d-Mannitol Metabolism by *Aspergillus candidus*

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Pathways of mannitol biosynthesis and utilization in *Aspergillus candidus* NRRL 305 were studied in cell-free extracts of washed mycelia prepared by sonic and French pressure cell treatments. A nicotinamide adenine dinucleotide-linked mannitol-1-phosphate (MIP) dehydrogenase was found in French pressure cell extracts of d-glucose-grown cells, whereas a specific mannitol-1-phosphatase was present in extracts prepared by both methods. The existence of these two enzymes indicated that mannitol may be synthesized in this organism by the reduction of fructose-6-phosphate. A specific nicotinamide adenine dinucleotide phosphate-linked mannitol dehydrogenase was also identified in both extracts. This enzyme may have been involved in mannitol utilization. However, the level of the mannitol dehydrogenase appeared to be substantially reduced in extracts from mannitol-grown cells, whereas the level of MIP dehydrogenase was increased. A hexokinase has been identified in this organism. Fructose-6-phosphatase, glucose isomerase, and mannitol kinase could not be demonstrated.

Pathways for the biosynthesis and utilization of mannitol have been described for many organisms. Basically, there are two mechanisms. In some organisms fructose is reduced directly by an NADPH (reduced nicotinamide adenine dinucleotide phosphate)-linked mannitol dehydrogenase. In a second mechanism, fructose-6-phosphate (F6P) is reduced to mannitol-1-phosphate (MIP) via an NADH (reduced nicotinamide adenine dinucleotide)-linked MIP dehydrogenase. MIP is acted upon by a specific phosphatase (mannitol-1-phosphatase) that releases inorganic phosphate and mannitol.

Mannitol utilization occurs by reversing these reactions; however, mannitol must be phosphorylated when MIP dehydrogenase is involved (3). Touster and Shaw (11) have reviewed the occurrence and function of polyol metabolizing systems in plants, animals, and microorganisms.

Lee (4) reported a biosynthetic pathway for mannitol in an *Aspergillus* sp. in which F6P was reduced to MIP by an NADH-linked MIP dehydrogenase. A mannitol-1-phosphatase was also found in this organism.

Since a mannitol fermentation was developed at the Northern Laboratory (10) with the white *Aspergillus, A. candidus* NRRL 305, we wanted to determine the pathway by which mannitol was synthesized in this organism. The results of this work, as well as studies on the pathway of mannitol utilization, are reported here.

**MATERIALS AND METHODS**

**Culture.** *A. candidus* NRRL 305 was grown in a medium consisting of Czapek’s salts plus 5% dextrose (Difco) or d-mannitol (Mann Research Laboratories, Inc., New York, N.Y.) and 1% Difco yeast extract. (The sugars and polyols used were of the D configuration.) The spores from a yeast extract-malt extract-agar slant were washed into 100 ml of medium in a 500-ml baffled Erlenmeyer flask. The flask was shaken in a New Brunswick rotary water bath shaker at 25° C (200 oscillations per min). After growth for 48 to 72 hr, the culture was added to 500 ml of the same medium in a baffled Fernbach flask which was shaken at 28 to 30° C in a New Brunswick Psychotherm shaker (200 oscillations per min). A 5-ml amount of a 1:6 dilution of Antifoam 60 (General Electric, Silicone Products Division, Waterford, N.Y.) was added to the larger cultures.

**Reagents.** MIP and sorbitol-6-phosphate were prepared by sodium borohydride reduction of mannose-6-phosphate and glucose-6-phosphate (G6P), respectively (Sigma Chemical Co., St. Louis, Mo.), according to the method of Wolff and Kaplan (12). The sodium salt of F6P was prepared by treating the barium salt (Sigma Chemical Co. or Schwarz Laboratories, Inc., New York, N.Y.) with Bio-Rad cationic exchange resin (50W-X2-H+ form; Bio-Rad Laboratories, Richmond, Calif.) and neutralizing with NaOH.

**Determinations.** The product from the mannitol dehydrogenase reaction was determined by descending chromatography on Whatman no. 1 paper after passing the reaction mixture through a 1- by 5-cm column of Bio-Rad cationic exchange resin (50W-X2-H+ form, 50 to 100-mesh) to stop the reaction and remove cations. Mannitol and free sugars were de-
developed in the methyl ethyl ketone-boric acid-acetic acid solvent (9:1:1) of Rees and Reynolds (8) or in a phenol–water solution (4:1, w/v). The sugars were detected by spraying the developed papers with 2% p-anisidine phthalate in 95% ethyl alcohol and heating at 110 °C for 10 min. The spots were observed and marked under ultraviolet light (366 nm). The paper was then sprayed with a 1% periodate solution. Polys were appeared as white spots on a purple background.

This chromatographic procedure was also used to confirm the identity of the sugar and polyol phosphates after treatment with alkaline phosphatase (EC 3.1.3.1).

Fructose and F6P were quantitatively determined by the method of Roe et al. (9). M1P was assayed by the periodate oxidation method (12). Glucose was measured by alkaline ferricyanide reduction with an autoanalyzer (10). Protein was assayed by the procedure of Lowry et al. (6).

Enzyme preparation and assays. The mycelia from 24-hr cultures were harvested by centrifugation or filtration and washed thoroughly with cold 0.1 M phosphate buffer (pH 7.0) or with cold distilled water (for phosphatase assay). The washed mycelia were broken by treatment with a prechilled French pressure cell (FPC; American Instrument Co., Inc., Silver Spring, Md.). The extracts were centrifuged at 0 to 4°C at 20,000 × g for 40 min, or as indicated. The supernatant fluid (crude extract) was used for enzyme assays.

Mannitol and M1P dehydrogenase activities were assayed by following nicotinamide adenine dinucleotide phosphate (NADP) and nicotinamide adenine dinucleotide (NAD) reductions, respectively, at 340 nm. The reaction mixture consisted of 1.0 ml of substrate, 0.2 ml of buffer (0.2 M glycine-NaOH, pH 9.8), 0.1 ml of NADP or NAD (0.01 M), and 0.05 ml of extract. The extract was added to start the reaction. NADPH and NADH oxidation were studied with 0.1 M sodium acetate (pH 6.0). The assays were carried out at 25°C.

Mannitol-1-phosphatase activity was determined by measuring the inorganic phosphate released by the Fiske and Subbarow method. M1P (19 μmoles) was incubated at 25°C with 0.7 ml of buffer, 0.2 ml of extract (water-washed cells), and distilled water to a volume of 2.0 ml. At 0 and 15 min, 0.4 ml was removed and pipetted into 1.0 ml of 10% trichloroacetic acid. The protein was removed by centrifugation.

NADP reduction was used to detect hexokinase. A reaction mixture containing 5 μmoles of MgCl2, 7H2O, 2 μmoles of adenosine triphosphate (ATP), 33 μmoles of glycine (0.1 M glycine-NaOH, pH 9.0), and 40 μmoles of fructose or glucose; the extract was incubated at 25°C for 15 min. NADP (1.0 μmole) was added and the increase in optical density at 340 nm was observed.

RESULTS AND DISCUSSION

Yamada et al. (13) reported finding both NAD-linked M1P dehydrogenase and mannitol-1-phosphatase in crude extracts of A. niger and A. oryzae. Lee (4) found the same enzymes in the Aspergillus strain used in his work.

An NAD-linked M1P dehydrogenase was detected in FPC extracts of glucose-grown A. candidus NRRL 305 as well (Fig. 1). It can be seen that the activity of this enzyme was much lower when the KH2PO4-EDTA (ethylenediaminetetraacetate) buffer (pH 7.5) of Lee (4) was used. There was no NAD reduction with mannitol as the substrate and no NADP reduction with M1P. In addition, little or no enzyme activity was demonstrable in extracts treated for 10 to 15 min in an ice salt-water bath with a Sonifier (Branson Instruments Inc., Stamford, Conn.). Possibly the sonic treatment destroyed the enzyme or FPC breakage solubilized the enzyme. However, no activity was found in the pellet obtained by centrifuging the crude sonic extract. The level of protein of FPC extracts was between 5 and 6 mg of protein/ml, whereas that in sonic extracts was around 2 to 3 mg of protein/ml. A high level of endogenous NADH oxidation prevented an assay of the reverse reaction.

In both sonic and FPC extracts, there was a specific mannitol-1-phosphatase. This enzyme...
was active in water or pH 6.0 acetate buffer, but inactive at pH 9.8 (Table 1). No cofactors, such as Mg$^{2+}$, appeared to be required. An enzyme fraction, precipitating between 0.3 and 0.7 saturation of ammonium sulfate and dialyzed 16 hr against water, still retained mannitol-1-phosphatase activity (as well as mannitol dehydrogenase activity). The crude enzyme was inactive with glucose-6-phosphate, F6P, sorbitol-6-phosphate, and mannose-6-phosphate.

Since both M1P dehydrogenase and mannitol-1-phosphatase were present in the extracts from glucose-grown cells, it seemed reasonable to assume that mannitol was being synthesized via F6P reduction as in other aspergilli (4, 13). However, a high level of NADP-linked mannitol dehydrogenase occurred in the extracts. The activity was present in both sonic and FPC extracts. Figure 2 shows that the enzyme is most active with glycine-NaOH buffer (pH 9.8). Its activity is diminished considerably in KHCO$_3$-EDTA buffer (pH 7.5). Enzyme activity was also reduced with 0.2 M carbonate-bicarbonate buffer (pH 9.8), 0.05 M tris(hydroxymethyl)amino-methane (pH 8.0 and 9.8), 0.2 M glycine-NaOH (pH 8.5), and 0.1 M sodium acetate (pH 6.0). Horikoshi et al. (1) used 0.2 N glycine-NaOH (pH 9.8) to detect mannitol dehydrogenase in conidia of _A. oryzae_.

The enzyme was specific for mannitol; NADP reduction did not occur with arabitol, sorbitol, dulcitol, or ribitol. No NAD-linked activity was noted. Horikoshi et al. (1) reported the enzyme from _A. oryzae_ conidia had both NADP and NAD activity.

To secure further evidence for the action of this enzyme, ketose production was examined. Table 2 contains the requirements for ketose formation, and a time-course experiment is plotted in Fig. 3. Fructose was identified chromatographically as the end product of the reaction. NADPH oxidation was also demonstrated with fructose as the substrate.

That the organism possessed a mannitol dehydrogenase as well as an M1P dehydrogenase raised the question as to which enzyme, if not both, is operable in mannitol biosynthesis. The logical synthetic pathway appeared to be the

### Table 1. Mannitol-1-phosphatase activity

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Time</th>
<th>Inorganic phosphate</th>
<th>Inorganic phosphate released</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M Sodium acetate (pH 6.0)</td>
<td>0</td>
<td>0.20</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1.40</td>
<td></td>
</tr>
<tr>
<td>0.2 M Glycine-NaOH (pH 9.8)</td>
<td>0</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>0</td>
<td>0.25</td>
<td>1.01</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1.26</td>
<td></td>
</tr>
</tbody>
</table>

* Extract (5.4 mg of protein/ml) used was prepared by FPC breakage of water-washed mycelia.

![Figure 2](http://www.jpg.com/fig2.png)

**Fig. 2. Mannitol dehydrogenase.** (A) Mannitol (0.1 μmole), NADP (1.0 μmole), and glycine-NaOH; (B) water, NaDP, and glycine-NaOH; (C) mannitol, NAD (1.0 μmole), and glycine-NaOH; (D) mannitol, NADP, and KHCO$_3$-EDTA; (E) water, NaDP, and KHCO$_3$-EDTA. The buffers were used in the same concentration as in the M1P dehydrogenase assay (see Fig. 1).

### Table 2. Mannitol dehydrogenase assay: requirements for ketose production

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ketose$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minus extract</td>
<td>3</td>
</tr>
<tr>
<td>Minus mannitol</td>
<td>12</td>
</tr>
<tr>
<td>Minus NADP</td>
<td>9</td>
</tr>
<tr>
<td>Minus buffer</td>
<td>13</td>
</tr>
<tr>
<td>Complete</td>
<td>34</td>
</tr>
<tr>
<td>Complete</td>
<td>35</td>
</tr>
</tbody>
</table>

* Expressed as micrograms of ketose per sample. A 1-ml amount of Resorcinol reagent (9) was added to stop the reaction after 30 min at 25 C.

$^b$ FPC extract contained 5.4 mg of protein/ml.
NADP-reduction assay has been along with the other extracts of the organism, Aspergillus (2), NRRL 305. Because there was no mannitol dehydrogenase, mannitol kinase, pyrophosphate phosphotransferase, or mannitol phosphoenolpyruvate phosphotransferase in the former organism, Lee (5) suggested that this new enzyme serves to phosphorylate mannitol so that it can be incorporated via MIP dehydrogenase. A Michaelis constant of 2.5 mM for mannitol was found. This unusually high value suggests that mannitol may not be the true substrate for the enzyme. We have been unsuccessful in our attempts to identify this enzymatic activity in extracts of mannitol-grown A. candidus NRRL 305.

The enzymes reported so far were demonstrated in glucose-grown cells. The question remained as to which pathway was operable in mannitol biosynthesis. An examination of extracts from man-
nitol-grown cells revealed both dehydrogenases and the phosphatase as well. Somewhat more perplexing was the finding that M1P dehydrogenase was present to a higher degree than in glucose-grown cells (Table 3).

The relationship of the two dehydrogenase activities is unusual. If M1P dehydrogenase is active in mannitol biosynthesis, then why is it present to a much greater extent in mannitol-grown cells? If it is active also in mannitol utilization, a satisfactory mechanism for mannitol phosphorylation remains to be established. Similarly, mannitol dehydrogenase is reduced in activity where it would be expected to be high (i.e., in mannitol-grown cells). Since a mechanism for fructose formation has not been found, there is no evidence that mannitol dehydrogenase is active in mannitol biosynthesis.

The mechanism controlling the production of these enzymes and the pathway of either biosynthesis or utilization of mannitol remain undetermined. The answers to these problems lie in the detection of those enzymes that introduce the proper substrates for the respective dehydrogenases. The metabolic regulation of these enzymes should be an interesting phenomenon as well.

**LITERATURE CITED**


**TABLE 3. Mannitol and M-1-P dehydrogenase activities in FPC extracts from glucose- and mannitol-grown cells**

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Enzyme assayed</th>
<th>Specific activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Mannitol de-</td>
<td>0.54 (0.45)a</td>
</tr>
<tr>
<td></td>
<td>hydrogenase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M1P dehydrogenase</td>
<td>0.18 (0.21)</td>
</tr>
<tr>
<td>Mannitol</td>
<td>Mannitol de-</td>
<td>0.10b</td>
</tr>
<tr>
<td></td>
<td>hydrogenase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M1P dehydrogenase</td>
<td>0.58b</td>
</tr>
</tbody>
</table>

a Expressed as Δ optical density per minute per milligram of protein. Data for glucose-grown cells was taken from Fig. 1 and 2. Values in parentheses are averages of three additional experiments.
b Average of four experiments.