Mannitol Metabolism in *Agaricus bisporus*: Purification and Properties of Mannitol Dehydrogenase

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Mannitol dehydrogenase (EC 1.1.1.138) has been purified to homogeneity from fruit bodies of *Agaricus bisporus*. M_r values of 115000 were determined by gel filtration and 130000 by rate zonal ultracentrifugation. The sedimentation coefficient is 6.5S. The native protein is composed of four subunits of M_r 29000. The enzyme is specific for NADP, and shows low activity with sorbitol. Normal Michaelis–Menten kinetics are exhibited for both mannitol and NADP, giving K_m values of 16.2 mM and 36 μ M respectively at pH 7.0. The K_m value for NADPH is 38.5 μ M and that for fructose approximately 1.2 M. The V_{max} is 591 μ M min⁻¹ (mg protein)⁻¹ for mannitol synthesis and 5 μ Mol min⁻¹ (mg protein)⁻¹ for fructose synthesis at pH 7.0. Inhibition of fructose synthesis by NADPH is stronger than inhibition of mannitol synthesis by NADP. The results are discussed with respect to the control of enzyme activity under physiological conditions.

INTRODUCTION

Mannitol synthesis and accumulation are major features of the growth of *Agaricus bisporus* fruit bodies (Rast, 1965; Hammond & Nichols, 1976). Mannitol is synthesized from fructose using NADPH as cofactor. The enzyme responsible, mannitol dehydrogenase (EC 1.1.1.138), has been partially purified and characterized previously (Edmundowicz & Wriston, 1963; Ruffner *et al.*, 1978). However, there are inconsistencies in the published results which may be due to incomplete purification. To advance our knowledge of the function and control of mannitol metabolism in fruit body growth, it was necessary to obtain accurate kinetic data and to assess the possibilities of *in vivo* regulation of the enzyme. The results of a programme of complete purification and characterization are reported here.

METHODS

A. bisporus (strain 'Darlington's 649') was grown in plastic buckets in compost prepared at the Glasshouse Crops Research Institute. Fruit bodies (stage 2–3; Hammond & Nichols, 1976) were thinly sliced, frozen in liquid nitrogen, and stored at –20 °C until used.

MTLDH purification. All operations were done at 4 °C. The procedure for a typical purification was as follows. Frozen slices of fruit body (340 g) were thawed and homogenized in 25 ml 10 mM-Tris/HCl buffer, pH 7.5, containing 50 mM-2-mercaptoethanol and 0.25 mM-phenylmethylsulphonyl fluoride (buffer 1). The homogenate was centrifuged at 15000 *g* for 30 min and the supernatant retained. Nucleic acids were precipitated by addition of excess protamine sulphate to give a final concentration of approximately 0.25% (w/v). The suspension was centrifuged as above. Protein was then precipitated from the supernatant by slow addition of ammonium sulphate. The fraction precipitating between 326 and 516 g ammonium sulphate l⁻¹ was centrifuged down and dissolved in a minimum volume (approx. 5 ml) of buffer 1. The preparation was applied to a Sephadex G100 column (33 × 2.5 cm) equilibrated with buffer 1, and was eluted with the same buffer. The active fractions, which eluted in the

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Abbreviation: MTLDH, mannitol dehydrogenase.

void volume, were pooled and concentrated to approximately 8 ml in an Amicon ultrafilter with a PM10 membrane. The concentrated sample was applied to a DEAE-Sephadex A-50 column (30×1.4 cm) equilibrated with buffer 1, and was eluted with the same buffer. The active fractions, which eluted in the void volume, were pooled and applied to a column (5×1 cm) of Matrex Gel Red A (Amicon) equilibrated with buffer 1. The column was washed with the same buffer until no further protein was eluted. The bound protein was eluted with a linear 100 ml gradient of 0.4 M-NaCl in buffer 1. The activity eluted as a single peak at 2.5–3.0 M-NaCl. The active fractions were pooled and dialysed against buffer 1 containing 10 mM-2-mercaptoethanol and 0.05 mM-phenylmethylsulphonyl fluoride. The retentate was concentrated by ultrafiltration. Glycerol was added to a concentration of 50% (v/v), and the preparation stored at -20°C . There was no significant loss of activity after storage for 1 month in this form. Protein content of solutions was determined by the method of Lowry.

MTLDH assay. MTLDH was assayed by monitoring oxidation of NADPH or reduction of NADP at 340 nm. Assays were done at 25°C in a total volume of 1 ml. The reduction of fructose was assayed by incubating a sample of extract in 50 mM-Tris/HCl buffer, pH 7.5, containing 800 mM-fructose and 0.25 mM-NADPH. The oxidation of mannitol was assayed by incubating extract in 50 mM- Na_2CO_3 buffer, pH 10.4, containing 200 mM-D-mannitol and 1 mM-NADP. One unit of activity is the amount of enzyme which catalyses the oxidation of $1\ \mu\text{mol}$ NADPH min^{-1} , or the reduction of $1\ \mu\text{mol}$ NADP min^{-1} in the above systems. Assays were done in the direction of fructose reduction except where stated otherwise.

Kinetics. For the determination of kinetic parameters, the above assay system was used with pure enzyme, except that while one substrate concentration was varied the other substrate was held at a saturating concentration (NADP = 1 mM; NADPH = 0.25 mM; mannitol = 200 mM). The fructose concentration was 800 mM since higher concentrations led to substrate inhibition (see Results). Kinetic parameters were determined between pH 6.5 and 11.0, in 50 mM Tris/maleate, Tris/HCl or Na_2CO_3 buffers as appropriate.

Determination of k_{eq} . Pure enzyme (10 μl , 4.4 μg) was incubated with 36.9 μM -NADP and 500 μM -D-mannitol in 1.0 ml 50 mM- Na_2CO_3 buffer, pH 10.18. The reaction was allowed to reach equilibrium as determined by a constant absorbance at 340 nm. The increase in concentration of NADPH was calculated from the increase in A_{340} , and the molar absorption coefficient of NADPH ($6220\ \text{M}^{-1}\ \text{cm}^{-1}$). Equimolar decreases in NADP and mannitol concentrations, and a molar stoichiometry of 1:1 between NADPH and fructose produced, were assumed (Edmundowicz & Wriston, 1963). The concentration of H^+ ions was calculated from the measured pH.

M_r determination. A sample of purified enzyme (0.25 ml; 0.33 mg ml^{-1}) was applied to a column (120×1.5 cm) of Sephadex G200 equilibrated with 50 mM-Tris/HCl buffer, pH 7.5. The enzyme was eluted with the same buffer. Protein in the fractions was detected by absorbance at 280 nm and enzyme activity by the assay described above. Standard proteins were: catalase (M_r 240000); horse liver alcohol dehydrogenase (150000); bovine serum albumin (66000); ovalbumin (45000); and horse myoglobin (17000). Analytical ultracentrifugation and rate zonal ultracentrifugation were done as described by Hammond (1985a).

Electrophoresis. Non-denaturing PAGE was done in 8% (w/v) polyacrylamide gels (1% crosslinked) with 40 mM-acetate buffer, pH 5.0. A reservoir buffer of 40 mM-acetate, pH 5.0, was used. MTLDH activity was detected by incubating the gels for 5–20 min at 25°C in 50 mM- Na_2CO_3 buffer, pH 10.4, containing 100 mM-D-mannitol, 1 mM-NADP, 0.002% phenazine methosulphate and 0.032% nitroblue tetrazolium. Protein bands were detected using Coomassie blue. SDS-PAGE was done in 8% polyacrylamide gels (1% crosslinked) containing 0.1% SDS, with 40 mM-potassium phosphate buffer, pH 7.2. The reservoir buffer was 0.1 M-potassium phosphate, pH 7.2, containing 0.01 M-EDTA and 0.1% SDS. Samples were prepared by heating to 100°C with reservoir buffer and 1% 2-mercaptoethanol (1:1, v/v). M_r standards were: *Bacillus stearothermophilus* methionyl-tRNA synthetase (90000); bovine serum albumin (66000); *Bacillus subtilis* tryptophanyl-tRNA synthetase (47000); rabbit muscle aldolase (40000); bovine chymotrypsinogen (25700) and lysozyme (14300). The *Bacillus* enzymes were gifts from Dr C. J. Bruton, Imperial College, London, UK.

Paper chromatography of reaction products. The incubation products of the mannitol dehydrogenase reaction were applied to Whatman no. 1 paper and separated by descending chromatography for 22 h at room temperature in ethyl methyl ketone/acetic acid/borate saturated water (9:1:1, by vol.). D-Fructose, D-glucose, D-mannitol and sorbitol markers were also run. After drying, the chromatogram was dipped in a saturated solution of lead tetraacetate in glacial acetic acid and allowed to dry. Sugars and sugar alcohols appeared as white spots against a brown background.

RESULTS

Purification of MTLDH. The results of a typical purification are shown in Table 1. A sample of 50 μg of the final preparation gave a single protein band after SDS-PAGE. PAGE of the native enzyme also gave a single protein band which corresponded with the band stained for MTLDH activity.

Table 1. *Purification procedure for mannitol dehydrogenase*

Fraction	Volume (ml)	Protein content (mg ml ⁻¹)	Specific activity (units mg ⁻¹)	Yield (%)	Purification (fold)
Crude extract	225	23.6	1.032	100	1.00
Protamine sulphate precipitate	213	21.7	1.128	95.2	1.09
45–80% (sat. (NH ₄) ₂ SO ₄) precipitate	8.7	65.4	2.388	24.8	2.31
Concentrated Sephadex G100 eluate	7.7	16.6	3.954	9.27	3.83
DEAE Sephadex A50 eluate	27.5	0.098	42.42	2.10	41.10
Concentrated Matrex Red eluate	2.1	0.44	194.40	3.27	188.40

Table 2. *Kinetic parameters of mannitol dehydrogenase at optimal pH and pH 7.0*

Values are means of at least two determinations with less than 10% variation between replicates.

Reaction	pH	K_m (mM)	V_{max} [$\mu\text{mol min}^{-1}$ (mg protein) ⁻¹]
Fructose reduction	7.0 (optimal)	Fructose 1.2 $\times 10^3$	591
		NADPH 3.85 $\times 10^{-2}$	
Mannitol oxidation	10.25 (optimal)	Mannitol 16.2	70.2
		NADP 8.2 $\times 10^{-2}$	
	7.0	Mannitol 16.2	5.0
		NADP 3.6 $\times 10^{-2}$	

M_r determination. SDS-PAGE of the purified enzyme gave a value of 29500 for the subunit *M_r*. The *M_r* of the native enzyme was 115000 by gel filtration and 130000 by rate zonal ultracentrifugation. The sedimentation coefficient (*s*_{20,w}) of the native molecule was 6.5S. Values are means of at least two determinations, showing less than 5% variation.

Specificity. No reaction was observed when D-fructose was replaced with D-glucose, D-galactose, L-sorbose or D-fructose 6-phosphate. When mannitol was replaced by sorbitol, the reaction rate was reduced to 7.7% of the value with mannitol. No reaction was observed with D-galactitol, ribitol, L-arabitol or erythritol. NADP and NADPH could not be replaced by NAD and NADH. With D-mannitol as substrate, D-fructose was identified as the sole product by paper chromatography; with D-fructose as substrate, D-mannitol was the sole product.

Kinetics. Linear Lineweaver–Burk and Eadie–Hofstee plots were obtained with all substrates in both directions of the reaction. The values for *V_{max}* and apparent *K_m* were calculated from the plots. The pH optima for fructose reduction and mannitol oxidation were 7.0 and 10.25 respectively. Table 2 summarizes the data for the reaction.

The *K_m* for mannitol was relatively constant at 16.2 mM over the pH range. Similarly, the *K_m* for NADPH varied between 17.0 μM at pH 7.25 and 19.2 μM at pH 10.25. The *K_m* for NADP rose from 36 μM at pH 7.5 to 86 μM at pH 10.5. Concentrations of NADP above 1.5 mM caused inhibition.

Product inhibition. With fructose as substrate and NADPH at a constant, non-saturating concentration (6.9 μM), added NADP inhibited the reaction. A NADP/NADPH ratio of approximately 38 was necessary for 50% inhibition of the reaction (Fig. 1). In the direction of mannitol oxidation, NADPH inhibited the reaction strongly (Fig. 1). With a saturating concentration of NADP (1 mM), 50% inhibition was seen at a NADPH/NADP ratio of 0.1, and with a non-saturating concentration of NADP (20 μM), 50% inhibition was seen at a ratio of 0.45. These data were obtained at pH 7.5.

Fig. 1. Effect of coenzyme product on MTLDH reaction rate. (a) Effect of increasing NADPH/NADP ratio on rate of mannitol oxidation at pH 7.5, with a saturating mannitol concentration (200 mM) and a saturating (1 mM; ○) or non-saturating (20 μM; □) NADP concentration. 100% = 0.61 μmol min⁻¹ (mg protein)⁻¹. (b) Effect of increasing NADP/NADPH ratio on the rate of fructose reduction at pH 7.5, with a saturating fructose concentration (800 mM) and non-saturating NADPH concentration (6.9 μM). 100% = 149 μmol min⁻¹ (mg protein)⁻¹.

Table 3. Concentrations of reactants and products to determine k_{eq} for mannitol dehydrogenase

Values are means of two determinations, showing less than 10% variation between replicates.

Reactant/product	Initial concn (M)	Final concn (M)
Mannitol	5.00×10^{-4}	4.70×10^{-4}
NADP	3.69×10^{-5}	6.52×10^{-6}
Fructose	0	3.04×10^{-5}
NADPH	0	3.04×10^{-5}
H ⁺	6.61×10^{-11}	6.61×10^{-11}

Determination of k_{eq} . The initial and final concentrations of reactants and products for the reaction are given in Table 3. The final concentrations obtained give:

$$k_{eq} = \frac{[\text{fructose}][\text{NADPH}][\text{H}^+]}{[\text{mannitol}][\text{NADP}]} = 1.99 \times 10^{-11} \text{ M}$$

DISCUSSION

A homogeneous preparation of MTLDH, as indicated by native and SDS-PAGE, has been isolated from *A. bisporus* fruit bodies. The subunit M_r of 29 500 and native M_r of 115 000–130 000 suggest that the enzyme is a tetramer. The value of 40 000 for the native M_r reported by Ruffner *et al.* (1978) thus appears to be based on the subunit value.

The high specificity of MTLDH for NADP(H) as coenzyme, and for D-fructose as substrate, confirms previous findings (Ruffner *et al.*, 1978), although differences in polyol specificity were found. No detectable activity was observed with L-arabitol in the present investigation, and activity with sorbitol was 7.7% of that with D-mannitol. Ruffner *et al.* (1978) reported values of 2% and 21% respectively for the same polyols; these observations may have resulted from contaminating enzyme activities. Since there have been no reports of significant levels of polyols other than mannitol in *A. bisporus*, it is unlikely that the activity of MTLDH against sorbitol is physiologically important.

Concentrations of fructose above 1.2 M at pH values below pH 9.9, and above 0.8 M above pH 9.9, resulted in substrate inhibition. Since substrate inhibition was seen at fructose concentrations similar in value to the K_m , saturation of the enzyme with this substrate was not possible under experimental conditions. Because of this, a fructose concentration of 800 mM was used in assays, which gave a rate of fructose reduction which was 90% of that at the optimum fructose concentration. Using this fructose concentration and varying the NADPH concentration gave apparent first order kinetics with respect to NADPH. Apparent values for K_m and V_{max} of 17 μ M and 240 μ mol min⁻¹ (mg protein)⁻¹ were calculated from these data. Double reciprocal plots of initial velocity against the concentration of one substrate at a series of fixed concentrations of a second substrate yield a set of parallel lines (see for example Velick & Vavra, 1962). By using the V_{max} obtained by varying the fructose concentration in the presence of a saturating concentration of NADPH [591 μ mol min⁻¹ (mg protein)⁻¹] as the intercept for the construction of a double reciprocal plot parallel to that obtained by varying the NADPH concentration in the presence of 800 mM-fructose, a corrected K_m of 38.5 μ M was obtained for NADPH.

The parallel double reciprocal plots imply a ping-pong mechanism with the Haldane relationship:

$$k_{eq} = \left(\frac{V^f}{V^r} \right)^2 \cdot \frac{K_m^P K_m^Q}{K_m^A K_m^B}$$

If the kinetic parameters for pH 7.0 are substituted and the H⁺ concentration is corrected for, the k_{eq} is calculated to be 5.7×10^{-10} . This is close to the value arrived at by equilibrium measurements, and thus supports the value obtained for the K_m for fructose.

The K_m values for coenzymes given here (Table 2) are similar to those reported previously. The K_m for mannitol is half that reported by Ruffner *et al.* (1978) (34 mM). The value of 0.46 mM found by Edmundowicz & Wriston (1963) may have been low because of the impurity of the enzyme preparation. The K_m for fructose shows larger discrepancies; the values of Ruffner *et al.* (1978) (190 mM) and Edmundowicz & Wriston (1963) (1.4 mM) are considerably lower than the 1.2 M found in this investigation. However, high K_m values have been reported for similar enzymes; a K_m of 1.0 M for fructose was reported for the MTLDH of *Cephalosporium chrysogenum* (Birken & Pisano, 1976). The high value obtained in the present study indicates that the physiological rate of mannitol production by the reaction must be directly proportional to the concentration of fructose, assuming that there are no other constraints on activity. As the overall fructose concentration in the cell appears to be no higher than 3 mM (Hammond, 1985*b*), compartmentation of mannitol synthesis must be presumed to exist; otherwise the physiological role of the enzyme would be in doubt since it would be operating at less than 1% of its V_{max} .

Physiological mannitol oxidation should occur at a rate almost independent of mannitol concentration in the fruit body, since the K_m is considerably lower than the observed intracellular concentration (Hammond, 1985*b*). Thus the physiologically important reaction, fructose reduction, can be subject to far greater rate control by substrate concentration than the reverse reaction.

Ruffner *et al.* (1978) placed emphasis on the sensitivity of fructose reduction to NADP concentration. Some sensitivity was observed here; however, a non-physiological NADP/NADPH ratio was necessary to give 50% inhibition. The NADP/NADPH ratio of approximately 1:1 estimated to be present *in vivo* (Hammond, 1985*b*) suggests that inhibition is unlikely to occur unless there is compartmentation of the oxidized and reduced forms of the coenzyme.

In contrast, the present study indicates that mannitol oxidation is very sensitive to NADPH concentration. The concentrations of NADP and NADPH in *A. bisporus* fruit bodies were reported to be between 50 and 180 μ M (Hammond, 1985*b*). At this NADP concentration, 50% inhibition of the reaction would be achieved with an NADPH/NADP ratio of about 0.25, and the *in vivo* ratio would give about 70% inhibition (see Fig. 1*a*). Hence in the physiological NADP(H) concentration range, small changes in the redox state of the coenzyme will have a considerable effect on the rate of mannitol oxidation.

Under normal conditions of fruit body growth, it seems likely that the rate of mannitol synthesis is governed by the supply of fructose and NADPH. The k_{eq} for the reaction shows that it is heavily displaced towards mannitol synthesis. Only under conditions of low NADPH relative to NADP would mannitol oxidation occur at a significant rate. Such conditions could occur after the fruit body has been harvested or during senescence, when mannitol concentrations in the fruit body are known to fall (Hammond & Nichols, 1975; 1976).

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