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# The enzymic determination of D-mannitol with mannitol dehydrogenase from *Agaricus bisporus**

Stephen Berezenko and Robert J. Sturgeon
*Department of Biological Sciences, Heriot-Watt University, Riccarton, Edinburgh EH14 4AS (Great Britain)*

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ABSTRACT

Mannitol dehydrogenase (D-mannitol:$NADP^+$ 2-oxidoreductase, EC 1.1.1.138), isolated from the sporocarps of *Agaricus bisporus*, has been purified 120-fold following fractionation with protamine sulphate, followed by hydrophobic and affinity chromatography. D-Mannitol and D-fructose appear to be the only substrate and products involved in the reaction, having $K_m$ values of 7.5 and 9.8mM, respectively. The purified enzyme has been used for the determination of D-mannitol and also the D-mannose content of glycoproteins and polysaccharides following the liberation of that hexose and reduction to D-mannitol.

## INTRODUCTION

Enzymes capable of catalysing the pyridine nucleotide-dependent formation of D-mannitol from D-fructose have been identified in various micro-organisms. These enzymes form part of a mannitol cycle which is an important NADPH regenerating system in many fungi imperfecti[1]. The distribution of enzymes of the mannitol cycle, including mannitol 1-phosphate dehydrogenase, mannitol 1-phosphatase, mannitol dehydrogenase, and hexokinase, have been reported[2]. In the fruit bodies of *Agaricus bisporus*, D-mannitol contributes up to 40% of the dry weight, with synthesis of the polyol occurring by reduction of free D-fructose in an NADPH-dependent dehydrogenase reaction, and is coupled to the dehydrogenase reactions of the hexose monophosphate shunt[3]. Three types of dehydrogenases have been classified for the interconversion of D-mannitol and D-fructose. Firstly, there are the polyol dehydrogenases with a wide range of substrate specificities for polyols in addition to D-mannitol. The enzymes from *Bacillus subtilis*[4], *Candida utilis*[5], *Gluconobacter oxydans*[6], and *Saccharomyces cerevisiae*[7] show various activities with each substrate, and exhibit either NAD- or NADP-dependence, but not both. Secondly, the mannitol dehydrogenase isolated from *Cephalosporium chrysogenum*[8] exhibits the same wide range of substrate specificities, but with the ability to use both NAD and NADP. In the third group, the mannitol dehydrogenases isolated from *Lactobacillus brevis*[9], *Leuconostoc mesenteroides*[10], *Agaricus campestris*[11], *Agaricus bisporus*[12,13], and *Aspergillus candidus*[14] have a specificity for only D-mannitol and either NAD or NADP.

* Dedicated to Professor Grant Buchanan on the occasion of his 65th birthday.

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In order to establish an enzymic method for the determination of a substrate, it is essential that the enzyme exhibits both a high degree of specificity, and a high affinity ($K_m$) for the substrate. We now describe an improved method for the purification of the mannitol dehydrogenase from *Agaricus bisporus*, and its use in the development of an enzymic method for the determination of D-mannitol.

## EXPERIMENTAL

Dithioerythritol, polyvinylpyrrolidone, protamine sulphate, Blue Sepharose, and Octyl-Sepharose were purchased from Sigma, and NADP from Boehringer. Edible mushrooms (*A. bisporus*) were purchased from local commercial sources.

*Hydrolysis of glycoproteins and polysaccharides.* — Samples (~5 mg) were hydrolysed in M $H_2SO_4$ for 1 h at 100° and the pH of each hydrolysate was adjusted to 8.5 with 2M NaOH. Sodium borohydride (0.1 mL, 10 mg.mL$^{-1}$) was added to each sample, and reduction was allowed to proceed for 2 h at room temperature. Excess of sodium borohyride was destroyed by the addition of 2M acetic acid (0.1 mL). Samples were adjusted to pH 8.5 (phenolphthalein end point) and kept at −10° until assayed.

*Analytical methods.* — Protein was measured by a modification of the Lowry method[15]. Polyphenol oxidase activity was determined by measuring the rate of formation of quinone from catechol, by the increase in absorbance at 420 nm[16].

*Enzymic estimations.* — Mannitol dehydrogenase activity was assayed spectrophotometrically by monitoring the increase in absorbance at 340 nm and 25° in a reaction mixture (final volume, 1 mL) containing 50mM Tris buffer (pH 8.8), 20mM D-mannitol, 1.2mM NADP, and enzyme. One unit of enzyme activity is the amount of enzyme that catalyses the formation of 1 μmol of NADPH per min at 25°.

*D-Mannitol.* — The initial absorbance at 340 nm of a mixture of 50mM Tris buffer (0.75 mL, pH 8.8), NADP (0.1 mL, 10 mg.mL$^{-1}$), and a solution of D-mannitol (either a standard or derived from a glycoprotein or polysaccharide hydrolysate, 0.15 mL) was measured. The reaction was initiated by the addition of mannitol dehydrogenase (0.02 mL, 0.25 U), and the change in absorbance at 340 nm was measured during 3 min. Using a standard solution of D-mannitol, a linear response for the rate of production of NADPH, and thus D-mannitol, was obtained in the range 0.1–2.0 μmol. D-Mannose was determined using the hexokinase assay[17].

*Purification of mannitol dehydrogenase.* — All operations were carried out at 4°. The gill tissue was removed from the underside of the sporocarps from *A. bisporus* (400 g). Sporocarps plus stalk tissue were then diced and homogenised with 50mM Tris buffer (pH 8.0) containing dithioerythritol (1mM) and polyvinylpyrrolidone (5% w/v) in a Waring Blendor (1 min). The homogenate was centrifuged (15,000*g*, 10 min), protamine sulphate was added to the supernatant solution (to a final concentration of 0.15%), and the precipitate was removed by centrifugation, as described above, and discarded. Polyphenol oxidases were then removed by adsorption on a column of Octyl-Sepharose (bed volume, 50 mL) equilibrated with the same buffer containing lactose (0.1% w/v) in order to stabilise the enzyme. Mannitol dehydrogenase does not bind to this support.

Elution with the buffer was continued until pigmented polyphenols just emerged in the eluate. Eluate containing mannitol dehydrogenase was then adsorbed on a column (12 × 1.5 cm.) of Blue Sepharose, equilibrated with the Tris–lactose buffer. The adsorbed enzyme was washed with buffer (3 vol.) and then eluted with 0.5M sodium chloride. Fractions containing the active enzyme were combined and dialysed against Tris lactose buffer. The preparation was stable in 3.2M ammonium sulphate for several months.

## RESULTS AND DISCUSSION

Initial experiments on the isolation of mannitol dehydrogenase from *A. bisporus* indicated a six-fold higher specific activity in the cap and stalk tissue compared with the gill tissue of the fungus. In addition, greater levels of polyphenol oxidase activity were detected in the gill tissue. It is essential in the purification scheme that polyphenol oxidase activity and phenolic contaminants are removed as efficiently and as quickly as possible, otherwise the final enzyme preparation gives unacceptably high absorbance readings in the spectrophotometric assay. Of the inhibitors of polyphenol oxidase which were examined (ascorbic acid, sodium cyanide, sodium azide, EDTA, and dithioerythritol), only dithioerythritol (1mM) was an efficient inhibitor of polyphenol oxidase, without inhibiting mannitol dehydrogenase activity (Table I). Dialysis of either the crude or purified mannitol dehydrogenase preparations resulted in rapid loss of enzymic activity. However, all enzymic activity was retained when dialysis was carried out in the presence of buffers containing 0.1% (w/v) of lactose. The lactose may be replaced by D-glucose, D-galactose, maltose, or D-glucitol. However, the use of D-glucitol is not recommended if the enzyme is to be used for analytical purposes since most commercial samples of D-glucitol are contaminated with D-mannitol. In the purification scheme used, the enzyme was obtained in ~50% yield and with 123-fold purification (Table II). The purified mannitol dehydrogenase is stable as a suspension in 3.2M ammonium sulphate at 4° for several months.

The enzyme catalysed the reversible oxidation of D-mannitol to D-fructose in the presence of NADP. No reduction of the cofactor occurred with the following polyols: glycerol, erythritol, D-glucitol, galactitol, D-arabinitol, L-arabinitol, ribitol, and D-mannitol 1-phosphate. The enzyme did not catalyse the reduction of D-glucose, D-mannose, D-galactose, D-xylose, or D-arabinose in the presence of NADPH. NADP could not be replaced by NAD in the enzymic reaction. The maximum activity of the enzyme was at pH 8.8–9.0 in the direction of D-mannitol oxidation when measured in 50mM Tris buffer, 20mM D-mannitol, and 1.2mM NADP. In the direction of D-fructose reduction, the maximum activity was between pH 6.5–7.5 when measured in 50mM phosphate buffer, 20mM D-fructose, and 85$\mu$m NADPH. The $K_m$ values of the purified enzyme for D-mannitol, D-fructose, and NADP, as determined by the Lineweaver–Burk method, were 7.2, 9.8, and 0.28mM, respectively. Other workers have reported a much lower affinity of the mannitol dehydrogenase of *A. bisporus*[12,13] for these substrates, namely, 34 and 190mM for D-mannitol and D-fructose, respectively, and 20 and 35mM, respectively, for the *Leuconostoc mesenteroides* enzyme[10].

TABLE I

Inhibition of mannitol dehydrogenase and polyphenol oxidase

| *Inhibitor* (*1mM*) | *Polyphenol oxidase* | D-*Mannitol dehydrogenase* |
|---|---|---|
| Dithioerythritol | 65[a] | 105 |
| Ascorbic acid | 64 | 70 |
| NaCN | 87 | 85 |
| $NaN_3$ | 77 | 95 |
| EDTA | 89 | 98 |

[a] All values are the % of enzyme activity remaining after incubation of the enzyme with inhibitor, as compared to the corresponding activities (100%) found in the absence of inhibitor

TABLE II

Purification of mannitol dehydrogenase from *Agaricus bisporus*

| *Fraction* | *Volume* (*mL*) | *Total protein* (*mg*) | *Total units* (*U*) | *Specific activity* (*U/mg*) | *Yield* (%) | *Purification factor* |
|---|---|---|---|---|---|---|
| Crude | 605 | 1143 | 128 | 0.11 | 100 | |
| Protamine sulphate | 645 | 922 | 133 | 0.14 | 104 | 1.3 |
| Octyl-Sepharose | 198 | 183 | 96 | 0.53 | 75 | 4.8 |
| Blue Sepharose | 13.7 | 4.8 | 65 | 13.60 | 51 | 123.0 |

In order to examine the usefulness of the purified mannitol dehydrogenase for the determination of $\mu$mol quantities of D-mannitol, a number of polysaccharides and glycoproteins containing D-mannose were examined. After acid hydrolysis, the products were reduced with sodium borohydride and the D-mannitol produced was determined. Close correlation was observed between the D-mannitol values obtained and the corresponding values for D-mannose on samples not treated with sodium borohydride (Table III). The method now reported has the advantage of requiring only one enzyme and one cofactor, as compared with four enzymes and one cofactor for the enzymic measurement of D-mannose[17].

TABLE III

Determination of the D-mannose content of polymers

| *Polymer* | D-*Mannose*[a] (%) | D*Mannitol* (%) (*derived from* D-*mannose*) |
|---|---|---|
| Fetuin | 3.0 | 3.0 |
| Invertase | 45.0 | 45.5 |
| Yeast mannan | 80.0 | 80.9 |
| Galactomannan (*Delonia regia*) | 79.6 | 80.5 |

[a] D-Mannose was determined by the hexokinase assay.

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