Phytochemistry, 1978, Vol 17, pp 865–868 © Pergamon Press Ltd. Printed in England 0031-9422/78/0501-0865 $02.00/0

PURIFICATION AND PROPERTIES OF MANNITOL DEHYDROGENASE FROM *AGARICUS BISPORUS* SPOROCARPS

HANS PETER RUFFNER, DORA RAST, HANSPETER TOBLER and HANS KARESCH
Institute of Plant Biology, University of Zürich, Switzerland

(*Received* 11 *October* 1977)

Key Word Index—*Agaricus bisporus*; Agaricaceae; cultivated mushroom; mannitol dehydrogenase; purification; properties.

Abstract—Mannitol dehydrogenase (mannitol: $NADP^+$ 2-oxidoreductase: EC 1.1.1.138) was isolated from *Agaricus bisporus* by fractionation with protamine sulphate and $(NH_4)_2SO_4$, followed by chromatography on DEAE-Sephadex, then by affinity and gel chromatography. The products of enzyme reaction were identified by GLC and TLC. K_m, optimum pH, MW and pI of the enzyme as well as the influence of temperature, ions and inhibitors on enzymic activity were determined. In the sugar reducing reaction, the enzyme was specific for fructose but, in the reverse direction, some structurally related polyols could substitute for mannitol. The enzyme was very sensitive to alterations in the $NADP^+$/NADPH ratio. The results are discussed in relation to the possible role of mannitol dehydrogenase in fungal metabolism.

INTRODUCTION

Although D-mannitol is a very common and abundant polyol in most higher fungi, its physiological role has not yet been elucidated and may vary among different types of fungi. The identification of specific synthetic and catabolic enzymes that metabolize mannitol also represents a still unresolved problem [1, 2].

In *Agaricus bisporus* fruit bodies, mannitol contributes up to 40% of the dry weight [3]. Synthesis of the polyol in mushroom fruit bodies occurs by reduction of free fructose in an NADP-dependent dehydrogenase reaction [4] and is coupled to the dehydrogenase reactions of the hexose monophosphate shunt [5] Using various sugars as carbon source, Dütsch and Rast [5, 6] furthermore found a direct relationship between mannitol formation and mycelial growth. These observations suggest an involvement of mannitol dehydrogenase in growth control. More recent results indicate a close correlation also between mannitol dehydrogenase activity and the growth rate of mushroom mycelium in submerged culture [7], thus supporting the hypothesis of growth regulation by mannitol synthesis.

Although mannitol dehydrogenase thus appears to play an important role in fungal metabolism, little is known about its characteristics. NADP-dependent mannitol dehydrogenases have been investigated by several authors [8–12] either in crude extracts or as partly purified preparations; Edmundowicz and Wriston [12] reported a 13-fold purification of the mannitol dehydrogenase from *Agaricus campestris*. The present paper describes an improved method for purification of mannitol dehydrogenase from *A. bisporus* and some of its characteristics.

RESULTS

Purification

Preliminary experiments had shown mannitol dehydrogenase to be most active in very young fruit bodies, and most stable in relatively concentrated form at a temperature below 4°. These conditions were therefore maintained and care has been taken to shorten or eliminate purification steps that expose the enzyme to excess dilution.

Mushrooms (50 g) were sliced and homogenized for 2 × 1 min in a cooled Sorvall Omnimixer with 50 ml 0.25 M Tris–HCl buffer, pH 8, containing 0.015 M cysteine-HCl. Insoluble material was removed by centrifugation at 15000 *g* for 10 min. Protamine sulphate solution (3%) was added dropwise with stirring to a final concentration of 0.125%. The protamine–nucleic acid complex was removed by centrifugation as above. The protein fraction precipitating between 55 and 80% saturation with $(NH_4)_2SO_4$ was redissolved after centrifugation (15000 *g*; 20 min) in 2 ml of 0.1 M Tris–HCl, pH 8 buffer. The extract was then desalted by passing it through a Sephadex G-25 column. The fractions containing the highest enzymic activities (a total of 8 ml) were pooled and reduced to 2 ml in an Amicon concentrator.

The concentrate was applied to a Sephadex A-25 anion exchanger column previously equilibrated with 0.1 M Tris–HCl, pH 8. The protein fraction emerging immediately after the void volume contained the mannitol dehydrogenase activity. Under these conditions the enzyme was not attached to the gel, whereas most of the phenolic contaminants were held back. The extract was then concentrated as above, immediately after collection. At this stage the enzyme could be stored conveniently overnight or longer in the cold room without loss of activity.

The concentrate was subsequently coupled to NADP-Sepharose in 0.1 M Tris–HCl, pH 8 [12], and washed extensively with portions of the same buffer. The enzyme was dissociated from the gel by addition of 2.5 ml 8 mM NADP solution. After 15 min the liquid was filtered, the gel washed with buffer and the combined

Table 1. Purification scheme for mannitol: $NADP^+$ 2-oxidoreductase from *Agaricus bisporus*

Fraction	Vol. (ml)	Protein (mg)	Activity		Yield	Purification
			Total (nkat)	Sp. (nkat)		
1 Crude extract	75	876	25785	29.4	100	
2 Protamine sulphate	74	640	26286	41.1	102	1.4
3. Ammonium sulphate (55–80% + G-25)	8.0	90	22629	155.0	88	5.3
4. DEAE-Sephadex	3.0	25	8250	332.3	32	11.3
5. NADP-Sepharose	2.6	2.80	7284	2580.8	28	87.8
6. Sephadex G-100	4.5	1.35	3340	2477.1	13	84 3

filtrates were concentrated. Approximately 90% of the enzymic activity was recovered by this method. In order to get regular conditions for the following enzyme assays, excess NADP was removed by gel filtration.

The protein–nucleotide mixture was applied to a 2.5 × 40 cm column of Sephadex G-100 and chromatographed. The fractions containing the enzyme were pooled and scanned in a spectrophotometer. The UV spectrum showed a sharp peak at 280 nm, whereas the nucleotide band at 260 nm had disappeared.

Properties

The identity of the enzyme under examination was confirmed by GLC of the product after the enzyme was incubated in a mixture of buffer and fructose, with and without addition of NADPH. The mixtures were then immediately frozen in liquid nitrogen, lyophilized, silylated in pyridine and chromatographed. The detector trace showed a marked decrease in fructose content within the sample containing NADPH, accompanied by an almost quantitative transformation into a new peak with retention characteristics of mannitol. For TLC analysis, the assay mixtures containing fructose or mannitol as substrates were boiled after incubation and chromatographed. Mannitol and fructose could be identified as the products of the respective reaction.

Maximum activity was found between pH 7.9 and 8.1 in the direction of fructose reduction and between pH 8.7 and 8.8 in the opposite direction. K_m values of the purified enzyme for D-fructose, D-mannitol and NADPH as determined by the Lineweaver–Burk method were 190, 34 and 0.525×10^{-2} mM, respectively. Optimum activity was observed at 47° in the fructose–mannitol direction, and 50% thermal inactivation occurred after 10 min of exposure at 45° and in less than 2 min at 55°.

Comparison of the migration distance in thin layer gel filtration of the enzyme with those of a mixture of standard proteins indicates a MW of *ca* 40000. The purified enzyme was subjected to isoelectric focusing in a pH gradient between 9 and 11, and its activity subsequently localized by means of a histochemical dehydrogenase test on a paper replica of the gel. Mannitol dehydrogenase formed two zones with pI values of 9.1 and 9.3.

In the sugar reducing reaction, L-sorbose, D-mannose, D-galactose, D-ribose, DL-arabinose, D-xylose, D-glucose, glucose-6-phosphate and fructose-6-phosphate (all 0.52 M) with NADPH and NADH separately as cosubstrates, were examined as potential alternative substrates for the mannitol dehydrogenase. Whereas in the crude extract very slight activity (1% and less, compared with fructose-NADPH) was found, the purified enzyme reduced none of these sugars or sugar phosphates, either in the presence of NADPH or of NADH.

As indicated in Table 2, the enzyme displays less specificity towards polyols in the oxidative reaction, but also in this case a strict requirement for NADP was observed.

We have tested the effect of PCMB, KCN, IA and NEM (all 10 mM) on mannitol dehydrogenase activity in the polyol synthesizing direction and found 94, 33 and 17% inhibition, respectively, for the first three reagents, whereas NEM had no effect. In routine tests with chlorides of manganese, magnesium, barium and calcium (5 mM), we could not detect any significant effects on the sugar reducing reaction. Interestingly however, in the other direction these cations do cause a marked increase in enzymic activity, viz. 115, 136, 137 and 152%, respectively, going up to a maximum of 240% with 10 mM Ca^{2+}. Finally, the enzyme is inhibited by addition of the respective antagonistic form of the nucleotide to the incubation mixture (Fig. 1). Half maximal activity in the fructose reducing reaction was reached at an $NADP^+$/NADPH ratio of 4:1 and in the polyol oxidizing direction at a ratio as low as 0.25.

DISCUSSION

Present knowledge of the characteristics of mannitol dehydrogenases that catalyse the reaction: fructose + NADPH $\rightleftharpoons$ mannitol + $NADP^+$, is very fragmentary and sometimes contradictory. Whereas enzyme

Table 2. Substrate specificity of mannitol dehydrogenase in the polyol oxidizing direction (expressed as percent of the activity with mannitol and NADP as substrates)

Substrates (concn 0.26 M)	Crude extract		Purified enzyme	
	NADP	NAD	NADP	NAD
D-Mannitol	100	10	100	0
D-Sorbitol	27	11	21	1
Perseitol	9	0	6	0
D-Arabitol	3	3	3	0
L-Arabitol	11	34	2	0
Adonitol	0	9	0	0
Xylitol	0	3	0	0
Galactitol	3	3	0	0
Mannitol-1-P	0	4	0	0

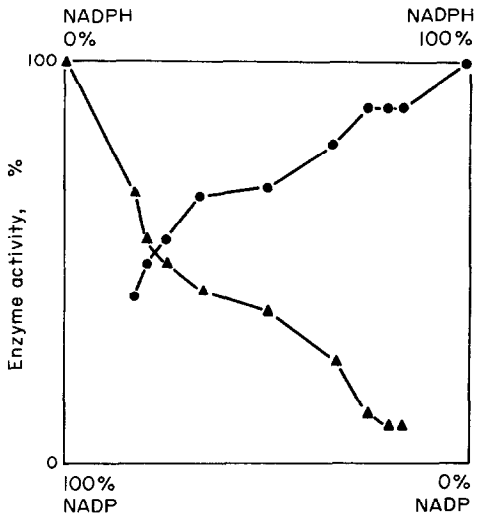

Fig. 1. Effect of alterations in the $NADP^+$/NADPH ratio on the activity of mannitol dehydrogenase (expressed as percent of activity under standard assay conditions). ●—● fructose → mannitol: 100% = 45.0 nkat. ▲—▲ mannitol → fructose: 100% = 10.5 nkat.

preparations from *Diplodia viticola* [8], *Dendryphiella salina* [10] and *Cephalosporium chrysogenum* [11] have been found to be relatively unspecific as to the substrate, those from *Agaricus campestris* [12] and *Aspergillus candidus* [9] are strictly specific for fructose or mannitol. A similar situation was observed in the enzyme nucleotide requirements, the *Diplodia* and *Aspergillus* preparations being exclusively NADP-dependent, whereas the enzymes from the other sources could substitute NAD for NADP with varying efficiency (8–40%). Purified mannitol dehydrogenase from *A. bisporus* is very specific for NADP and fructose in the reductive reaction, but in the reverse direction some polyols are metabolized at least to some extent (Table 2). The degree to which polyol oxidation can be catalysed by mannitol dehydrogenase seems to depend largely on the steric configuration of the molecule. Evidently, sugar alcohols with a chain length of 5–7 carbon atoms are transformed, provided they contain: hydroxyl groups in *cis*-vicinal positions at C-2 and C-3; and hydroxyl groups in *trans*-configuration at C-3 and C-4. It appears to be of minor importance whether the reactants have D- or L-configuration. Among the polyols meeting the necessary requirements, mannitol takes a prime position, which might be due to its symmetrical structure allowing enzymic attack from either end of the molecule.

The K_m values of 1.9×10^{-1} M for fructose and 3.4×10^{-2} M for mannitol indicate a very low affinity for the substrates. Values in the same order of magnitude have, however, been described for the *C. chrysogenum* [11] and *D. viticola* enzymes [8]. On the other hand, the mannitol dehydrogenase of *A. campestris* [12], which is supposed to be very closely related to *A. bisporus*, had approximately 100 × higher affinities. The relationship between the *Diplodia* and *A. bisporus* mannitol dehydrogenases is further emphasized by the fact that both enzymes oxidize to some extent sorbitol and arabitol as alternative substrates. In *D. viticola* a close correlation between glucose-6-phosphate dehydrogenase activity and mannitol accumulation was found [8]. Mannitol synthesis in *A. bisporus* has been shown to involve reoxidation of NADPH generated in the pentose phosphate cycle, into which flows a large proportion of the glucose-6-phosphate [5]. The NADP thus regenerated would then again become available for the oxidative reactions of the pentose phosphate shunt, which are known to be controlled by the $NADP^+$/NADPH ratio [14]. Our results demonstrate that *A. bisporus* mannitol dehydrogenase is very sensitive towards changes in the redox status of the nucleotide (Fig. 1), particularly in the polyol oxidizing direction, in which enzyme activity is severely inhibited even in the presence of comparatively small amounts of NADPH.

Because of these facts and the observation that vegetative hyphae and fruit bodies accumulate mannitol throughout their life [3, 14], which is not used except under severe stress, such as post-harvest storage [6, 15], we believe that mannitol dehydrogenase from *A. bisporus* functions as a synthesizing enzyme under physiological conditions. The above arguments do not, however, exclude the possibility that the enzyme acts catabolically when the $NADP^+$/NADPH ratio is high, which presumably occurs during starvation. The influence of metal ions, especially of Ca^{2+}, may or may not play an important part in mannitol catabolism, but since no data on the existence and distribution of these ions within the fungus are available, conclusions as to their role in fungal biochemistry would certainly be premature.

EXPERIMENTAL

The enzyme was extracted from young fruit bodies of *A. bisporus* (cap diam. 1–2 cm) immediately after harvest.

Enzyme assays. Mannitol dehydrogenase was assayed by following the oxidation–reduction of $NADP^+$/NADPH in a Beckman double-beam spectrophotometer at 340 nm and 25°. Optimum conditions (buffer, pH, substrate and cosubstrate concs) were determined. Accordingly, the incubation mixtures for routine tests contained (concn mM in parentheses): fructose (520), NADPH (0.1), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) pH 8 (90); or D-mannitol (260), NADP (0.1), Tris–HCl, pH 8.8 (90); and enzyme to a total vol. of 2.3 ml. For product identification the enzyme (30 μg) was incubated for 30 min in a mixture of the respective buffer (90), substrate (50) and cosubstrate (25).

GLC. Samples were analyzed after silylation in 0.75 ml Py with 0.2 ml HMDS and 0.05 ml TMCS on a 3 m × 2 mm glass column (3% SE-30, temp. program 160–250° at 3°/min; N_2, 30 ml/min; FID; injector + detector, 270°).

TLC. The sugar–polyol mixtures were separated on Si gel 0.2 mm (Merck 60 F 254) with $CHCl_3$–MeOH–HOAc (70:30:15); staining, 0.5% $NaIO_4$ in 0.2 M HOAc followed by 0.5% benzidine in EtOH–HOAc (4:1).

MW. The MW of the enzyme was determined in a Pharmacia thin-layer gel filtration apparatus on Sephadex G-200 with Tris–HCl, 0.05 M, pH 8, as solvent and cytochrome *c*, chymotrypsinogen A, ovalbumin, BSA, catalase and ferritin as reference proteins.

Detection of enzyme activity by staining. After separation a paper replica (Whatman 3 MM) of the gel was prepared and sprayed with a mixture of 5 mg nitro blue tetrazolium, 2.5 mg phenazine methosulfate, 2.5 mg D-mannitol and 5 mg NADP in 25 ml Tris–HCl, 0.1 M, pH 8. The reference proteins were made visible with Coomassie BB.

Isoelectric focusing. IEF was done in an Ampholine pH gradient (nominal range pH 9–11) on granulated gel (Ultrodex),

essentially as prescribed by the manufacturer (LKB Produkter, Sweden). About 25 μg of the purified enzyme were applied to the gel by means of small paper wicks. Enzymic activity was localized after the run by pressing a paper sheet, soaked with staining soln (see above), on to the gel.

Protein determination. To minimize interference by phenolics and Tris, aliquots of the extract were treated as suggested by Kluge and Osmond [16], the washed ppts redissolved in M NaOH and the protein content was determined according to ref. [17].

Acknowledgements—Financial support from the Swiss National Science Foundation (Grant Nr. 3.001–076) is gratefully acknowledged. We also thank Dr G. A Dütsch, University of Zürich, for helpful suggestions and discussions. Mushrooms were kindly supplied by the E. Hauser-Champignonkulturen AG, Gossau-Zürich, Switzerland.

REFERENCES

1. Lewis, D. H. and Smith, D C. (1967) *New Phytologist* **66**, 143.
2. Blumenthal, H. J. (1976) in *The Filamentous Fungi* (Smith, J. E. and Berry, D R. eds) Vol. 2, pp. 293–307 Edward Arnold, London.
3. Rast, D. (1965) *Planta* **64**, 81.
4. Dütsch, G. A. and Rast, D. (1969) *Arch Mikrobiol.* **65**, 195.
5. Dütsch, G. A. and Rast, D. (1972) *Phytochemistry* **11**, 2677
6. Dütsch, G A. and Rast, D. (1972) *Mushroom Sci* **8**, 387
7. Lendenmann, J. (1978) Ph.D Thesis, Univ of Zürich.
8. Strobel, G A. and Kosuge, T. (1965) *Arch Biochem Biophys.* **109**, 622.
9. Strandberg, G W. (1969) *J. Bacteriol* **97**, 1305.
10. Holligan, P M. and Jennings, D. H (1972) *New Phytologist* **71**, 583.
11. Birken, S. and Pisano, M. A. (1976) *J. Bacteriol* **125**, 225
12. Edmundowicz, J. M and Wriston, J C. (1963) *J. Biol. Chem* **238**, 3539.
13. Lamed, R., Levin, Y. and Wilchek, M. (1973) *Biochim Biophys. Acta* **304**, 231
14. Eggleston, L V. and Krebs, H. A. (1974) *Biochem J.* **138**, 425
15. Hammond, J B. W. and Nichols, R. (1975) *J Sci. Food Agr.* **26**, 835.
16. Kluge, M. and Osmond, C. B. (1972) *Z. Pflanzenphysiol* **66**, 97
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol Chem.* **193**, 265