Studies on the Isomerization of Sugars by Bacteria

Part VIII. Purification and Some Properties of Mannose Isomerase from *Xanthomonas rubrilineans* S-48

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Crude mannose isomerase preparation from *Xanthomonas rubrilineans* S-48 which converts D-mannose to D-fructose was further purified by ammonium sulfate fractionation and DEAE-cellulose column chromatography. The specific activity of the purified enzyme solution was about 35-fold of original crude preparation. By using this purified enzyme solution, several enzymatic properties were investigated.

(1) The Michaelis constant was 1.2×10^{-2} M. (2) The enzyme was sensitive against temperature, but Ca^{++} protected the enzyme to some extent from the effect of temperature. (3) The enzyme was stable in the pH range from 6 to 9. (4) The enzyme was not inhibited by glucose, xylose, mannitol, sorbitol, mannonic acid, mannuronic acid and so on, but strongly inhibited by D-arabinose and L-fucose, and K_i values of these inhibitors were 1.1×10^{-2} M and 7.1×10^{-4} M respectively.

INTRODUCTION

In the previous paper^{1,2)}, authors reported that a newly isolated bacterium having mannose isomerase was identified as *Xanthomonas rubrilineans*; the enzyme was constitutive and widely distributed in bacteria which belong to *Xanthomonas*.

The present report deals with purification and some properties of mannose isomerase from *Xanthomonas rubrilineans* S-48.

EXPERIMENTAL

Organism.

The organism used in this paper was *Xanthomonas rubrilineans* S-48 isolated by the author.

Assay of mannose isomerase activity.

The assay of mannose isomerase was carried out as follows: The reaction mixture was prepared by adding 1 ml of 0.1 M phosphate buffer solution (pH 8) and 0.2

ml of 0.1 M mannose to the enzyme solution to make the total volume 2 ml. The reaction mixture was incubated for thirty to sixty minutes at 35°C. The reaction was stopped by the addition of 2 ml of 0.5 M perchloric acid. Fructose formed was determined by the cysteine-carbazole method.

The amount of enzyme which will produce 1 μ M of fructose from mannose per hour at 35°C under the above assay condition is defined as a unit of the enzyme.

Determination of protein.

Protein was determined by the Lowry's method.

Purification of enzyme.

X. rubrilineans S-48 was grown in the synthetic medium containing 1% glucose as a sole carbon source as previously described. The cells were centrifuged, washed twice with 0.02 M phosphate buffer solution (pH 7), suspended in the same buffer solution and then the cell suspension was sonicated at a frequency of 10 KC for fifteen to twenty minutes. After centrifugation at 10,000 rpm for ten minutes, the supernatant (80 ml) was brought to 40% saturation by the addition of 19.2 g of solid ammonium

1) Y. Takasaki, O. Tanabe, *J. Agr. Chem. Soc. Japan*, **37**, 524 (1963).

2) Y. Takasaki, O. Tanabe, *This Journal*, **28**, 601 (1964).

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sulfate and was adjusted to pH 7 by the addition of ammonia. After being stirred for ten minutes under low temperature, the mixture was centrifuged and the supernatant brought to 60% saturation by adding 10.6 g of solid ammonium sulfate. After ten minutes, the suspension was centrifuged and the precipitate was dissolved in 20 ml of water and treated with 1.8 ml of 2% protamin. The 40~60% fraction by the addition of solid ammonium sulfate was collected in the same way as above. The precipitate was dissolved in 10 ml of water and the centrifuged supernatant was then dialyzed overnight against 1 l of 0.02 M phosphate buffer solution (pH 7) containing 5×10^{-3} M cysteine. The precipitate in the dialyzed bag was centrifuged and the supernatant poured onto a DEAE-cellulose column (1.3 $\times$ 18 cm) which was pretreated with 0.02 M phosphate buffer solution (pH 7). The enzyme absorbed by the column was eluted with 0.02 M, 0.05 M, 0.1 M and 0.2 M phosphate buffer solution (pH 7). Flow rate was approximately 5 ml per hour and the elute was collected every 5 ml.

The results of the purification processes are shown in Table I. Fig. 1 shows the result of the enzyme elution from this column.

RESULTS AND DISCUSSION

Influence of Mannose Concentration

The results of an experiment with different concentration of mannose is shown in Fig. 2. Fig. 2 also shows the reciprocal of velocity against the reciprocal of substrate concentration, and the calculation of apparent Michaelis constant gave a value of 1.2×10^{-2} M. It is observed that the enzyme was slightly inhibited by high substrate concentration.

Stability Against Heat

One tenth ml of enzyme solution and 1 ml of buffer solution (0.1 M phosphate buffer and 0.1 M phosphate buffer containing 1×10^{-2} M CaCl_2)

FIG. 1. Separation of Mannose Isomerase by DEAE-Cellulose Column Chromatography.

Column; 1.3 $\times$ 18 cm, Flow rate; 5 ml/hr, Fraction; 5 ml
●—● Protein, ×—× Activity

were mixed and treated at various temperature. After five minutes treating, the reaction mixtures were immediately cooled and added with 0.2 ml of 0.1 M mannose to make the final volume 2 ml and the residual enzyme activities were measured. The results are shown in Fig. 3. The enzyme is unstable above 40°C, but is stabilized in the presence of Ca^{++} .

pH stability

To 1 ml of each buffer solution (MacIlvane buffer and Sørensen buffer), 0.2 ml of enzyme solution was added and the mixture was kept at room temperature (27°C) for five hours. After adjustment of pH to 8, 0.2 ml of 0.1 M mannose was added to make the final volume 2 ml and the residual enzyme activities were measured. The result shown in Fig. 4 indicates that the mannose isomerase is stable under the pH range

TABLE I. SUMMARY OF THE PURIFICATION PROCEDURE OF MANNOSE ISOMERASE

	Total-volume (ml.)	Protein (mg)	Total activity (unit)	Specific activity	Yield (%)
Wet cells (6.2 g)					
Cell extracts	80	620	2,356	3.8	100
Amm. sulfate (40~60%)	20	115	1,111	9.7	47.6
Protamine treatment	40	69	996	14.4	42.2
Amm. sulfate (40~60%)	10	56	1,040	18.6	44.1
Dialysis		43	917	21.1	38.9
DEAE-cellulose		4.4	576	130.8	24.5

FIG. 2. Influence of Mannose Concentration.

FIG. 3. Stability against Heat

×—× None,   o—o CaCl_2 (1×10^{-2} M)

from 6 to 9, but is unstable below pH 6 or above pH 9.

Effect of other Substance

To the reaction mixture containing 0.02 M of mannose, 0.01 M of one of other substance such as D-glucose, D-xylose, D-mannitol, D-mannonic-

FIG. 4. pH Stability

×—× MacIlvaine buffer (citrate- Na_2HPO_4)
o—o Sørensen buffer (KH_2PO_4 - Na_2HPO_4)
Δ—Δ Sørensen buffer (glycol- NaOH)

acid and so on was added in order to test their effect. Of them, D-arabinose and L-fucose showed strong inhibitory effect, however, D-glucose, D-galactose D-xylose, D-mannitol, D-sorbitol, D-mannonic acid and D-mannuronic acid etc. did not inhibit the enzyme. The results are shown in Table II.

In order to determine whether these inhibitions by D-arabinose and L-fucose are competitive or

TABLE II. EFFECT OF OTHER SUBSTANCE

Substance	Relative activity
None	100
D-Glucose	98.2
D-Galactose	100
D-Sorbose	100
D-Mannitol	100
D-Sorbitol	100
D-Gluconic acid	98.2
D-Mannonic acid	100
D-Glucuronic acid	100
D-Mannuronic acid	100
D-Xylose	100
D-Ribose	100
L-Rhamnose	98.8
D-Arabinose	51.5
L-Arabinose	100
L-Fucose	23.5
D-Glucosamine	94.5
D-Glucosone	94.2

Reaction mixture was prepared by adding 1 ml of 0.1 M phosphate buffer solution (pH 8), 0.4 ml of 0.1 M mannose and 0.2 ml of 0.1 M other substance to the 0.2 ml of enzyme solution to make the total volume 2 ml and incubated for 30 minutes at 35°C.

FIG. 5. Competitive Inhibition of Mannose Isomerase by D-Arabinose and L-Fucose.

—△— L-Fucose —×— D-Arabinose —○— None

not, the reciprocals of velocities in the presence and absence of inhibitors (1×10^{-2} M) were plotted against the reciprocals of substrate concentrations. The results shown in Fig. 5 suggest that these inhibitions are competitive since the intercept does not change. From the inclination of the lines, the K_i values were calculated to be 1.1×10^{-2} M for D-arabinose and 7.1×10^{-4} M for L-fucose.

Palleroni and Doudroff³⁾ reported that the structure of sugar, being necessarily be the substrate for mannose isomerase, should be the same as that of mannose in the structural configuration of the first five carbon atoms, since D-lyxose and D-rhamnose were also attacked by this iso-

merase (Fig. 6). In our experiment, although D-lyxose and D-rhamnose were not tested, it was found that D-glucose, D-galactose, L-rhamnose, D-xylose, D-arabinose and L-fucose were not attacked by the mannose isomerase, and furthermore found that D-arabinose and L-fucose showed strong inhibitory action for the mannose isomerase. The structures of these two sugars differ from that of mannose, which can be attacked by the enzyme, from a view-point of the structural configuration of the OH group at the third carbon atom within the first five carbon atoms (Fig. 6). These facts indicate that D-arabinose and L-fucose have the structures which are capable to bound to mannose isomerase, though these sugars are not attacked by mannose isomerase

3) N.J. Palleroni, M. Doudroff, *J. Biol. Chem.*, **218**, 535 (1956)

FIG. 6. Structure of Sugar Capable of Combining with Mannose Isomerase

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