Mannitol Dehydrogenase from *Agaricus campestris*

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(Received for publication, June 24, 1963)

Enzymes capable of catalyzing D-mannitol formation from D-fructose or D-fructose-6-phosphate have been identified in various microorganisms. Substrate and coenzyme specificity have been established in only a few instances, but there appear to be enzymes, dependent upon nicotinamide adenine dinucleotide and upon nicotinamide adenine dinucleotide phosphate, which reduce fructose directly to mannitol. An example of the former, a specific mannitol dehydrogenase from *Lactobacillus brevis*, has been described recently by Martinez, Barker, and Horecker (1). Mannitol formation in another group of microorganisms has been shown to be brought about indirectly by a phosphatase-catalyzed hydrolysis of mannitol-1-P formed from fructose-6-P by an NAD-dependent dehydrogenase (2-4). D-Mannitol, and other polyols as well, are widely distributed in plants, and it has generally been assumed that they originate from sugars by reduction. The simultaneous occurrence of D-mannitol and D-fructose in the brown algae, for example, has led to the suggestion that an enzyme catalyzing their interconversion may be present. Aside from the observation that extracts of *Sorbus aucaparia* berries possess NAD-linked dehydrogenase activity toward sorbitol and D-mannitol (6), no work has been reported on mannitol dehydrogenases in plants or higher fungi. This, together with the recent demonstration by Hughes, Lynch, and Somers (7) that D-mannitol is present to the extent of some 18% of dry weight in the cultivated mushroom, *Agaricus campestris*, led us to examine this system.

**EXPERIMENTAL PROCEDURE**

**Materials**—NAD, NADP, and NADPH were purchased from California Corporation for Biochemical Research. Glucose-6-P, fructose-6-P, and mannose-6-P were obtained from Mann Research Laboratories. D-Fructose and D-mannitol (reagent grade) were obtained from the General Chemical Division of Allied Chemical Corporation. We wish to thank Dr. N. O. Kaplan for a gift of mannitol-1-P. The mushrooms used were obtained from commercial growers in southeastern Pennsylvania, and in most cases were obtained on the day of harvesting. They were washed with cold tap water, rinsed with cold distilled water, and stored in the cold room overnight prior to use.

**Methods**—Washed, chilled mushrooms, 200 g, were diced and then homogenized for 20 to 30 seconds in a Waring Blender with 250 ml of Tris buffer (0.05 M, pH 8.05). The homogenate was centrifuged at 10,000 r.p.m. (Spinco rotor No. 30) and the clear supernatant dialyzed against Tris buffer. Solid ammonium sulfate was added to bring the solution to 65% saturation, and the precipitate was removed by centrifugation and discarded. The supernatant was then brought to 75% saturation with solid ammonium sulfate and the resulting precipitate dissolved in a minimum of Tris buffer and dialyzed against the same buffer. The specific activity of this preparation, which was used for all the studies to be described unless otherwise stated, was 13 times that of the original extract. The enzyme may be stored at -20° for at least 1 month without any significant loss of activity, but activity is completely destroyed by heating at 70° for 10 minutes.

All spectrophotometric assays were made with a Zeiss PMQ-II spectrophotometer with quartz cuvettes, 3.0-ml capacity, having a 1.0-cm light path. Mannitol dehydrogenase was assayed spectrophotometrically, as indicated in the legend for Fig. 1. A unit of mannitol dehydrogenase is that amount of enzyme which will produce an initial rate of change of optical density of 0.001 per minute under the standard assay conditions at 25°. Specific activity is expressed as units per mg of protein.

Mannitol was determined by the method of Burton (9) after the removal of Tris, which interferes with the assay, by dialysis against 0.05 M phosphate buffer, pH 8.05. Fructose was determined by the method of Roe (10). NADPH was assayed spectrophotometrically according to the method of Ciotti and Kaplan (11) and was found to be 92.9% pure. NADP was assayed spectrophotometrically from the known extinction coefficient of the cyanide complex in 1.0 M KCN at 327 mp (11). The commercial material was found to be 97.3% pure. In the presence of NADPH, which also absorbs at 327 mp, the concentration of NADP was determined from the absorption of the cyanide complex at the isosbestic point, 332 mp.

**RESULTS**

Fig. 1 shows the change in optical density with time when D-fructose and NADPH were incubated with the 65 to 75% ammonium sulfate fraction. The addition of mannitol after most of the NADPH had been oxidized led to an immediate increase in optical density, as shown in the same figure. The observation that neither sorbitol nor mannitol-1-P produced a similar increase, and the identification of D-fructose by paper chromatography (in 80% phenol-water, using the aniline-hydrogen phthalate reagent of Partridge (12)) when mannitol and NADP were incubated with the 65 to 75% ammonium sulfate fraction, confirmed the identity of the enzyme under examination.

The reaction did not proceed without added enzyme or with boiled enzyme. With the original crude extract, there was a slight oxidation of NADPH in the absence of added substrate,
Enzymatic reduction of fructose by 65 to 75% ammonium sulfate fraction of *Agaricus campestris*. Enzyme, 60 unit (0.91 mg of protein), was added to 0.3 μmole of NADPH, 10 μmoles of d-fructose, and Tris buffer in a total volume of 2.7 ml. d-Mannitol, 100 μmoles, was added at the point shown.

The stoichiometry and the equilibrium constant of the reaction were determined by studying the reaction in the direction of fructose formation, as shown below.

\[ \text{Mannitol} + \text{NADP} \rightarrow \text{d-fructose} + \text{NADPH} + \text{H}^+ \]

The enzyme was added in aliquot portions so as to minimize the loss of activity during the incubations. After all of the enzyme had been added, 0.1-ml aliquots of the incubation mixture were removed at 15-minute intervals. When the optical density at 340 mμ was constant for two consecutive determinations, equilibrium was considered to have been attained, and aliquots were then assayed for NADPH, NAD, mannitol, and fructose. The mannitol values were corrected for fructose, which causes some interference. The results are shown in Table I, and indicate a one-to-one relationship between mannitol and NADP disappearance, and fructose and NADPH formation.

### Table I

<table>
<thead>
<tr>
<th>ΔMannitol (μ mole)</th>
<th>−ΔNADP (μ mole)</th>
<th>+ΔNADPH (μ mole)</th>
<th>+ΔFructose (μ mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.68</td>
<td>0.75</td>
<td>0.73</td>
<td>0.76</td>
</tr>
</tbody>
</table>

Fig. 1. Enzymatic reduction of fructose by 65 to 75% ammonium sulfate fraction of *Agaricus campestris*. Enzyme, 60 unit (0.91 mg of protein), was added to 0.3 μmole of NADPH, 10 μmoles of d-fructose, and Tris buffer in a total volume of 2.7 ml. d-Mannitol, 100 μmoles, was added at the point shown.
with mannitol, small changes in optical density, amounting to 5 to 10% of the normal activity, were observed.

DISCUSSION

Of the systems previously examined for mannitol dehydrogenase activity, Piricularia oryzae, a fungus causing the blast disease of rice (4), would appear to stand closest phylogenetically to A. campestris. Nevertheless, the enzyme from A. campestris catalyzes the oxidation of mannitol, does not catalyze the oxidation of mannitol-1-P, and appears to resemble the enzyme present in various acetobacters, for example, Acetobacter acetii, studied by De Ley and Schell (14). The ability of mannitol, but not sorbitol, to bring about the reduction of NADP formed from fructose and NADPH in the presence of the 65 to 75% ammonium sulfate fraction, and the identification of fructose as the product of this reaction, indicate the specificity of the enzyme.

SUMMARY

Mannitol has been shown to be formed directly from d-fructose in extracts from the cultivated mushroom Agaricus campestris by a reaction catalyzed by mannitol dehydrogenase, and dependent upon reduced nicotinamide adenine dinucleotide phosphate, as shown below.

Fructose + NADPH + H+ = mannitol + NAD

With a preparation purified approximately 13-fold by salt fractionation, the specificity of the enzyme has been examined; the stoichiometry and equilibrium constant were established; the Michaelis-Menten constants were determined; and the effect of certain inhibitors studied.

REFERENCES

Mannitol Dehydrogenase from Agaricus campestris
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