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Crystalline D-mannitol:NAD$^+$ oxidoreductase from *Leuconostoc mesenteroides*

The specific D-mannitol dehydrogenase was first obtained as crystals from the D-fructose-grown cells of *Lactobacillus brevis*, ATCC 367 by MARTINEZ, BARKER AND HORECKER[1]. This enzyme was specific for the reversible reduction of D-fructose to D-mannitol. Other ketoses were not reduced by this enzyme preparation. Of lactic acid bacteria, we found that one strain of *Leuconostoc mesenteroides* produced D-mannitol dehydrogenase in approximately equal amounts from D-fructose and from D-glucose or sucrose[2]. It is of interest that glucose is effective in the production of D-mannitol dehydrogenase, whereas glucose is not a good substrate for this enzyme.

In this paper, we report the purification procedures of D-mannitol dehydrogenase from *Leuconostoc mesenteroides*.

Culture. Leuconostoc mesenteroides, ATCC 9135 (Institute for Fermentation, Osaka, Japan, 3426) was used throughout the experiments[2]. Culture medium contained 1% sodium acetate, 1% peptone, 0.2% yeast extract, 0.02% $MgSO_4 \cdot 7H_2O$, 0.001% NaCl, 0.0002% $MnSO_4 \cdot 4H_2O$ and 1% D-glucose. An inoculum was made in 8 ml of the above medium in a test tube and incubated overnight at 32°. The whole culture was transferred to 400 ml of the same medium and incubated for 24 h, then transferred to 10 l of medium and incubated for 16 h. Cells were harvested by a Sharples centrifuge and collected cells were suspended in chilled 0.05 M acetate buffer (pH 6.0) containing 10^{-3} M of mercaptoethanol. After centrifugation, the washed cells were kept in a deep freeze.

Enzymatic assay. The activity of D-mannitol dehydrogenase was determined by the rate of consumption of NADH by fructose at 340 mμ in the reaction system described below. The reaction mixture (0.3 ml) contained 6 μmoles of acetate buffer (pH 5.35), 0.03 μmole of NADH, 30 μmoles of D-fructose and enzyme. The enzyme was sufficiently diluted in acetate buffer (pH 5.35) to give an absorbance change of about 0.10 per min. The activity of NADH oxidase was measured without addition of D-fructose. The reaction was started by addition of substrate. D-Mannitol dehydrogenase activity was determined after correction for NADH oxidase activity. Disappearance of NADH was followed at 340 mμ with a Model 139 Hitachi Perkin–Elmer spectrophotometer equipped with a microcuvette chamber and with a thermospacer for the cell compartment. The temperature was maintained at 30° through by means of a constant-temperature circulating-water bath. The microcuvettes have a 1.0-cm light path and a 0.45-ml capacity. They were purchased from the Pyrocell Manufacturing Co., Westwood, N.J., U.S.A. Protein was determined from $A_{260\ m\mu}$ and $A_{280\ m\mu}$ (ref. 3).

Unit of enzyme and specific activity. A unit of enzyme is defined as the amount of enzyme required to produce a change in $A_{340\ m\mu}$ of 1.0 per min in the standard assay with D-fructose. Specific activity is defined as a unit of enzyme per mg of protein.

Purification. All procedures were carried out at 2° unless otherwise stated. The washed cells (94 g in wet weight from 50 l of medium) were disrupted by grinding with alumina in a mortar and the enzyme was extracted with 1000 ml of 0.05 M acetate buffer (pH 6.0) containing 10^{-3} M mercaptoethanol. Cell debris and alumina were removed by centrifugation (crude extract). Protamine sulfate solution (2%, 46 ml)

was added dropwise with stirring. After standing 1 h, precipitates were removed by centrifugation (protamine supernatant). Solid $(NH_4)_2SO_4$ (674 g) was added to give 100% satn. The precipitate was collected, dissolved in 0.05 M phosphate buffer (pH 7.0) containing 10^{-3} M mercaptoethanol and dialyzed overnight against the same buffer ($(NH_4)_2SO_4$ Fraction I). $(NH_4)_2SO_4$ Fraction I was treated again by solid $(NH_4)_2SO_4$ and obtained the precipitates from 0.50 to 0.65 of saturation and dialyzed ($(NH_4)_2SO_4$ Fraction II). The second $(NH_4)_2SO_4$ fraction was adjusted to pH 6.0 by 0.2 M acetic acid and was chilled. Acetone (3.4 ml), previously chilled to $-20°$, was added dropwise with stirring (30% acetone). The precipitated protein was removed by centrifugation at $-10°$ and discarded. To the supernatant (9.8 ml) was added 4.3 ml of acetone to give 50%. The precipitated protein was dissolved in 0.05 M phosphate buffer (pH 7.0) containing 10^{-3} M mercaptoethanol. The solution was treated by $(NH_4)_2SO_4$ fractionation, from 0.50 to 0.80 satn. The precipitate was dissolved and dialyzed overnight (acetone fraction).

TABLE I

PURIFICATION OF D-MANNITOL DEHYDROGENASE

Fraction	*Vol. (ml)*	*Protein (mg)*	D-*Mannitol dehydrogenase*		
			Total units ($\times 10^3$)	*Specific activity (units/mg)*	*Yield (%)*
Crude extract	930	3810	1003	263	
Protamine	950	1070	691	643	69
$(NH_4)_2SO_4$ (I)	28.5	1130	691	611	69
$(NH_4)_2SO_4$ (II)	7.4	612	614	1004	61
Acetone 30–50%	7.7	241	566	2350	56
First crystals	(2.9)	86.7	532	6140	53
Second crystals	(1.1)	43.2	312	7220	31
Third crystals	(1.5)	34.1	278	8140	28

Crystallization. To the acetone fraction was added, dropwise, the same volume of a saturated $(NH_4)_2SO_4$ solution (7.65 ml). The slightly turbid solution was clarified by centrifugation and kept overnight at 5°. Upon swirling, the solution showed the silky sheen characteristic of crystalline enzyme. The first crystals were collected, dissolved in 0.05 M phosphate buffer (pH 7.0) containing 10^{-3} M mercaptoethanol and dialyzed against the same buffer overnight (first crystals). Recrystallization was carried out by the same procedure without difficulty. The purification of the enzyme is summerized in Table I and the second crystals are shown in Fig. 1. The overall purification is approx. 30-fold in specific activity.

Analysis of the recrystallized enzyme showed a single, symmetric peak on ultracentrifugation. Properties of this crystalline enzyme, effect of pH on enzyme activity, substrate specificity and coenzyme specificity, seemed to be similar to the crystalline D-mannitol dehydrogenase from *Lactobacillus brevis*[1].

The crystalline D-mannitol dehydrogenase reported in this paper seems to be similar to that of *L. brevis*[1]. Specific activity of the present crystalline enzyme however, is about twelve to fourteen-fold higher than that of the *L. brevis* enzyme. It is also an

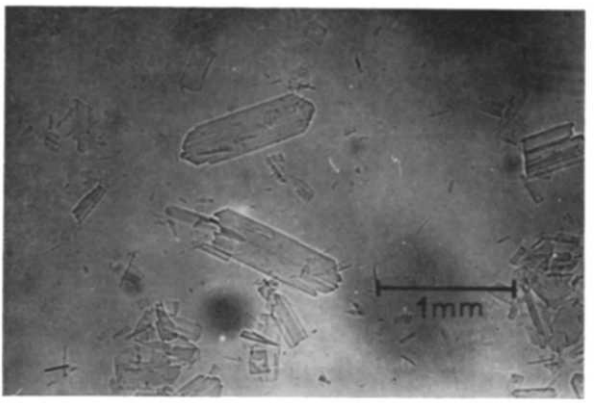

Fig. 1. Crystals of D-mannitol dehydrogenase. Recrystallized enzyme from the glucose-grown cells of *Leuconostoc mesenteroides*.

advantage to be able to prepare the crystalline enzyme from glucose medium by very convenient purification steps.

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Electrophoresis of glutathione reductase from human red blood cells

Glutathione reductase from human red blood cells can be separated into two distinct isozymes (GR I, GR II) by high-voltage electrophoresis. Cases of partial deficiency of the enzyme are frequently associated with a hemolytic, non-spherocytic anemia[1] or pancytopenia[2]. In one family[2] with this genetically determined polymorphism oligophrenia and neurological disorders were found (spastic signs of the Babinski group and pathologically altered electroencephalograms). Simultaneous occurrence of partial deficiency of glutathione reductase with hemoglobin C disease[3] and β-thalassemia minor (G. W. LÖHR, unpublished results) have been observed. Occasionally we have noticed the development of acute leukemia in cases of partial deficiency of glutathione reductase (G. W. LÖHR, unpublished results). Frequently