose membranes used for immunoblot analysis were supplied by Micron Separations, Inc. (Westboro, MA). Fractogel EMD DEAE (M) ion-exchange resin, Superose 12 HR 10/30, and gel filtration molecular mass standards were obtained from Pharmacia. Alkaline phosphatase-linked anti-IgG was purchased from Promega, as were chemicals for visualization. All buffers used for FPLC were degassed with helium and filtered through 0.22-μm MF-Millipore type GS membranes and used for FPLC were degassed with helium and filtered through 0.22-μm MF-Millipore type GS membranes and gel filtration molecular mass standards were obtained from Pharmacia. Alkaline phosphatase-linked anti-IgG was purchased from Promega, as were chemicals for visualization. All buffers used for FPLC were degassed with helium and filtered through 0.22-μm MF-Millipore type GS membranes and adjusted to the respective pH at 25°C. Celery (Apium graveolens L. var dulce [Mill.] Pers.) suspension cultures were maintained by subculture every 14 d on Murashige-Skoog (Murashige and Skoog, 1962) medium with mannitol as the carbon source (Stoop and Pharr, 1993).

**MTD Purification**

**Crude Extract**

All purification procedures were carried out at 4°C. Celery suspension cultures were harvested 4 d after subculture. Cells were vacuum filtered on Whatman No. 1 paper, rinsed with deionized H2O, immediately frozen in liquid nitrogen, and stored at −80°C until used for extraction. Cells were ground with a chilled Polytron using a 1:4 tissue to buffer ratio. The extraction buffer contained 50 mM Mops (pH 7.5), 5 mM MgCl2, 5 mM DTT, 1 mM EDTA, 1% Triton X-100, 1 mM PMSE, and 13% (w/v) Dowex-I anion exchanger. Homogenates were centrifuged at 20,000g for 20 min at 4°C. Supernatant fractions were pooled and desalted as the crude extract.

**PEG Fractionation**

Crude extract was brought to 15% (w/v) saturation with PEG (average molecular mass 8 kD; 50% [w/v] dissolved in 50 mM Mops [pH 7.5], 5 mM MgCl2, 5 mM DTT) at 4°C, gently stirred for 1 h, and centrifuged at 20,000g for 20 min. The supernatant was retained and brought to 30% (w/v) saturation by further addition of PEG and stirred for an additional 1 h. After the sample was centrifuged as above, the supernatant was discarded and the pellet was suspended in a minimum volume of 20 mM Mops (pH 7.5), 2 mM MgCl2, and 1 mM DTT.

**DEAE Ion-Exchange Chromatography**

The dissolved pellet was applied at 4 mL/min onto a Fractogel EMD DEAE (M) ion-exchange column (5.31 × 26 cm, 138-mL bed volume) equilibrated with 20 mM Mops (pH 7.5), 2 mM MgCl2, and 1 mM DTT (buffer A). The column was connected to an FPLC system (Perkin-Elmer Cetus), and washed with buffer A until the A285 decreased to baseline levels. Proteins were eluted with a linear 0 to 400 mM KCl gradient (752 mL) in buffer A. The flow rate was 4 mL/min, and 12-mL fractions were collected. Fractions containing MTD eluted at approximately 125 mM KCl. Fractions that were free of ADH and PMI were pooled. The pooled DEAE fraction was then concentrated and desalted by buffer exchange in 20 mM Mops at pH 7.5 containing 1 mM DTT using Centricon-10 centrifugal concentrators (Amicon, Beverly, MA).

**NAD-Agarose Affinity Chromatography**

The concentrated, desalted DEAE fraction was loaded onto a 28-mL (1.54 × 18 cm) NAD-agarose column (Sigma catalog No. N1008) equilibrated with 20 mM Mops (pH 7.5) containing 1 mM DTT (buffer B). The column was connected to an FPLC system, and proteins were eluted with a 60-mL linear gradient of 0 to 0.6 mM NAD in buffer B. The flow rate was 1 mL/min, and 2-mL fractions were collected. Peak activity fractions containing MTD eluted at approximately 0.19 mM NAD. Individual active fractions were subjected to native and SDS-PAGE, and only fractions containing a single 40-kD peptide on an SDS gel and a single peptide on a native gel were pooled. MTD activity remained constant for at least 5 months when the final preparation was stored at −80°C. In initial purifications, proteins were eluted from the NAD-agarose column by a single 2 mM NAD pulse.

**Enzyme Activity and Protein Assays**

Crude extracts were desalted by centrifugal filtration on a Sephadex G-25–50 column equilibrated with 50 mM Mops-NaOH (pH 7.5) containing 1 mM DTT prior to assay for MTD activity. Fractions from purification steps were desalted in 20 mM Mops (pH 7.5) containing 1 mM DTT. MTD activity was measured by monitoring the reduction of NAD spectrophotometrically at 340 nm. The assay mixture contained 100 mM Bis-Tris propane (pH 9.0), 2 mM NAD, enzyme extract, and 150 mM D-mannitol in a total volume of 1 mL. The reactions were initiated with D-mannitol. The presence of MTD activity in column fractions during purification was also assayed using a protocol adapted for a UV-max kinetic microplate reader (Molecular Devices, Menlo Park, CA). The intact assay for the microplate reader was identical with that of the spectrophotometer assay except that all reaction components were proportionally reduced so that the reaction volume was 300 μL. The microtiter plate MTD activity assay was used only to qualitatively locate fractions with MTD activity, because the absolute MTD activity varied from that observed spectrophotometrically. PMI and ADH activity assays were as described by Stoop and Pharr (1992). One unit of activity was defined as the amount of enzyme that catalyzed the reduction of 1 μmol NAD h−1. Protein concentrations were determined spectrophotometrically by the Bradford method (Bradford, 1976) using BSA as a standard.


**Purification of NAD-Dependent Mannitol Dehydrogenase**

Estimation of pI of MTD

The pI of purified MTD was estimated by IEF using the Rotofor system (Bio-Rad). A total of 1 mg of purified MTD in 18 mL of buffer containing 4.5 mM Mops (pH 7.5), 0.04 mM NAD, 0.2 mM DTT, 3 mM urea, and 2% Biolyte 5/7 (Bio-Rad) was loaded into the minichamber. Focusing of the Rotofor cell required 3.5 h at 12 W of constant power. The coolant temperature was set at -6°C to obtain a temperature of approximately 4°C inside the chamber. The initial conditions were 1120 V and 11 mA, and at equilibrium the values were 2030 V and 6 mA. Electrolytes in the anode and cathode chambers were 0.1 M H₃PO₄ and 0.1 M NaOH, respectively. Twenty fractions were collected, their pH values were measured, and aliquots were analyzed for the presence of peptides using native gels stained with silver (Bio-Rad).

PAGE

Denaturing SDS-PAGE was carried out according to the method of Laemmli (1970). The final acrylamide concentration was 12% (w/v) for the separating gel and 4.5% (w/v) for the stacking gel. All samples were preincubated in the presence of SDS-sample buffer (62 mM Tris-HCl, pH 6.8, 2% [w/v] SDS, 5% [v/v] 2-mercaptoethanol, 5% [v/v] glycerol, and 0.002% [w/v] bromphenol blue) for 10 min at 100°C prior to being loaded on the gels. Gels were run at a constant 200 V for 45 min at room temperature on a Mini-Protean II gel apparatus (Bio-Rad). Gels were subsequently stained for proteins with silver or 0.25% (w/v) Coomassie brilliant blue R250.

Nondenaturing PAGE was performed at 4°C using a 7.5% separating and 4.5% stacking gel at a constant 200 V applied for 55 min. Gels were precooled to 4°C prior to electrophoresis. Gels were either stained for protein (see above) or for MTD activity. MTD activity was visualized by staining with phenazine methosulfate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as described by Stoop and Pharr (1992). For the determination of molecular mass by SDS-PAGE, a plot of relative mobility versus logarithm (molecular mass) was constructed with protein molecular mass standards ranging from 200 to 14.3 kD (GIBCO-BRL).

Native Molecular Mass Determination

The relative molecular mass of the purified MTD was estimated by gel filtration on a 24-mL FPLC Superose 12 HR 10/30 column (Pharmacia-LKB) equilibrated with 50 mM Mops, pH 7.5, 10 mM MgCl₂, and 100 mM NaCl. The flow rate was 0.4 mL/min, and 1-mL fractions were collected. Aldolase (158 kD), BSA (67 kD), ovalbumin (43 kD), and RNase A (13.7 kD) were used as molecular mass standards to calibrate the column. Fractions were monitored at A₂₈₀ and assayed for MTD activity.

Peptide Mapping with Clostripain

Proteins that eluted in a linear 0 to 0.6 mM NAD gradient from an NAD-agarose column were cleaved with clostripain. Five hundred nanograms of protein were incubated with a 1:5 clostripain to protein ratio on ice overnight. The cleavage products were analyzed on a 1.5-mm-thick, 10 to 20% (w/v) gradient SDS gel (Bio-Rad) and stained with silver.

Characterization of Polyclonal Antibodies and Immunoblotting

Polyclonal antibodies were raised in rabbits against an MTD protein fraction that eluted from an NAD-agarose column using a single 2 mM NAD pulse, and that was further purified by SDS-PAGE. A total of 200 µg of protein were subjected to electrophoresis on a 12% preparative SDS-polyacrylamide gel and stained with an aqueous solution of Coomassie blue. A band representing a single major 40-kD species was sliced out of the gel and used for immunizations. Immunizations and bleedings were performed by HRP, Inc. (Denver, PA). Protein samples were subjected to SDS-PAGE as described above and blotted onto a nitrocellulose membrane using a Bio-Rad semidymanalysis apparatus and the manufacturer’s protocol. Immunodetection of the antigen was carried out using the Protoblott western blot AP systems kit (Promega).

Immunotitration of MTD Activity

One-tenth unit of purified MTD liquid chromatography was incubated overnight on ice with increasing amounts of anti-MTD serum or preimmune serum. Incubation mixtures contained BSA for equal total protein concentrations in each sample. After incubation, the mixture (45 µL total) was transferred to a 1-mL cuvette and MTD was assayed using the spectrophotometric assay described above.

Protein Sequencing

A tryptic digest of the purified MTD was separated by microbore HPLC on a 0.8- × 250-mm liquid chromatography packing column, and individual fragments were sequenced using a Perkin-Elmer Cetus Applied Biosystems Division model 494 (Michigan State University, East Lansing, MI).

RESULTS

Purification of MTD

The NAD-dependent MTD activity of crude extracts from mannitol-grown celery suspension cultures was 25 units g⁻¹ fresh weight and was higher than the MTD activity of 7 units g⁻¹ fresh weight measured in crude extracts from celeriac root tips. Furthermore, crude extracts from cells grown on mannitol had a slightly higher specific activity compared to celeriac root tips, 0.62 versus 0.54 unit mg⁻¹ protein, respectively, making the cultured cells the preferred source from which to isolate and purify MTD.

The MTD protein was purified 589-fold to a specific activity of 365 units mg⁻¹ protein with an overall recovery of 37%, using PEG fractionation, followed by anion-exchange chromatography on a Fractogel EMD DEAE, and...
affinity chromatography on an NAD-agarose column using NAD gradient elution (Table I). Crude extracts of MTD were obtained from cells on the 4th d after subculture, when the enzymatic activity of MTD is high. Fractionation with PEG increased the specific activity 4-fold, with 87% recovery of the MTD activity. PEG fractionation was used instead of ammonium sulfate fractionation, as used previously in partially purifying MTD from celeriac roots (Stoop and Pharr, 1992), because ammonium sulfate fractionation of extracts from celery suspension cells resulted in a low recovery (25%) of MTD activity (data not shown). The Fractogel EMD DEAE ion-exchange step resulted in a purification of MTD away from ADH and PMI, in a manner similar to that described for celeriac roots (Stoop and Pharr, 1992). In initial purifications, the ion-exchange fraction was loaded on an NAD-agarose affinity column, and MTD was eluted in a single step with 2 mM NAD added to the running buffer. When peptides in this fraction were separated by SDS-PAGE, one dominant band was observed (data not shown), whereas separation by native PAGE, resulted in two distinct protein bands (Fig. 1, lane A). When the native gel was stained for MTD activity, only the lower mobility protein stained (Fig. 1). We were able to separate the protein with MTD activity (low mobility on native gel) from the protein without MTD activity (high mobility on native gel) using a 0 to 0.6 mM NAD linear gradient on the NAD-agarose affinity column. Patterns of these two proteins on native PAGE are shown in Figure 1, lanes B and C. Molecular mass determination using MALDI (Michigan State University, East Lansing, MI) indicated that the protein with MTD activity had a molecular mass of 40.35 kD and the other protein had a molecular mass of 42.00 kD. The relatedness of the two proteins was investigated by peptide mapping of their clostripain cleavage fragments. As shown in Figure 2, the cleavage patterns were not similar, indicating that the 40.35- and 42.00-kD proteins may not be related.

The SDS-PAGE protein patterns of various steps of the purification are shown in Figure 3. Throughout the purification, a protein with an apparent molecular mass of 40 kD became more predominant. Lane D represents the MTD fraction eluted with a linear gradient from an NAD-agarose column. When excess amounts of purified MTD, up to 1.3 μg, were subjected to electrophoresis on SDS-PAGE and stained with silver, no contaminating peptides could be observed in the range of detectability (5–10 ng of protein) (data not shown).

### Characteristics of MTD

Native molecular mass was determined by calibrated gel filtration to be approximately 43 kD. This result together with the MALDI and SDS-PAGE data suggests that MTD is monomeric. The MTD was blotted onto nitrocellulose paper and subjected to N-terminal amino acid sequencing, which indicated that the N terminal was blocked. The amino acid sequence was obtained from three peptides generated by tryptic digests of the purified MTD protein and purified by microbore HPLC. The following sequences were obtained: peptide 1, AFGWAAR; peptide 2, VLF/C/SVCHSDHMMHNNWGF (manually terminated); peptide 3, LLGTTINGGIK.

The pl of the MTD was estimated to be 6.5 using the Rotofor system (Bio-Rad).

### Immunological Characterization

Polyclonal antibodies against MTD were raised in rabbits immunized with SDS gel-purified MTD from initial purifications in which the MTD was eluted from an NAD-agarose column in a single 2 mM NAD pulse, described above. Subsequent analyses showed that this MTD fraction contained 90% MTD. These analyses were based on determination of protein concentration of the fractions from an NAD gradient elution and native PAGE. Immunoblot analysis of crude extracts from mannitol-grown cells and NAD-agarose affinity column under different elution conditions. Lane A, Protein eluted by a single 2 mM NAD pulse. Lanes B and C, Two protein fractions eluted using a linear 0 to 0.6 mM NAD gradient. When this gel was stained for MTD activity, the protein with low mobility (Active) stained for MTD activity, whereas the protein with high mobility (Inactive).

### Table 1. Purification of NAD-dependent MTD from celery suspension cultures

<table>
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<tr>
<th>Purification Step</th>
<th>Total Protein</th>
<th>Specific Activity</th>
<th>Recovery</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>7061</td>
<td>0.62</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>15-30% PEG fractionation</td>
<td>1384</td>
<td>2.69</td>
<td>87</td>
<td>4.3</td>
</tr>
<tr>
<td>Fractogel DEAE ion-exchange</td>
<td>146.6</td>
<td>17.05</td>
<td>57</td>
<td>27.5</td>
</tr>
<tr>
<td>NAD-agarose</td>
<td>4.4</td>
<td>365</td>
<td>37</td>
<td>589</td>
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Purification Step: Crude extract was derived from 260 g fresh weight of cells.

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Cell extracts were obtained from cells on the 4th d after subculture,

*Figure 1. Native PAGE of proteins eluted from an NAD-agarose affinity column under different elution conditions. Lane A, Protein eluted by a single 2 mM NAD pulse. Lanes B and C, Two protein fractions eluted using a linear 0 to 0.6 mM NAD gradient. When this gel was stained for MTD activity, the protein with low mobility (Active) stained for MTD activity, whereas the protein with high mobility (Inactive).*
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Figure 2. Electrophoretic patterns of clostripain cleavage products of a 40-kD protein with MTD activity (B) and a 42-kD protein with no MTD activity (C). Clostripain fragments were prepared from proteins separated on an NAD-agarose affinity column by gradient elution and were electrophoresed on a 10 to 20% gradient SDS minigel. Lane A contains low-range molecular mass markers (GIBCO-BRL).

agarose-purified MTD probed with anti-MTD serum showed a single major immunoreactive band corresponding to an apparent molecular mass of 40 kD (Fig. 4). Blots probed with preimmune serum had no immunoreactive bands (Fig. 4). Immunoblot analyses of crude extract from celery and celeriac root tissue also showed a single major cross-reactive band corresponding to a molecular mass of 40 kD (Fig. 5). The anti-MTD serum was also cross-reactive with a 40-kD protein in extracts from the innermost leaves of parsley (Fig. 5). MTD activity measured in parsley sink (innermost) leaves was 3.9 units g⁻¹ fresh weight, whereas no MTD activity was detected in source leaves of parsley.

Immunotitration of MTD with preimmune and anti-MTD sera indicated that anti-MTD serum inhibits enzymatic activity of the purified MTD, whereas preimmune serum has no effect on the MTD activity (Fig. 6).

DISCUSSION

In this paper we describe a rapid and efficient protocol for purifying the NAD-dependent MTD, previously referred to as mannitol:Man 1-oxidoreductase (Stoop and Pharr, 1992), from celery suspension cultures grown on D-mannitol as the sole carbon source. This enzyme catalyzes the oxidation of D-mannitol to D-Man. MTD was purified 589-fold to apparent electrophoretic homogeneity and a final specific activity of 365 units mg⁻¹ protein. The three-step protocol yielded 4.4 mg of protein from 260 g of cells, representing 37% of the initial activity in the crude homogenate. Previously, the MTD was partially purified to a specific activity of 201 units mg⁻¹ protein using celeriac roots (Stoop and Pharr, 1992). Cell-suspension cultures were chosen for this study because of the higher specific activity and ease of obtaining large quantities of cells. The high purity of MTD is indicated by the presence of only a single polypeptide of 40 kD on SDS gels together with the presence of a single polypeptide after native PAGE. MALDI analysis of the purified MTD also gave a signal consistent with a single protein. We were also able to obtain defined peptide sequences from the final purifica-
placed to SDS gel electrophoresis. These antibodies were tested against the gel-purified MTD reacted with a protein region from the cDNA clone, indicating that the cDNA clone encoded the MTD protein (Williamson et al., 1995).

Immunoblot analysis of SDS-PAGE-separated proteins of crude extracts from celery and celeriac showed a single dominant immunoreactive band, demonstrating that the antiserum was cross-reactive with root tissue of celery and celeriac. This allowed us to determine the amount of MTD in salt-stressed celery plants, and the results indicated that the amount of MTD in root extracts was proportional to the MTD activity in these extracts (Pharr et al., 1995).

MTD activity was also observed in parsley (*Petroselinum crispum* L.), which is a member of the Apiaceae and closely related to celery and celeriac. As in celery, the MTD activity observed in parsley leaves was dependent on the developmental stage of the tissue analyzed, with high activity expressed in sink leaves. Antibodies raised against the celery MTD were also cross-reactive with a 40-kD protein in extracts from sink leaves (Fig. 5). Further investigations of the presence of the mannitol catabolic pathway in plants will be useful in determining how widespread the use of MTD is in the plant kingdom.

Continued work toward a more detailed analysis of the physical and kinetics properties of the MTD, as well as a more thorough understanding of the regulation of this catabolic enzyme, is presently underway. Further areas that need attention are the determination of the intracellular localization of the MTD to better understand the transport physiology of mannitol under normal and stressed conditions.

ACKNOWLEDGMENTS

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LITERATURE CITED


![Figure 6. Immunotitration of MTD activity of the NAD-agarose-purified fraction with preimmune serum or anti-MTD serum.](image-url)


