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Purification and characterization of mannitol dehydrogenase and identification of the corresponding cDNA from the head blight fungus, *Gibberella zeae* (*Fusarium graminearum*)

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Abstract

The mannitol-2-dehydrogenase (MtDH) from *Gibberella zeae* was purified and the corresponding cDNA identified. Purification of MtDH was accomplished using a combination of ammonium sulfate fractionation, anion exchange and dye-ligand chromatography. Final purification was achieved following electroelution from a native gel. Molecular mass determination based on SDS–PAGE indicated that the denatured protein was 29 kDa. Native protein mass was determined to be 110 kDa using gel permeation chromatography, indicating a tetrameric form. The pH optima for mannitol oxidation and fructose reductase activities were 9.0, and 7.0, respectively. Activity with sorbitol as the substrate was 21% of activity with mannitol. Kinetic parameters were determined by direct-linear plots of enzyme activity vs. substrate concentrations. Fructose concentrations above 600 mM and NADPH concentrations above 0.3 mM caused substrate inhibition. Comparisons of predicted amino acid sequences of several fungal MtDHs indicated high conservation within the phyla. A possible role for MtDH in generation of turgor pressure for forcible ascospore discharge is discussed.

Keywords: *Gibberella zeae*; *Fusarium graminearum*; Mannitol; Fructose; Sorbitol; Dehydrogenase; NADP; NADPH; Ascospores; Perithecia; Inoculum

1. Introduction

Studies on the roles of mannitol in fungal biology have increased in recent years with the isolation and characterization of several mannitol synthesizing enzymes and associated genes in fungi from different phyla. Fungi use mannitol as a common storage carbon (Jennings, 1995). It has been shown to accumulate in *Agaricus bisporus* during salt stress as an osmoprotectant (Stoop and Mooibroek, 1998). More recently, mannitol was proposed to have a role in fungal–plant interactions. Pathogen-produced mannitol was hypothesized to serve as a quencher of reactive oxygen species of the plant defense response, possibly aiding in pathogen colonization (Jennings et al., 1998). This polyol is also a major component of the Buller's drop, part of the mechanism to dislodge basidiospores from basidia (Webster et al., 1995). Finally, mannitol has been suggested to be an important osmoticum that generates turgor pressure during mycelial growth (Jennings, 1995).

Despite the apparent importance of this polyol in fungal biology, relatively little is known about mannitol metabolism. Mannitol may be synthesized by one of two pathways in fungi: fructose is converted to mannitol by mannitol-2-dehydrogenase (MtDH; EC 1.1.1.138), and fructose-6-phosphate is converted to mannitol-1-phosphate by mannitol-1-phosphate dehydrogenase (M1PDH; EC 1.1.1.17; Jennings, 1995). Whether or not one of these enzymes is dedicated to catabolism and the other to biosynthesis of mannitol is still a matter of discussion. Mannitol oxidation to fructose generates NADPH, and has been proposed to be a source of reducing power. Conversion of fructose to mannitol generates NADP, which may be used for oxidative reactions in the cell (Jennings, 1995).

Gibberella zeae (Schw.) Petch (asexual state = *Fusarium graminearum* Schwabe) causes Fusarium head blight or scab of wheat and barley, a disease which has reached epidemic proportions in the United States (Windels, 2000). We are studying perithecium development (Trail and

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Common, 2000) and ascospore discharge in *G. zeae* (Trail et al., 2002). Recently, we reported that MtDH activity in perithecia from *G. zeae* was detected at highest levels in mature perithecia actively discharging ascospores. M1PDH activity was not detected in any tissue of several tissues tested (Trail et al., 2002). Therefore, we have pursued the purification and characterization of the MtDH and isolation of the MtDH cDNA to determine the role of mannitol in discharge of ascospores. Our future goal is to specifically disrupt the gene for MtDH in the fungus and determine the role for mannitol in ascospore discharge.

2. Results and discussion

2.1. Purification of MtDH and determination of molecular mass

The purification of MtDH from hyphal tissue was achieved using a combination of ammonium sulfate fractionation, anion exchange and dye-ligand chromatography. Despite our interest in MtDH activity in perithecia, purification was instead performed using actively growing hyphae due to the difficulty in obtaining sufficient tissue from sporulating cultures. Final purification from hyphal tissue was achieved following electroelution from a native gel. Fig. 1A shows the protein patterns from sequential stages of purification. Throughout the purification process, a single peak of MtDH activity was observed, indicating only one protein with this activity was present. In activity gel assays, a single band of similar size appeared in the crude culture filtrates derived from perithecia and from hyphae (Fig. 1B), supporting the likelihood that only one form of the enzyme is produced by this fungus. A substantial amount of protease activity was present subsequent to the ammonium sulfate fractionation and remained partially active despite the introduction of inhibitors. The activity was eliminated by the anion exchange step. The enzyme did not elute from the Affi-Blue matrix with 10 mM NADP, indicating that the binding was not entirely based on ligand affinity. However, active enzyme was eluted with addition of buffer at pH 9.0 and was fairly concentrated. The purified protein was sensitive to freeze–thaw cycles, but was stable when stored at

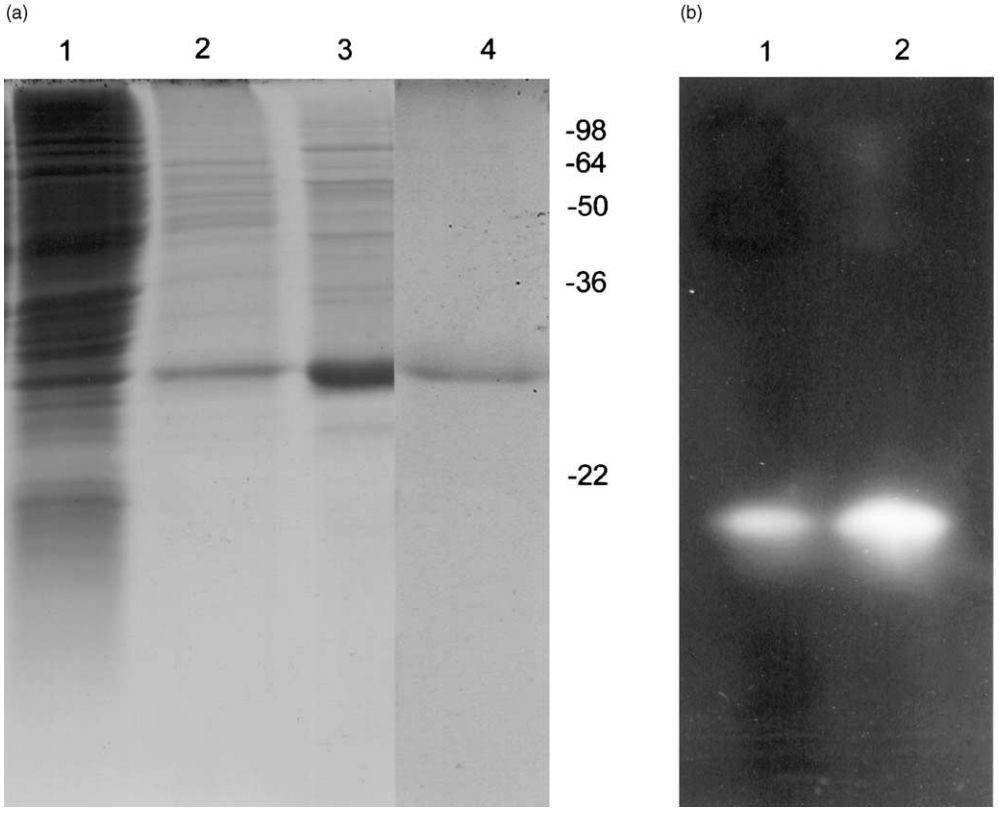

Fig. 1. SDS and native polyacrylamide gel electrophoresis of active fractions from stages of purification of mannitol dehydrogenase. (A) SDS–PAGE separation of samples: after G-10 (60 μg ; lane 1); after anion exchange (6 μg, lane 2); after Affi-Blue (5 μg; lane 3); purified protein (2 μg; lane 4). Gel was stained with Coomassie blue. Size markers (kDa) indicated at right. (B) Native PAGE of crude extracts of mycelia (60 μg; lane 1) and perithecia (60 μg; lane 2). Activity was detected under UV light as described in text.

−80 °C. Enzyme activity could not be recovered following exposure to SDS. Table 1 summarizes the purification procedure for the mannitol dehydrogenase.

Molecular mass on SDS–PAGE indicated that the denatured protein was 29 kDa. Molecular mass as determined by predicted amino acid sequence (Fig. 2) was 28.4 kDa. These sizes are similar to those previously reported for other MtDH enzymes (Morton et al., 1985; Noeldner et al., 1994). Native protein mass was determined by gel permeation chromatography to be 110 kDa, indicating the protein is likely in tetrameric form. Recently, the crystallographic structure of the mannitol dehydrogenase from *Agaricus bisporus* confirmed this prediction (Horer et al., 2001). This mannitol dehydrogenase has been classified as a member of the short-chain dehydrogenase family and the catalytic triad consisting of Ser, Tyr and Lys has been identified. These amino acids are conserved in all MtDH enzymes (Fig. 3). The NADP binding site is present in the MtDH sequences, but is missing a conserved arginine or lysine in the sorbitol dehydrogenase (Fig. 3), in support of the use of NAD by the latter (Janbon et al., 1998).

2.2. Kinetic analysis and enzymatic activity

The pH optima for mannitol dehydrogenase and fructose reductase activities were determined. In repeated experiments, the optimal pH for mannitol oxidation was 9.0, and fructose reduction was 7.0. No activity was observed in the crude mycelial extract or purified protein when NADP and NADPH were replaced with NAD and NADH, respectively. Activity with sorbitol as the substrate was 21% of activity with mannitol at concentrations of 0.8 M. Activity decreased with decreasing sorbitol concentrations.

Kinetic parameters were determined by direct-linear plots of enzyme activity vs. substrate concentrations. The K_m and V_{max} values were determined from these plots. Fructose concentrations above 600 mM and NADPH concentrations above 0.3 mM caused substrate inhibition. Table 2 summarizes the kinetic data.

The high K_m values and pH optima for both the mannitol oxidation reaction and fructose reduction are characteristic of other MtDHs (Morton et al., 1985) and have been structurally related to active site binding of the substrate in these enzymes (Horer et al., 2001). It has been suggested that very low affinity for the substrate facilitates the accumulation of large amounts of mannitol in the mycelium (Niehaus and Dilts, 1982). This would be a requirement if the mannitol was to accumulate in the ascus to generate sufficient turgor pressure to discharge ascospores.

2.3. Protein sequencing

The purified protein was excised from the native gel, subjected to trypsin digestion and three peptides were sequenced (Fig. 2). These peptide sequences were subsequently identified in the predicted amino acid sequence of the cDNA from *G. zeae,* supporting the suggestion that the clone encodes the MtDH.

2.4. Identification and sequence analysis of the MtDH cDNA

Two full-length cDNA clones were identified (Fig. 2) from a library of nitrogen-starved cultures through EST sequencing (Trail et al., 2002). The dbEST Id numbers are 13380737 and 13381710. The cDNA predicts a protein of 266 amino acids with a molecular mass of 28.4 kDa. PAUP* (Swofford, 2002) was used to generate a neighbor-joining tree for the mannitol and sorbitol dehydrogenase sequences. One thousand bootstrap replicates were run, and confirmed the clustering of the ascomycete MtDH sequences with 100% support. Basidiomycete MtDH protein sequence clustered as a separate group, as did the sorbitol dehydrogenase which was used as an outgroup.

We are generating transgenic isolates in which the MtDH is disrupted. We will use these mutants to assess the potential role of MtDH activity in ascospore discharge and in pathogenicity. Should gene disruption show that MtDH is important in the process of ascospore discharge, we will develop antibodies for labeling the protein in vivo. Localization of the MtDH will be important in understanding the mechanism of forcible discharge.

Table 1
Purification of NADP-dependent mannitol dehydrogenase from hyphae of *Gibberella zeae*

Purification step	Total volume (ml)	Total activity (nkat)	Total protein (mg)	Specific activity [nkat (mg protein)^{1}]
Mycelial extract	1.00×10^{2}	18.96	2.35×10^{2}	8.07×10^{-2}
Ammonium sulfate fractionation	1.00×10	5.40	5.68×10	9.51×10^{-2}
G-10	2.25×10	4.75	4.34×10	$1.09\text{x}10^{-1}$
DEAE Sepharose (×3)[a]	3.20×10	4.96	8.50	5.84×10^{-1}
Affi-GelBlue (×2)[a]	1.32×10	2.85	7.1×10^{-1}	4.0
Electroelution (×2)[a]	1.60	2.34	2.6×10^{-1}	9.0

[a] Combined results from all runs.

3. Experimental

3.1. Strains and culture conditions

G. zeae strain NRRL 31084 (PH-1; Trail and Common, 2000) was used during this study. Soil stocks were maintained at −20 °C, and fresh cultures initiated as needed.

```
                                                        M  V
   1 GCACGAGGATCTTTACCACATCTACTTCCACTACCGCAACCATGG

   3   Q  Q  I  P  K  A  N  H  I  L  D  L  L  S  L
  46 TTCAACAAATCCCCAAGGCCAACCACATCCTCGACCTCCTCAGCC

  18   K  G  K  V  V  V  V  T  G  A  S  G  P  R  G
  91 TCAAGGGCAAGGTCGTCGTCGTCACCGGCGCTTCCGGCCCCCGAG

  33   M  G  I  E  A  A  R  G  A  A  E  M  G  A  D
 136 GCATGGGTATCGAGGCCGCCCGCGGTGCCGCTGAGATGGGCGCCG

  48   V  A  I  T  Y  A  S  R  K  E  G  A  D  K  N
 181 ACGTCGCCATCACCTACGCTTCCCGCAAGGAGGGCGCCGACAAGA

  63   V  E  E  L  V  K  E  Y  G  V  K  A  K  A  Y
 226 ACGTCGAGGAGCTCGTCAAGGAGTACGGCGTCAAGGCCAAGGCTT

  78   K  L  N  A  A  D  Y  N  D  V  E  R  F  V  G
 271 ACAAGCTCAACGCTGCCGACTACAACGATGTTGAGCGATTTGTTG

  93   E  V  V  K  D  F  G  K  I  D  G  F  V  A  N
 316 GCGAGGTTGTCAAGGACTTTGGCAAGATTGACGGATTTGTCGCCA

 108   A  G  A  T  A  N  A  G  V  I  D  G  S  A  A
 361 ACGCCGGTGCTACTGCCAACGCTGGTGTCATTGACGGCTCCGCTG

 123   D  W  D  H  V  I  Q  I  D  L  N  G  T  A  Y
 406 CCGACTGGGACCACGTTATCCAGATCGACCTCAACGGCACCGCCT

 138   C  A  K  A  V  G  A  L  F  R  K  Q  G  H  G
 451 ACTGCGCCAAGGCCGTCGGCGCTCTCTTCCGCAAGCAAGGCCACG

 153   S  F  V  I  T  S  S  M  S  G  H  I  A  N  Y
 496 GCTCCTTCGTTATTACCTCCTCCATGTCCGGCCACATCGCCAACT

 168   P  Q  E  Q  T  S  Y  N  V  A  K  A  G  C  I
 541 ACCCTCAAGAGCAGACCTCTTACAACGTCGCCAAGGCGGGCTGCA

 183   H  M  A  R  S  L  A  N  E  W  R  D  F  A  R
 586 TCCACATGGCACGCTCTCTCGCCAACGAGTGGCGCGACTTTGCCC

 198   V  N  S  I  S  P  G  Y  I  D  T  G  L  S  D
 631 GCGTCAACTCCATCTCTCCCGGCTACATCGACACCGGCCTGTCTG

 213   F  I  D  A  E  T  Q  E  L  W  R  S  M  I  P
 676 ACTTTATCGACGCCGAGACCCAGGAGCTGTGGAGGAGCATGATCC

 228   M  G  R  N  G  D  A  K  E  L  K  A  A  Y  V
 721 CCATGGGCCGAAACGGAGATGCCAAGGAGCTCAAGGCTGCTTATG

 243   Y  F  L  S  D  A  S  T  Y  T  T  G  S  D  L
 766 TTTACTTCTTGTCTGATGCTAGTACTTATACTACTGGTAGCGATC

 258   V  I  D  G  G  Y  T  C  R  *
 811 TCGTCATTGATGGTGGATACACTTGCCGATAGATGGGGGATGGTT
 856 TTAATGACCTTGATGATACCAAAATTAAATAAATAAATAAATAAT
 901 ATATAGTAATGACAATTACATGATCCAAAAAAAAAA
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Fig. 2. The cDNA sequence encoding the *Gibberella zeae* mannitol dehydrogenase and the deduced amino acid sequence (266 amino acids). Translation termination site is indicated (*). Amino acids in bold type correspond to sequenced peptides from the purified protein.

3.2. Purification of the MtDH

Mycelia from a vigorously growing culture on carrot agar (approximately 5 days old) were used to inoculate 2 × 100 ml YES (2% yeast extract, 20% sucrose) and grown for 4 days at room temp. shaking at 180 rpm. Mycelia (approximately 20 g wet weight) were harvested by filtration, rinsed with distilled water, and stored at −80 °C prior to use. Collected tissue was transferred to liquid nitrogen and ground to a fine powder with a mortar and pestle. The pulverized mycelia were suspended in 100 ml 50 mM MOPS buffer (pH 4.5, 0.5 mM dithiothreitol, 1 mM EDTA, 5 mM phenylmethylsulfonyl fluoride). Undissolved components were removed from the homogenate by centrifugation (20,000 × *g* for 20 min) at 4 °C. The supernatant from mycelia was collected and brought to 45% saturation with $(NH_4)_2SO_4$, stirred for 15 min at 4 °C, and centrifuged as described previously. The supernatant was collected, brought to 80% saturation with $(NH_4)_2SO_4$, stirred for 15 min at 4 °C, and centrifuged for 20 min as described above. The 80% pellet was dissolved in 10 ml HEPES buffer [25 mM HEPES (pH 7.5) 0.5 mM dithiothreitol] in which was dissolved one Complete Mini Protease Inhibitor Cocktail™ tablet (Roche Diagnostics GmbH, Mannheim, Germany).

The mycelial extract was desalted by passage through a G-10 Sephadex column (3×28 cm) equilibrated with HEPES buffer. Active fractions were divided into three aliquots of approximately 7 ml each and frozen at −80 °C. Single aliquots were thawed and applied to a DEAE Sepharose CL-6B anion exchange column (1.5×18 cm). The column was washed with three column volumes of HEPES buffer and MtDH was eluted with a linear gradient (100 mls) of 0–0.5 M NaCl in HEPES buffer. Active fractions (2.5 ml) were collected and pooled.

Active fractions from three ion exchange columns were combined, divided into two equal portions and applied separately to an Affi-Gel Blue (Bio-Rad, Richmond, CA) column (1.5×18 cm) equilibrated with HEPES buffer. The column was washed with six column volumes of HEPES buffer and the protein was then eluted with the application of 20 mM Tris–HCl pH 9.0. Final purification was achieved through separation of proteins from active fractions from the Affi-Gel Blue columns by native PAGE in 7% polyacrylamide (Ornstein and Davis, 1964). Native PAGE gels were run overnight at 4 °C at 5 mA. Location of the active protein was determined by an in-gel activity assay modified from a previously published procedure (Morton et al., 1985). The gel was equilibrated in 100 mM Tris, pH 9.0, 50 mM mannitol, then transferred to fresh buffer containing 50 mM NADP for 10 min at 37 °C. Active bands were identified by UV exposure and removed from the gel. Protein was electroeluted in the Native gel buffer system for 4 h at 10 mA at 4 °C.

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Cl MPQRIPEAEHLLDLLSLKGRVVVVTGASGPKGMGIEAARGCAEMGADLAITYASRAEGGL 60
Ne ----IPTASKLSDLFSLKGKVVVITGASGPRGMGIEAARGCAEMGASVAITYASRADGAQ 56
Gz MVQQIPKANHILDLLSLKGKVVVVTGASGPRGMGIEAARGAAEMGADVAITYASRKEGAD 60
Ca ---QLP--SNVLDLFSLKGKVASVTGSSG--GIGWAVAEAFAQAGADVAIWYNSKP--AD 51
Ag --------------ISFVNKTIIVTG--GNRGIGLAFTRAVAAAGANVAVIYRSAKDAVE 44
Ur --------------IDLKDQCIIVTG--GNRGIGLAMSQACADAGAAVGIIYNSAKDAED 44
                  :.: .:   :**  *  *:*   :.. *  ** :.: * *

Cl KNAEELSKQYGIKCKAYKCQVDKYESVEQLVKDVIQDFGKIDAFIANAGATANSG-ILDG 119
Ne KNVAELEKEYGIKAKAYKLNVADYAECEKLVKDVIADFGQIDAFIASAGATAKSG-VLDG 115
Gz KNVEELVKEYGVKAKAYKLNAADYNDVERFVGEVVKDFGKIDGFVANAGATANAG-VIDG 119
Ca AKAEYLTEKYGVKAKAYKCNVTDPNDVSKVINEIEKDFGTIDIFVANAGVAWTDGPEIDV 111
Ag VT-EKVGKEFGVKTKAYQCDVSNTDIVTKTIQQIDADLGAISGLIANAGVSVVKP-ATEL 102
Ur R-ASEISKKYGVKCKAYQCDVGQQHKVKEVFKKINEELGPVTGLIANAGVSVVKE-ALQY 102
        : :::*:* ***: :. .     . . .:  ::* :  ::*.**.:       :

Cl S-VEDWNHVVQVDLNGTFHCAKAVGHHF-KE-RGTGSFVITSSMSGHIANYPQ-----EQ 171
Ne S-KEEWDRVIETDLNGTAYCAKAVGPHF-KE-RGRGSFVITSSISGHIANYPQ-----EQ 167
Gz S-AADWDHVIQIDLNGTAYCAKAVGALF-RK-QGHGSFVITSSMSGHIANYPQ-----EQ 171
Ca QGYDQWKKIVDCDLNGVYYCAHTVGQIF-KK-NKSGSLIITSSMSGTIVNIPQ-----LQ 164
Ag T-HEDFKFVYDVNVFGVFNTCRAVAKLWLQK-QQKGSIVVTSSMSSQIINQSSLNGSLTQ 160
Ur N-KDDFNKIFDVNVFGVFNCAQAMAQIWTDTGFQRGSVVIISSMSSQICNR-----PLTQ 156
       ::. : : :: *.   .:::.  :       **.:: **:*. * *         *

Cl TSYNVAKAGCIHMARSLANEWRDFA-RVNSISPGYIDTGLSDFVAKDIQKLWHSMIPLGR 230
Ne TSYNVAKAGCIHMARSLANEWRDFA-RVNSISPGYIDTGLSDFVDQKTQDLWKSMIPLGR 226
Gz TSYNVAKAGCIHMARSLANEWRDFA-RVNSISPGYIDTGLSDFIDAETQELWRSMIPMGR 230
Ca APYNAAKAACTHLAKSLSVEWASFGARVNSISPGYILTDIADFADPEMKKKWWQLTPLGR 224
Ag VFYNSSKAACSNLVKGLAAEWASAGIRVNALSPGYVNTDQTAHMDKKIRDHQASNIPLNR 220
Ur CFYNSSKAAVSNLGKCLAAEWAEKSIRVNMLSPGYVKTDQTSHMDQKLRDFQADGVPLKR 216
     ** :**.  :: : *: ** . . *** :****: *. : .   . :.   .  *: *

Cl DGLAKELKGAYVYLVSDASTYTTGADIVIDGGYTCR 266
Ne NGDAKELKGAYVYLVSDASSYTTGADILIDGGYTVR 262
Gz NGDAKELKAAYVYFLSDASTYTTGSDLVIDGGYTCR 262
Ca EGLPQELVGAYLYLASNASTYTTGSNIAVDGGYTC? 259
Ag FAQPEEMTGQAILLLSDHATYMTGGEYFIDGG???? 252
Ur FAEPEEMAGQAILLLSPKASYMTGGEYFVDGG???? 248
    . .:*: .  : : *  ::* **.:  :***
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Fig. 3. Alignment of protein sequences of mannitol dehydrogenases [Cl: *Cladosporium fulvum* (Pike et al., unpublished), Ne: *Neurospora crassa* (Whitehead Institute Center for Genome Research, Neurospora genomic sequence), Gz: *Gibberella zeae*, Ag: *Agaricus bisporus* (Stoop and Mooibroek, 1998), Ur: *Uromyces* (Hahn and Mendgen, 1997) and sorbitol dehydrogenases [Ca: *Candida albicans* (Janbon et al., 1998)]. The MtDH from Ne was identified by homology of the genomic sequence. The MtDH from Ur was identified by homology of EST sequence to Ag. The consensus line: "*" = identical or conserved residues in all sequences in the alignment; ":" = indicates conserved substitutions; "." = indicates semi-conserved substitutions. Catalytic triad S, Y and K are in bold. The NADP-binding motif is underlined.

Gel permeation chromatography was accomplished using G150 Sephadex on a 1×90 cm equilibrated with 20 mM Tris–HCl, 10 mM KCl, pH 7.5, run at 17.3 ml/h. Protein size was determined by comparison to standard proteins: carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), and alcohol dehydrogenase (150 kDa). Protein concentration was determined spectrophotometrically using the BioRad Protein Assay system (BioRad Laboratories, Hercules, CA) with bovine serum albumin as a standard.

Table 2
Kinetic arameters of mannitol dehydrogenase

Reaction	pH	K_m	V_{max} [nkat (mg protein)$^{-1}$]
Fructose reduction	7.0 (optimal)	Fructose 290 mM NADPH 70 μM	3.3
Mannitol oxidation	9.0 (optimal)	Mannitol 40 mM NADP 78μM	2.5
	7.0	Mannitol 57mM NADP 140 μM	1.0

Mature perithecia were generated on carrot agar as previously described (Trail and Common, 2000). Culture extracts from perithecia were prepared by scraping 6-day-old perithecia from the surface of the agar, rapidly freezing the tissue in liquid nitrogen and grounding to a fine powder with a mortar and pestle. The pulverized mycelia were suspended in a minimal volume of 50 mM MOPS buffer and undissolved components were removed as earlier.

3.3. MtDH activity assays and kinetics

MtDH assays were performed using modifications of a previously published procedure (Stoop et al., 1995). Enzyme activity (reactions in both directions) was assayed spectrophotometrically (340 nm) by monitoring the reduction/oxidation of NADP/NADPH (MtDH) or of NAD + /NADH (M1PDH) in 500 μl reaction mixtures. Activity was assayed as follows: oxidation of mannitol was performed in 100 mM Tris buffer (pH 9.0) containing 2 mM NADP, an appropriate amount of enzyme

(up to 5 μl), and 400 mM mannitol; reduction of fructose was similarly performed in 100 mM MOPS (pH 7.0) containing 0.3 mM NADPH, and 600 mM fructose. Oxidation of mannitol-1-phosphate by M1PDH was assayed in 10 mM HEPES (pH 9.0), 0.5 mM NAD, 10 mM mannitol-1-phosphate. Reduction of fructose-6-phosphate by M1PDH was assayed in 10 mM HEPES (pH 7.0), 0.5 mM NADH, 10 mM fructose-6-phosphate. Oxidation of mannitol was used to follow enzyme purification.

Kinetic parameters were determined by direct-linear plots of enzyme activity vs. substrate concentration. Measurements were taken by changing the concentration of one substrate in the presence of 0.3 mM NADPH, 2 mM NADP, 600 mM fructose or 400 mM mannitol, as appropriate, at 30 °C. A minimum of nine different substrate concentrations were tested to determine K_m and V_{max} and critical concentrations were done in triplicate. Standard deviations between trials were less than 10%.

3.4. SDS–polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis under denaturing conditions followed the standard procedure of Laemmli (1970). The final acrylamide concentration was 10% for the separating gel and 4.5% for the stacking gel. Staining was done with Coomassie brilliant blue R250.

3.5. Protein and DNA sequencing and analysis

The band containing the MtDH was identified by activity staining and was excised from the gel. A tryptic digest of purified MtDH was separated by reversed phase HPLC on an octoadecyl (C18) silica gel column. Peptide sequencing was performed using a Perkin-Elmer Cetus Applied Biosystems Division model 494 (Stanford University PAN facility, Stanford CA).

cDNA sequencing was performed on an ABI PRISM® 3100 Genetic Analyzer (Genomics Technology Support Facility at Michigan State University).

Sequences for MtDH proteins were obtained from the NCBI Website (http://www.ncbi.nlm.nih.gov/) and the Neurospora genome database (http://wwwgenome.wi.-mit.edu/annotation/fungi/neurospora/). Protein sequences were aligned with ClustalW (Thompson et al., 1994) and compared using PAUP* (Phylogenetic Analysis Using Parsimony, Version 4.0b10. (Swofford, 2002).

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