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CRYSTALLIZATION AND PROPERTIES OF L-ARABINOSE ISOMERASE FROM *LACTOBACILLUS GAYONII*

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SUMMARY

1. L-Arabinose isomerase (L-arabinose ketol-isomerase, EC 5.3.1.4) was isolated in the crystalline state in 20% yield from the extracts of L-arabinose-grown cells of *Lactobacillus gayonii*. The molecular weight of the crystalline enzyme was estimated as 271 000 with the method of the sucrose density gradient centrifugation.

2. The crystalline enzyme is specific for L-arabinose and L-ribulose. It is slightly active on D-galactose. The optimal pH for the isomerization of L-arabinose is 6.0–7.0, and the Michaelis constants are: 55 mM for L-arabinose and 5 mM for L-ribulose.

3. The enzyme specifically requires Mn^{2+} for activity and the Michaelis constant is 5.25 μM.

4. The enzyme activity is inhibited competitively by L-arabitol, ribitol or xylitol and the inhibition constants are: 7.5 mM for L-arabitol, 6 mM for ribitol and 38 mM for xylitol.

INTRODUCTION

L-Arabinose isomerase (L-arabinose ketol-isomerase, EC 5.3.1.4) was first demonstrated in the L-arabinose-grown cells of *Lactobacillus pentosus* by LAMPEN[1] in 1954. It was purified 7.7-fold in regard to specific activity by fractionation with $(NH_4)_2SO_4$ and with cold acetone by HEATH *et al.*[2] (1958). Many strains of lactic acid bacteria which are able to ferment L-arabinose produce L-arabinose isomerase[3]. The enzyme was purified from the L-arabinose-grown cells of *L. gayonii* by column chromatography on DEAE-cellulose[4] (1962). The L-arabinose-grown cells of *Aerobacter aerogenes* PRL-R3 also produced the L-arabinose isomerase[5] (1958), and the purification of this enzyme was carried out by YAMANAKA AND WOOD[6] to 83-fold in the specific activity. The isolation of this enzyme in the crystalline state, however, was not achieved.

One of the pentose isomerases, D-xylose isomerase, was purified and crystallized by YAMANAKA[7] from the D-xylose-grown cells of *L. brevis*. Similar techniques were

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applied for the purification of L-arabinose isomerase from *L. gayonii* and the crystals of the enzyme were obtained by adding the saturated $(NH_4)_2SO_4$ solution.

The purification and crystallization of the L-arabinose isomerase and some properties of the crystalline enzyme are described. Differences of some properties of the two crystalline enzymes, D-xylose isomerase and L-arabinose isomerase, are also discussed.

MATERIALS AND METHODS

Chemicals

L-Arabinose was obtained from General Biochemicals, Ohio, U.S.A. D-Xylulose, D- and L-ribuloses were prepared and purified as described[7]. Potassium salt of L- and D-arabonates and of D-ribonate were prepared from L- and D-arabinoses and D-ribose with the oxidation by iodine at alkaline pH. Potassium salt of pentonates was crystallized from methanol solution and recrystallized from aqueous solution by methanol.

Culture

L. gayonii, ATCC 8289 was grown at 30° in a 10-l bottle containing a medium consisting of 1% sodium acetate, 1% peptone, 0.2% yeast extract, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% $MnSO_4 \cdot 4H_2O$, 0.01% $CoCl_2 \cdot 6H_2O$, 1% D-glucose and 0.1% L-arabinose. Culture was made as reported previously[7].

Enzymatic assay

L-Arabinose-isomerase activity was assayed by measuring the increase of ribulose[4,6]. Unless otherwise specified, the standard reaction mixture (1.0 ml) contained 25 μmoles Tris–maleate buffer (pH 7.0), 0.5 μmole $MnCl_2$, 0.01 to 0.20 ml of enzyme preparation with suitable dilution, and distilled water to a final volume of 0.95 ml. After equilibration for 5 min at 35°, 0.05 ml of 0.1 M L-arabinose was added and incubated at 35° for 10 min. Ribulose was determined by the cysteine–carbazole reaction[8]. 1 unit of enzyme was defined as the amount required to produce 1 μmole of ribulose under these conditions. Specific activity was expressed as units of enzyme per mg of protein. Protein was assayed spectrophotometrically with the aid of a nomogram based on the data of WARBURG AND CHRISTIAN[9].

Sucrose density gradient centrifugation

Sucrose density gradients were prepared according to MARTIN AND AMES[10]. The gradients were linear with 5–20% sucrose in 0.05 M Tris–HCl buffer (pH 7.5). Sedimentation was performed in a swing-out rotor, Hitachi RPS 40 A, in an Hitachi model 40P ultracentrifuge for 4, 8, 12, 16 and 20 h at 35 000 rev./min. Samples were collected by punching a hole in the bottom of the tube with a needle. Each fraction has 20 drops. Protein was assayed at 215 mμ. As a standard protein, catalase of beef liver from Worthington Biochemicals was used. Activity of catalase was assayed by spectrophotometry at 240 mμ (ref. 11). The distance from meniscus to the peak of the moved protein during centrifugation was calculated from counting drops and expressed in mm.

RESULTS

Purification procedures

All operations for purification of the L-arabinose isomerase were carried out at 2°. All precipitates from $(NH_4)_2SO_4$ were dissolved in 0.02 M Tris–HCl buffer (pH 7.5) and dialyzed against the same buffer containing 5 mM $MnSO_4$. The washed L-arabinose-grown cells (60 g in wet wt. from 40 l of medium) were disrupted by grinding with about 150–200 g of levigated alumina, and the enzyme was extracted with about 650 ml of 0.02 M Tris–HCl buffer (pH 7.5). Alumina and cell debris were removed by centrifugation (crude extract, 594 ml). To the crude extract, 30 ml of 1 M $MnCl_2$ were added dropwise and pH was maintained at 7.0–7.5 by adjusting with 1 M NaOH. The precipitate was centrifuged and discarded ($MnCl_2$ supernatant, 610 ml). Solid $(NH_4)_2SO_4$ (176 g) was added to the supernatant (0.5 satn.). The precipitate was discarded. $(NH_4)_2SO_4$ (195 g) was then added to the supernatant (0.95 satn.). After dialysis of the enzyme solution overnight, 14 200 units of enzyme activity were recovered ($(NH_4)_2SO_4$ fraction, 102 ml). The solution was treated with 5 ml of 1 M $MnCl_2$ and placed in a water bath at 80°. When the temperature of the enzyme solution reached 47°, the flask was transferred to another water bath at 50°, and kept for 5 min at the same temperature. The fraction was cooled in an ice bath, and the coagulated protein was removed by centrifugation and discarded. To the supernatant (98 ml) was added 70 g of $(NH_4)_2SO_4$ to obtain 1.0 satn. The precipitate was dissolved and dialyzed overnight with the same procedure as described above ($MnCl_2$-treated, 80 ml). The enzyme solution was applied to a column of DEAE-cellulose (3.0 cm × 70 cm) which had been equilibrated with 0.02 M Tris–HCl buffer (pH 7.5). The protein was eluted with a KCl gradient, 0 to 0.6 M, at pH 7.5. The fractions (No. 164–180) were pooled (88.4 ml, 7500 units). After precipitating the protein by $(NH_4)_2SO_4$, the enzyme was dissolved in 4 ml of 0.02 M Tris–HCl buffer (pH 7.5) and passed through the column of Sephadex G-200 (1.5 cm × 90 cm). The enzyme was eluted by 0.02 M Tris–HCl buffer (pH 7.5). Fractions of 2.2 ml were collected. The active fractions (No. 24–30) were combined (17.6 ml, 3640 units). The enzyme was precipitated with $(NH_4)_2SO_4$.

Crystallization

The crystallization of the enzyme was carried out by the addition of saturated $(NH_4)_2SO_4$ solution. After being dialyzed overnight, to the enzyme solution (final protein concentration was about 3.5%) was added 0.98 ml of the saturated $(NH_4)_2SO_4$ solution (0.46 satn.). The mixture was kept at 5° overnight. The resulting precipitate was removed by centrifugation. The $(NH_4)_2SO_4$ saturation was slowly increased at a rate of not more than 0.05 per day until 0.56 satn. was reached. For each time, some turbidity occurred and a trace of precipitate was removed by centrifugation. The amorphous precipitates between 0.51 and 0.558 satn. contained a small amount of the L-arabinose-isomerase activity. The solution was brought finally to 0.63 satn. The clear supernatant was kept at 5° for 4 days. The first crystalline L-arabinose isomerase thus obtained was collected and dissolved in 0.01 M Tris–HCl buffer (pH 7.5). Recrystallization was performed with the same procedure at 0.63 $(NH_4)_2SO_4$ satn., but the activity and protein content were not analyzed owing to the small amount of crystals. Fig. 1 showed the photomicrograph of the recrystallized enzyme. The crystals have

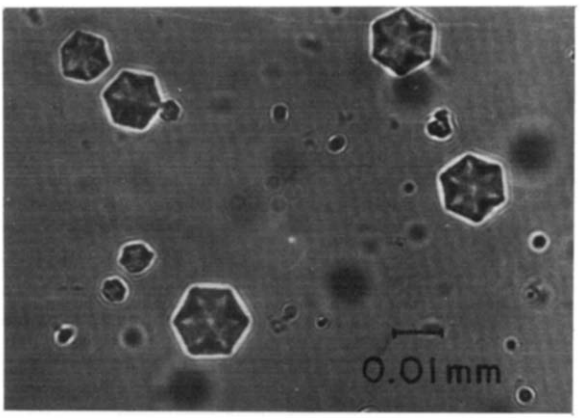
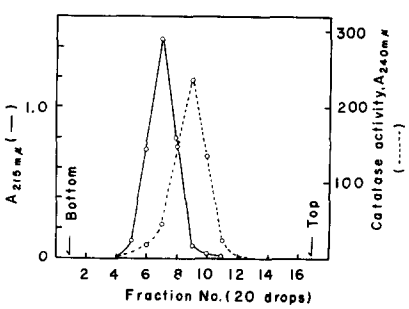


Fig. 1. Photomicrograph of the crystalline L-arabinose isomerase. (The second crystals.)

TABLE I

PURIFICATION OF L-ARABINOSE ISOMERASE

Fraction	*Protein (mg)*	*L-Arabinose isomerase*		*Recovery (%)*
		Total units	*Specific activity*	
Crude extract	2380	11 880	5.0	100
$MnCl_2$ supernatant	1340	13 900	10.3	117
$(NH_4)_2SO_4$ 0.5–0.95 ppt.	1400	14 200	10.1	119
$MnCl_2$-heated fraction	1360	11 900	8.8	100
DEAE-cellulose eluate	158	5 300	33.2	45
Sephadex G-200 eluate	67	3 640	54.5	33
Crystalline enzyme	45	2 390	53.1	20



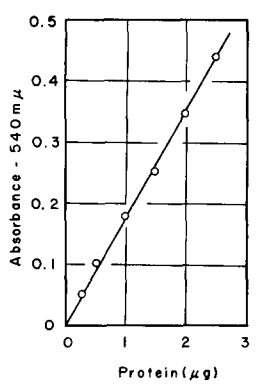
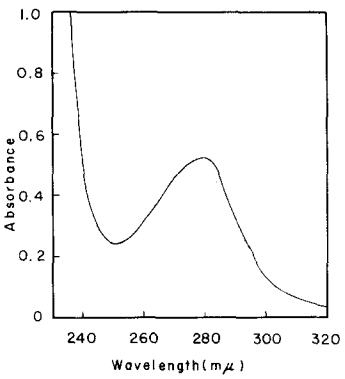


Fig. 2. Sedimentation pattern of L-arabinose isomerase and catalase on a sucrose density gradient centrifugation. Enzyme solution (0.01 ml) was layered on a gradient. The rotor was run at 35 000 rev./min for 12 h.

Fig. 3. Linearity of the L-arabinose isomerase assay. The conditions are described in the text.



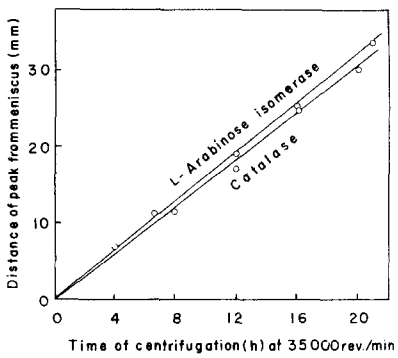


Fig. 4. Ultraviolet absorption spectrum of the L-arabinose isomerase.

Fig. 5. Sedimentation behavior of L-arabinose isomerase and catalase in a 5–20% sucrose density gradient.

regular hexagonal system. This is summarized in Table I. This procedure yielded 20% of the initial activity as crystals with a purification of slightly over 10-fold in the specific activity. The second crystals showed a single symmetrical moving peak on sucrose density gradient centrifugation (Fig. 2). A linear relationship was obtained between the absorbance at 540 mμ by cysteine–carbazole test and the amount of enzyme protein up to 3 μg (Fig. 3).

Properties

Absorption spectrum. The absorption spectrum of the crystalline enzyme showed a maximum at 280 mμ (Fig. 4). The ratio $A_{280\ m\mu}/A_{260\ m\mu}$ was found to be 1.60.

Molecular weight. Since sufficient crystalline enzyme was not available for molecular-weight determinations by methods involving schlieren optics, an estimation of the molecular weight was obtained by centrifugation in a sucrose density gradient by the method of MARTIN AND AMES[10]. L-Arabinose isomerase (0.01 ml) was layered on one gradient and 0.5 mg of catalase (in 0.01 ml buffer) was layered on a second gradient. An estimation of the molecular weight can be obtained from the sedimentation constant alone[10].

$$R = \frac{\text{distance travelled from meniscus}_1}{\text{distance travelled from meniscus}_2} = \frac{s_1}{s_2} = \left(\frac{\text{mol. wt}_1}{\text{mol. wt}_2}\right)^{\frac{2}{3}}$$

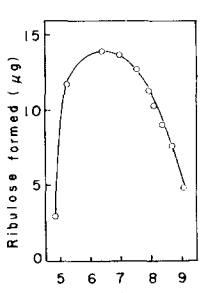


Fig. 6. Effect of pH on enzyme activity. Tris–maleate buffer for pH 4.9–8.0; Tris–HCl buffer for pH 7.0–9.1. Reaction proceeds at 35° for 10 min.

TABLE II

SUBSTRATE SPECIFICITY OF THE ENZYME

The reaction took place at pH 7.0 in the presence of 5 mM $MnCl_2$ at 40° for 20 min. The amount of crystalline enzyme used was 0.76 μg for L-arabinose and L-ribulose; 7.6 μg for D-galactose; 152 μg for other sugars. Ketoses were assayed by the cysteine–carbazole method at 35° for 20 min for ketopentose and at 50° for 30 min for ketohexose. Ketoses were expressed as ribulose or fructose.

Substrate (0.05 M)	*Ketose (μmoles)*
L-Arabinose*	5.48
D-Arabinose	0
D-Xylose	0
L-Xylose	0
D-Ribose	0
L-Ribulose*	−23.4
D-Ribulose	−0
D-Xylulose	−0
D-Glucose	0
D-Galactose	0.09
D-Fructose	−0
L-Sorbose	−0

* Concentration was 5 mM.

Molecular weight of catalase was adopted as 244 000 (ref. 14). R was calculated from the slopes obtained after 4, 6.5, 12, 16 and 21 h centrifugation (Fig. 5). The molecular weight of L-arabinose isomerase was thus calculated as $2.71 \cdot 10^5$.

Effect of pH on the activity. In Tris–maleate and Tris–HCl buffers, isomerization of L-arabinose to L-ribulose proceeded maximally between pH 6 and 7 at 35° (Fig. 6). The enzyme was stable at pH 5.5–9.0 for 10 min incubation at 50°.

TABLE III

REQUIREMENT OF METAL

EDTA-dialyzed enzyme preparation (0.01 ml) was incubated with 0.5 ml of 0.05 M Tris–HCl buffer (pH 8.0), 5 μmoles L-arabinose and metal solution of concentration indicated in the table, for 10 min at 35° (total volume, 1.0 ml). Activity was expressed as the amount of ribulose formed in μg.

Addition	*Metal (mM)*	*Activity (L-ribulose formed (μg))*			
		0	*0.05*	*0.1*	*1*
0		8.5			
$MnCl_2$			20.1	19.6	21.2
KCl			11.6	10.9	7.3
NaCl			9.6	8.2	7.6
$MgSO_4$			7.4	7.5	9.4
$ZnSO_4$			4.9	4.9	4.3
$CoCl_2$			12.1	10.6	12.9
$CuSO_4$			2.6	2.8	3.3
$SrCl_2$			8.9	11.4	10.9
$BaCl_2$			8.3	8.8	10.2
$FeSO_4$			6.7	3.4	3.8
Li_2SO_4			8.4	8.0	7.3
$HgCl_2$			2.5	2.5	1.7

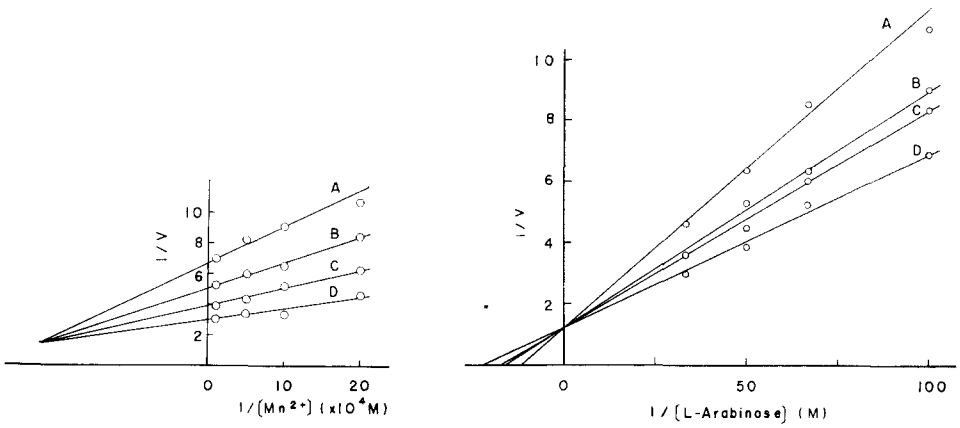
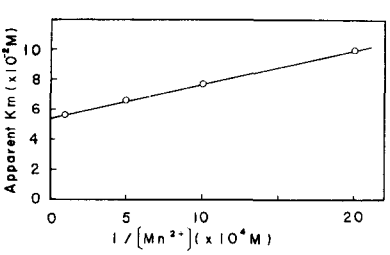


Fig. 7. Effect of concentration of Mn^{2+} on L-arabinose-isomerase activity. The reaction system was the same as in Table III with Mn^{2+} (5–100 μM) and 0.01 M (A), 0.01 M (B), 0.02 M (C) and 0.03 M (D) of L-arabinose.

Fig. 8. Effect of concentration of L-arabinose. The reaction system was the same as in Table III with L-arabinose (0.01–0.03 M) and 5 μM (A), 10 μM (B), 20 μM (C) and 100 μM (D) of Mn^{2+}.

Substrate specificity. The enzyme is specific for L-arabinose and L-ribulose (Table II). Among those tested, D-galactose was also found to serve as substrate, as was noted by SIMPSON *et al.*[5] on the L-arabinose isomerase from the crude extracts of *A. aerogenes*. D-Fucose is expected to serve as substrate, but is not available. The crystalline enzyme is devoid of the pentitol-dehydrogenase activities with NAD^+ or $NADP^+$ and with L-arabitol, D-arabitol, xylitol or ribitol at pH 7.5–9.0. There is no decrease of absorbance at 340 mμ of NADH or NADPH with L-ribulose, D-ribulose, D-xylulose or L-arabinose at pH 7.5. Therefore, the contribution of two pentitol dehydrogenases in the reversible isomerization of L-arabinose to L-ribulose can be neglected.

Metal requirement. The requirement of Mn^{2+} for the enzyme activity is evident. The enzyme solution was dialyzed against 0.01 M Tris–HCl buffer (pH 8.0) containing 5 mM EDTA for 48 h at 2°, then dialyzed against the same buffer without EDTA for 24 h. The activity of the EDTA-dialyzed enzyme preparation can be recovered by the addition of Mn^{2+} specifically. Mg^{2+} was ineffective, but Co^{2+} showed slight activation of this enzyme (Table III). The Michaelis constant for Mn^{2+} was 5.25 μM (Fig. 7).



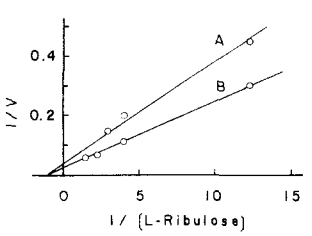


Fig. 9. Effect of Mn^{2+} on the K_m for L-arabinose.

Fig. 10. Effect of concentration of L-ribulose. The standard reactions condition used were as described in text with L-ribulose (0.8–10 mM). Enzyme protein, 9.6 μg (A) and 19.2 μg (B).

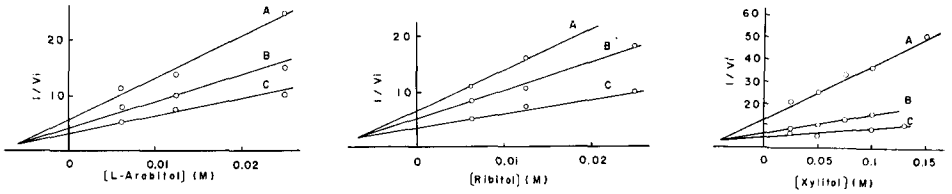


Fig. 11. Inhibition by L-arabitol of L-arabinose-isomerase activity. The reaction system was the same as in Table III with 0–0.025 M of L-arabitol and 2.5 mM (A), 3.3 mM (B) and 5 mM (C) of L-arabinose.

Fig. 12. Inhibition by ribitol of L-arabinose-isomerase activity. The reaction system was the same as in Table III with 0–0.025 M of ribitol and 2.5 mM (A), 3.3 mM (B) and 5 mM (C) of L-arabinose.

Fig. 13. Inhibition by xylitol of L-arabinose-isomerase activity. The reaction system was the same as in Table III with 0–0.15 M of xylitol and 2 mM (A), 4 mM (B) and 8 mM (C) of L-arabinose.

Effect of substrate concentration. As the enzyme requires Mn^{2+} for its activity, the apparent Michaelis constant for L-arabinose should vary depending on the Mn^{2+} concentration (Fig. 8). The calculated apparent Michaelis constants were then plotted *versus* the reciprocal of the Mn^{2+} concentration. The intercept should be the true Michaelis constant. This is 55 mM for L-arabinose (Fig. 9). The affinity for L-ribulose calculated from the conventional Lineweaver–Burk equation was 5.0 mM (Fig. 10). Owing to the inaccuracy of determination of remaining ribulose by colorimetric method, it is difficult to obtain the K_m by the above-mentioned method.

Inhibition by pentitols. The activity of the crystalline L-arabinose isomerase is strongly inhibited by L-arabitol, ribitol and xylitol, but not by D-arabitol (0.005–0.2 M). The inhibition by these pentitols was found to be competitive against the concentration of L-arabinose. The K_i was 7.5, 6, and 38 mM for L-arabitol, ribitol, and xylitol, respectively (Figs. 11–13). Pentonates (L-arabonate, D-arabonate and D-ribonate, 0.005–0.1 M) showed neither an inhibitory nor stimulatory effect on the L-arabinose-isomerase activity.

DISCUSSION

The presence of the L-arabinose isomerase was first reported by LAMPEN[1] in 1954. The role of the enzyme was clearly elucidated in the metabolic pathway of L-arabinose fermentation by *L. plantarum*[2] and *A. aerogenes*[5]. The enzyme was later partially purified[2,4], but the isolation of the enzyme and its reaction mechanism received relatively little attention. D-Xylose isomerase was first isolated in the crystalline form from *L. brevis* by YAMANAKA[7] (1968). L-Arabinose isomerase is here reported to be obtained in pure state as crystals. Recently, PATRICK AND LEE[13] reported on the purification of L-arabinose isomerase from a mutant strain of *Escherichia coli*. From their abstract, the L-arabinose isomerase was not isolated in a crystalline form yet, but was homogeneous in the ultracentrifuge. The molecular weight was estimated as $3.62 \cdot 10^5$. This compares with the *L. gayonii* enzyme. The molecular weight of the latter was estimated as $2.71 \cdot 10^5$. In spite of the different origin, it was found there were some similarities between the two enzymes. Both enzymes required Mn^{2+} for their activation.

Evidence presented in Figs. 7, 8 and 9 indicated that the apparent K_m for L-arabinose decreased when the Mn^{2+} concentration was increased, while the v_{max} remained constant. This is the same with the D-xylose isomerase of *L. brevis*[7,14]. This implies that Mn^{2+} would participate in the enzyme–substrate complex formation as enzyme–Mn^{2+}–substrate (L-arabinose or L-ribulose). This is in contrast to the Mn^{2+}-requiring D-lyxose isomerase of *A. aerogenes* found by ANDERSON AND ALLISON[15]. The Michaelis constant for Mn^{2+} was found to be 5.25 μM from Fig. 7. The K_m for Mn^{2+} was 6.1 μM for the crystalline D-xylose isomerase[7]. These data coincided well and would imply a similar role of Mn^{2+} for the activation of L-arabinose isomerase and D-xylose isomerase. For the D-xylose isomerase, a compulsory order of substrate binding is proposed in which the substrate binds only to the Mn^{2+}–enzyme complex to form ternary complex, D-xylose Mn^{2+}–enzyme complex[16]. A similar mechanism is expected for the enzyme–substrate complex formation by L-arabinose isomerase.

Several pentitols were found to be the effective inhibitors of the enzyme, and the inhibition was competitive. The K_i for L-arabitol or ribitol were almost equal to the Michaelis constant for L-ribulose, but smaller than the Michaelis constant for L-arabinose. This evidence would imply the specificity of the enzyme affinity for the substrate molecule. L-Arabitol and ribitol were also reported to be competitive inhibitors of the L-arabinose isomerase from *E. coli*[13].

The differences between D-xylose isomerase and L-arabinose isomerase, especially concerning their substrate specificity, are clear, but there is much similarity between the enzymes. Similarities or differences between the enzymes, for example, the amino acid sequence around the active site of the enzyme, will give more valuable suggestions to elucidate the reaction mechanism of enzymatic isomerization of pentose. The availability of large quantities of the crystalline enzyme should facilitate an approach to the problem.

ACKNOWLEDGEMENT

We are grateful to Dr. H. Katagiri, emeritus professor of Kyoto University for his continued encouragement.

ADDENDUM

After submittance of this manuscript, a paper on the L-arabinose isomerase from *E. coli* by PATRICK AND LEE was published[17]. It is now clear that there is much similarity between the actions of L-arabinose isomerases of *E. coli* and *L. gayonii*. The Michaelis constant for L-arabinose is 55 mM for *L. gayonii*, and 60 mM for *E. coli*. The K_i for ribitol is the same, 6 mM, for both enzymes, but *L. gayonii* enzyme is more sensitive to the inhibition by L-arabitol. The K_i is 7.5 mM for *L. gayonii* enzyme, and 18 mM for *E. coli* enzyme. Xylitol also inhibits the enzyme activity in both cases, but its K_i is not reported for *E. coli* enzyme.

REFERENCES

1 J. O. LAMPEN, *Abstr. Am. Chem. Soc.*, (1954) 44C.
2 E. C. HEATH, B. L. HORECKER, P. Z. SMYRNIOTIS AND Y. TAKAGI, *J. Biol. Chem.*, 231 (1958) 1031.

3 K. YAMANAKA, *Bull. Agr. Chem. Soc. Japan*, 24 (1960) 310.
4 K. YAMANAKA, *Agr. Biol. Chem. Tokyo*, 26 (1962) 167.
5 F. J. SIMPSON, M. J. WOLIN AND W. A. WOOD, *J. Biol. Chem.*, 230 (1958) 457.
6 K. YAMANAKA AND W. A. WOOD, in W. A. WOOD, *Methods in Enzymology*, Vol. 9, Academic Press, New York, 1967, p. 596.
7 K. YAMANAKA, *Biochim. Biophys. Acta*, 151 (1968) 670.
8 Z. DISCHE AND E. BORENFREUND, *J. Biol. Chem.*, 192 (1941) 583.
9 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1941) 384.
10 R. G. MARTIN AND B. N. AMES, *J. Biol. Chem.*, 236 (1961) 1372.
11 B. CHANCE AND A. C. MAEHLY, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 2, Academic Press, New York, 1955, p. 764.
12 T. SAMEJIMA, M. KAMATA AND K. SHIBATA, *J. Biochem. Tokyo*, 51 (1962) 181.
13 J. W. PATRICK AND N. LEE, *Bacteriol. Proc.*, (1968) 123.
14 K. YAMANAKA, *Kagawa Daigaku Nogakubu Kiyo*, 16 (1963) 1.
15 R. L. ANDERSON AND D. P. ALLISON, *J. Biol. Chem.*, 240 (1965) 2367.
16 K. YAMANAKA, *Arch. Biochem. Biophys.*, submitted for publication.
17 J. W. PATRICK AND N. LEE, *J. Biol. Chem.*, 234 (1968) 4312.