JOURNAL OF BACTERIOLOGY, Mar. 1970, p. 777-780

Vol. 101, No. 3
Printed in U.S.A.

Purification of a D-Mannose Isomerase from *Mycobacterium smegmatis*[1]

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Received for publication 22 September 1969

An enzyme, D-mannose ketol isomerase, catalyzing the isomerization of D-mannose and D-fructose was purified approximately 60-fold from cells of *Mycobacterium smegmatis* grown on mannose as the sole carbon source. This enzyme was shown to catalyze the conversion of D-mannose and D-lyxose to ketoses. The ketose produced from mannose was identified as fructose by chemical and chromatographic methods. The reaction was shown to be reversible, the equilibrium ratio of fructose to mannose being approximately 65 to 35. The *p*H optimum was about 7.5, and the K_m for mannose was estimated to be 7×10^{-3} M. Mannose isomerase activity was greatest in cells grown on mannose, whereas cells grown on fructose had about 30% as much activity. Very low levels of activity were detected in cells grown on other substrates. There was an immediate increase in enzyme activity on transfer of cells from nutrient broth to a mannose mineral salts medium.

During the course of studies on the metabolism of trehalose in *Mycobacterium smegmatis*, it was of interest to obtain some radioactive trehalose for these studies. Thus *M. smegmatis* was grown in a mineral salts medium with various radioactive sugars as carbon sources. During these studies, it was found that *M. smegmatis* grew much more rapidly on mannose and fructose than on glucose. These organisms were then found to contain a mannose isomerase which is apparently responsible for this growth response.

The present report describes the purification of this enzyme from cells of *M. smegmatis* grown on mannose. The enzyme was also present in cells grown on fructose but was virtually absent in cells grown on other substrates. Some of the properties of the enzyme are presented.

MATERIALS AND METHODS

Methods. All chemicals were obtained from commercial sources unless otherwise indicated. Calcium phosphate gel was prepared as described by Keilin and Hartree (5).

Fructose was determined by the method of Roe (8). Other ketoses were determined by the cysteine-carbazole reaction (3). Protein was determined by the method of Sutherland et al. (9).

Descending paper chromatography was performed with Whatman no. 1 filter paper by using solvent 1 [phenol-water (4:1, v/v)], solvent 2 [propanol-ethyl acetate-water (7:1:2)], and solvent 3 [ethyl acetate-acetic acid-water (3:3:1)]. Sugars were detected on paper with the alkaline silver nitrate reagent (11) or the aniline acid phthalate spray reagent (7). Ketoses were detected with the orcinol-trichloroacetic acid spray (2).

Growth of the organism. *M. smegmatis* was maintained on slants of tryptic soy agar. These slants were used to inoculate 125-ml flasks containing 25 ml of tryptic soy broth. After 48 hr of growth at 37 C, 5 ml of this culture was used to inoculate 2-liter flasks containing 1 liter of the mineral salts medium of Heath and Ghalambour (4), except that various carbon sources were used as indicated. Sugars and sugar alcohols were autoclaved separately and added to a final concentration of 0.25%. Cells were grown in mineral salts medium for 72 hr at 37 C.

Assay of enzyme. For routine assay, incubation mixtures contained the following components in a final volume of 0.2 ml: tris(hydroxymethyl)aminomethane (Tris)-maleate buffer (*p*H 7.5), 5 μmoles; D-mannose, 6 μmoles; and an appropriate amount of enzymatic extract. Mixtures were incubated for 30 min at 37 C. The reactions were stopped by heating the tubes for 5 min in a boiling-water bath. A 4-ml amount of water was added, and 1 ml was taken for the assay of fructose by the Roe method. One unit of enzyme activity is defined as that amount which will convert 1 μmole of mannose to fructose per min at 37 C.

Purification of D-mannose isomerase. For purification of D-mannose isomerase, the following procedures were used.

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Preparation of extract. A 20-g amount of cell paste of *M. smegmatis* grown on mannose-mineral salts medium was suspended in 100 ml of 0.005 M Tris-maleate buffer (*p*H 7.5) and was sonically disrupted. This suspension was centrifuged at 30,000 × *g* for 10 min, and the supernatant liquid was used in the following experiments.

Streptomycin sulfate treatment. To 100 ml of the above extract was added, slowly with stirring, 10 ml of an aqueous solution of streptomycin sulfate (100 mg/ml). The mixture was allowed to stand for 10 min at 0 C, and the precipitate was removed by centrifugation and discarded.

pH and heat treatment. Portions (25 ml) of the above supernatant liquid were placed in each of four 125-ml Erlenmeyer flasks. To each flask was added 1.7 ml of 1 M sodium acetate buffer, *p*H 5.0. Each flask was then shaken gently in a water bath at 55 C for 90 sec and cooled rapidly in ice. The precipitate from this step was removed by centrifugation.

Treatment with calcium phosphate gel. To 100 ml of supernatant liquid remaining from the above step was added, slowly with stirring, 100 ml of calcium phosphate gel (15 mg/ml). The mixture was allowed to stand for 10 min, and the gel was removed by centrifugation. The enzymatic activity was in the supernatant liquid.

Ammonium sulfate fractionation. To 180 ml of the supernatant liquid was added, slowly with stirring, 57.9 g of solid ammonium sulfate (50% saturation). The precipitate was removed by centrifugation and discarded, and to the supernatant liquid was added an additional 28.8 g of solid ammonium sulfate (70% saturation). The precipitate was isolated by centrifugation, dissolved in 12 ml of 0.01 M Tris-maleate buffer (pH 7.5), and dialyzed overnight against 2 liters of the same buffer.

Diethyaminoethyl (DEAE)-cellulose column chromatography. DEAE-cellulose (Sigma Chemical Co.) was purified by consecutive treatments with 1 N NaOH and 1 N HCl. It was washed with distilled water until neutral and was then suspended in 1 M KCl until used. Before use, the DEAE-cellulose was washed with 0.01 M Tris-maleate buffer (*p*H 7.5) until the effluent was free from chloride ions.

The ammonium sulfate fraction was applied to a column (2 by 24 cm) of DEAE-cellulose. The column was washed with 250 ml of 0.01 M Tris-maleate buffer (*p*H 7.5) and was eluted first with 250 ml of 0.1 M KCl in 0.01 M Tris-maleate buffer (*p*H 7.5) and then in 250 ml of 0.2 M KCl in Tris-maleate buffer (*p*H 7.5). The enzyme was eluted between 75 and 115 ml of 0.2 M KCl. The fractions containing enzymatic activity were pooled and concentrated by using the Amicon Diaflow apparatus, with a UM-1 filter (molecular weight cut off of 10,000). Any residual KCl was removed by overnight dialysis against 0.01 M Tris-maleate, *p*H 7.5.

With these procedures, the enzyme was purified approximately 60-fold, with 16% recovery (Table 1). This enzyme fraction was used in the following experiments.

The enzyme was found to be stable to freezing at all stages of purification and could also be stored overnight in ice without any loss in activity. The purified enzyme could be stored at −10 C for at least 2 weeks and could be frozen and thawed several times without loss in activity.

RESULTS

Effect of growth substrate on mannose isomerase activity. Figure 1 presents the growth curves of *M. smegmatis* on mineral salts medium with various sugars as carbon sources. Growth was much more rapid, that is the lag period was shorter, when mannose and fructose rather than glucose were used as carbon sources.

The effect of these various substrates on mannose isomerase activity was also determined. Mannose isomerase activity was greatest in cells grown on mannose, whereas cells grown on fructose had about 30% as much activity. Cells grown on a mineral salts medium with glucose, mannitol, or sorbitol as the carbon source or on nutrient broth contained little or no mannose isomerase activity

Effect of time, protein concentration, substrate concentration, and pH on the isomerization of mannose and fructose. The rate of isomerization of mannose to fructose was linear with time and enzyme concentration for at least 30 min and over a fourfold range of protein concentration provided that a large excess of mannose was present. However, it could be shown that eventually an equilibrium between mannose and fructose was established. At equilibrium, the ratio of mannose to fructose was about 35 to 65. The K_m for mannose was estimated to be approximately 7×10^{-3} M. The optimum *p*H in Tris-maleate buffer was approximately 7.5.

Substrate specificity and effect of other monosaccharides on mannose isomerase activity. At the sugar concentration used routinely (0.03 M), the purified enzyme was found to be active towards D-mannose and D-lyxose. No activity could be detected with D-glucose or D-arabinose. In addition, no activity could be detected towards D-mannitol, showing than mannose was not reduced to mannitol and then reoxidized to fructose.

The activity of mannose isomerase on 0.005 M mannose was examined in the presence of various sugars. The presence of glucose, galactose, and *N*-acetylglucosamine at concentrations as high as 0.05 M did not affect enzyme activity, whereas arabinose at this concentration did inhibit enzyme activity. Glucuronic acid at concentrations as low as 0.005 M significantly inhibited enzyme activity.

Charatcerization of the products. The formation of a ketose upon incubation of mannose

TABLE 1. *Purification of mannose isomerase*

Fraction	Total units	Specific activity (units/mg of protein)
Crude extract	30.60	0.039
Streptomycin sulfate, *p*H and heat	28.30	0.044
Calcium phosphate gel	25.00	0.064
Ammonium sulfate	10.35	0.396
DEAE-cellulose	4.86	2.250

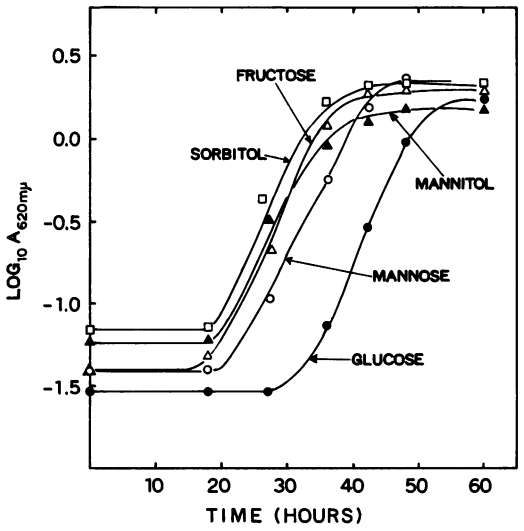

FIG. 1. *Growth of Mycobacterium smegmatis on a mineral salts medium with various sugars as carbon sources. Each sugar was added at a final concentration of 0.25%. Growth was followed in a Zeiss spectrophotometer at 620 nm. The ordinate is the logarithm of the absorbancy.*

with the purified enzyme was indicated by the positive cysteine-carbazole reaction. Further, the positive reaction with the Roe method indicated the formation of fructose. Also, when fructose was incubated with the purified enzyme, a disappearance of this compound could be detected by using the Roe method. Paper chromatography in solvent 1 of the reaction mixtures, when either D-mannose or D-fructose was used as the substrate, revealed the presence of two and only two compounds which had the same mobilities as authentic mannose and fructose (Table 2). Development with the silver nitrate reagent of paper chromatograms of the same reaction mixtures in solvents 2 and 3 showed only one spot with the same mobility as authentic fructose and mannose.

Effect of transferring M. smegmatis from nutrient broth to mannose-mineral salts medium. Figure 2 shows the effect of transferring *M. smegmatis* from nutrient broth to a mannose-mineral salts medium. It can be seen that there is an immediate increase in enzyme activity on transfer to the mannose-containing medium.

DISCUSSION

The isolation of enzymes capable of catalyzing the direct isomerization of mannose and fructose has been described by Palleroni and Doudoroff (6) and by Takasaki (10). Palleroni and Doudoroff isolated a mannose isomerase from *Pseudomonas saccharophila* grown on fructose as

TABLE 2. *Paper chromatographic identification of the products formed by incubation of mannose and fructose with mannose isomerase*

Compound	$R_{(glucose)}$[a] in solvent 1
Glucose	1.00
Mannose	1.23
Fructose	1.45
Unknowns 1[b]	1.24
	1.45
Unknowns 2[c]	1.25
	1.47

[a] Mobility with respect to glucose.
[b] From incubation of mannose with purified mannose isomerase.
[c] From incubation of fructose with purified mannose isomerase.

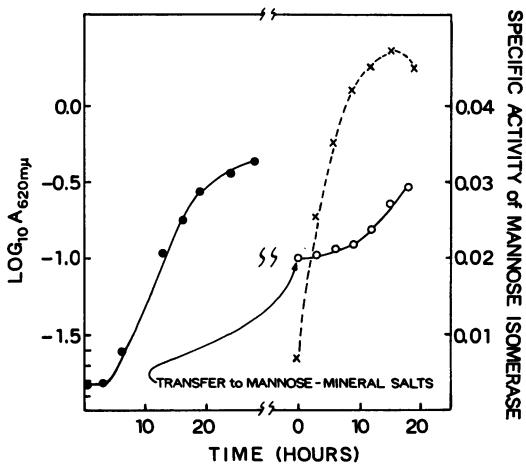

FIG. 2. *Effect of transferring Mycobacterium smegmatis grown on nutrient broth to mannose-mineral salts medium. Solid lines indicate growth on nutrient broth (●) and mannose-mineral salts (○). The broken line represents mannose isomerase activity (×).*

the sole carbon source. The enzyme was found to be active towards D-lyxose and D-rhamnose as well as D-mannose. The product of the reaction with D-lyxose was shown to be D-xylulose. The product of the reaction with D-rhamnose was presumed to be D-rhamnulose. More recently, Anderson and Allison (1) isolated a D-lyxose isomerase from *Aerobacter aerogenes* grown on lyxose as the sole carbon source. The enzyme was capable of catalyzing the isomerization of mannose and fructose but was not induced by growing the cells on mannose. The mannose isomerase isolated from *M. smegmatis* is also capable of catalyzing the conversion of D-lyxose to a ketose, presumably D-xylulose.

The fact that the purified mannose isomerase will catalyze the conversion of only mannose to fructose makes this an ideal reaction for the determination of mannose in mixtures of sugars. In addition, the enzyme is easy to prepare and is relatively stable. The enzyme activity is affected only by glucuronic acid and high concentrations of arabinose. Since the smallest amount of fructose that can be detected by the Roe method is approximately 0.02 μmole and the equilibrium position is 65:35, it should be theoretically possible to determine quantities of mannose as low as 0.03 μmole.

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