Studies on the Pentose Isomerases of Lactic Acid Bacteria

Part VII. Chromatography of Pentose Isomerases*

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Column chromatographic procedures for the purification of pentose isomerases were carried out. Cation and anion exchange resins were not suitable because of their low exchange capacities and instability of the enzyme at acidic pH range. But the isomerases were successfully purified by DEAE-cellulose column chromatography with high recovery (85~90%). Using a Tris buffer, KCl concentration was increased in gradient. D-Xylose isomerase was eluted at pH 7.0 at 0~0.2 M KCl, and L-arabinose isomerase at pH 8.0 at 0~0.4 M KCl. The purified isomerases, D-xylose isomerase and L-arabinose isomerase, both required manganese ions specifically for their activities.

INTRODUCTION

Pentose isomerases (D-xylose and L-arabinose isomerases) which catalyze the interconversion of aldopentoses and corresponding ketopentoses were found in several bacteria^{1~11}. L-Arabinose isomerase from *Lactobacillus plantarum*, a homofermenter, was purified 7.7 fold by Heath et al. in the specific activity⁷), and D-xylose and L-arabinose isomerases from heterofermenters were also purified to about 8 fold in the specific activity¹²).

In the present paper, further purifications of the enzyme using column chromatography were carried out from the heterofermenters grown in a pentose-medium containing 5×10^{-3} M MnSO_4 .

METHODS

Culture.

D-Xylose and L-arabinose isomerases were obtained from the cells of *Lactobacillus brevis* and *L. gayonii* grown in D-xylose or L-arabinose, respectively¹². In order to increase the yield of the enzyme, the cultural medium was modified as reported previously¹³. Micro-organisms were grown for 16 hours, at which time the enzyme activity was the highest (Table I).

Preparation of Enzymes.

Partial purification of isomerase from pentose-grown cells was carried out as reported in the previous paper¹² with several modifications. Precipitate by ammonium sulfate at 0.6 to 0.9 saturation was dissolved in 0.05 M Tris* buffer (pH 7.4 for D-xylose isomerase, pH 8.0 for L-arabinose isomerase) and

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* The following abbreviations are used: Tris, Tris-(hydroxymethyl)-aminomethane; EDTA, disodium salt of ethylenediamine tetraacetic acid; CM-cellulose, carboxymethyl-cellulose; DEAE-cellulose, diethylaminoethyl-cellulose; FMN, flavin mononucleotide; and FAD, flavin adenine dinucleotide.

TABLE I. EFFECT OF CULTURAL TIME ON ISOMERASE PRODUCTION

Culture (hr.)	Cell growth (O.D. ₅₃₀)	Cell yield (g/3 l)	Enzyme preparation	Protein (mg)	D-Xylose isomerase	
					Specific activity	Total units
12	0.77	7.0	{Crude extracts	332	10.3	3415
			{(NH ₄) ₂ SO ₄ 0.6~0.9 sat.	50.4	29.1	1455
16	0.87	10.0	{Crude extracts	480	7.8	3755
			{(NH ₄) ₂ SO ₄ 0.6~0.9 sat.	54.0	33.4	1810
18	0.91	9.4	{Crude extracts	483	5.2	2540
			{(NH ₄) ₂ SO ₄ 0.6~0.9 sat.	58.4	30.1	1750
22	0.96	8.0	{Crude extracts	378	4.5	1710
			{(NH ₄) ₂ SO ₄ 0.6~0.9 sat.	55.7	26.8	1490
24	0.97	7.8	{Crude extracts	328	3.4	1100
			{(NH ₄) ₂ SO ₄ 0.6~0.9 sat.	43.5	18.8	820
45	0.98	7.4	{Crude extracts	347	0.0	trace
			{(NH ₄) ₂ SO ₄ 0.6~0.9 sat.	50.5	0.0	trace

L. brevis, D-xylose isomerase.

dialyzed against 0.025 M Tris buffer (pH 7.4) at 6°C for 40 hours. Further purification was carried out with acetone. The pH of ammonium sulfate fraction was adjusted to 4.8 to 5.0 with 0.05 M acetic acid. Acetone previously cooled at -20°C was added dropwise to the fraction at -10~-15°C with stirring and precipitates were collected at acetone concentrations of 0~40% and 40~60%. Isomerase was extracted with chilled water from these precipitates. After centrifugation, the clear supernatant was obtained which was used mainly in this experiment. The precipitate was reextracted with 0.05 M Tris buffer, but only a trace of enzyme activity was recovered. Typical data for preparation of enzymes are shown

TABLE II. PREPARATION OF PENTOSE ISOMERASE*

A. D-Xylose isomerase (*L. brevis*)

Fraction	Volume (ml)	Protein (mg)	Specific activity	Total units	Yield (%)
Crude extracts	50	480	7.8	3755	—
Mn-treated	50	237	12.5	2960	79.0
Ammonium sulfate ppt. 0.6~0.9 sat.	10.6	54	33.4	1805	51.0
Acetone 40~60% ppt.	7.0	23.3	75.9	1330	35.4

B. L-Arabinose isomerase (*L. gayonii*)

Fraction	Volume (ml)	Protein (mg)	Specific activity	Total units	Yield (%)
Crude extracts	50	255	5.9	1670	—
Mn-treated	50	162	13.1	2115	126
Ammonium sulfate ppt. 0.6~0.9 sat.	9.0	24.3	46.6	1110	66.5
Acetone 40~60% ppt.	6.0	14.5	85.5	1024	61.4

* 3 l, 16 hours old culture.

in Table II. Fractionation with ethanol at various pH range was not successful. Enzyme preparations were stored at -20°C.

Chromatography on Ion Exchange Resins.

Cation and anion exchange resins were bufferized with citrate-phosphate buffer at pH 4.0 to 8.0 according to the method of Hirs, Moore and Stein¹⁴. The enzyme solution was treated with bufferized resins for thirty minutes at 0°C in a test tube. After separation of resins by centrifugation or filtration, the enzyme was eluted from the resins with two 5.0 ml portions of 0.2 M NaH₂PO₄-Na₂HPO₄ buffer (pH 7.0 for D-xylose isomerase and pH 8.0 for L-arabinose isomerase). After adjustment of the pH of effluents to 7.0 or 8.0, the enzyme activity was determined.

Chromatography on DEAE-Cellulose.

CM- and DEAE-celluloses, Serva Products, were kindly supplied by Prof. Y. Morita, Kyoto University. Enzyme was applied on a 10~14 cm × 0.8 cm column which contained about 0.5 g of DEAE-cellulose in equilibrium with the starting buffer. For most experiments, the initial buffer was 0.025 M in respect to Tris concentration. Column was cooled by passing cold water at 2~4°C through a jacket tube. A gradient elution with KCl was carried out at a constants pH. As a starting buffer, 100 ml of 0.025 M Tris buffer were placed in a mixing chamber (A). In another 100 ml Erlenmyer flask (B), 100 ml of the same buffer containing KCl 0.2 or 0.4 M were added. These are connected by a syphone of polyethylene tubing (0.4 cm internal diameter), and the bottom

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of two flasks were kept at the same level and mixed by a magnetic stirrer. The flow rate through column was regulated at 0.5 ml per minute, and effluent fractions of 20 ml were collected. The optical density of each fraction was measured at 280 $m\mu$.

RESULTS AND DISCUSSIONS

Some Properties of Pentose Isomerases.

Effects of temperature, pH and buffers on their activities and stabilities were determined as shown in Figs. 1~3 and in Table III. With ten minutes' incubation time, the optimum temperature was 45°C for both isomerases. In the presence of 10^{-3} M $MnSO_4$, however, enzyme activities were strongly accelerated at higher temperatures and attained maximum at 60°C. Manganese ions apparently served as a protective agent against thermal inactivation of the enzyme¹²). In this experiment, activities were measured at pH 7.4 for both isomerases after the preincubation at a temperature 10°C lower than the reaction temperature for five minutes. Optimum pH for activity was determined in ten minutes' in-

cubation and found to be at pH 5.8~7.0 for D-xylose isomerase, and at pH 7.8~8.0 for L-arabinose isomerase. Effects of pH on enzyme stability were examined by incubating the enzyme at various pH's at 20°C for 150 minutes. After adjusting pH's to 7.0 and 8.0 for D-xylose isomerase and L-arabinose isomerase respectively, remaining activities were determined and expressed as per cent of original activity. D-Xylose isomerase was most stable at pH 7.0~7.4 and L-arabinose isomerase at pH 8.2.

Furthermore, effects of buffers and of dilution of enzyme for stability were also determined. Enzyme was diluted 80 fold and stored at 0°C in 0.1 M NaH_2PO_4 - Na_2HPO_4 or in 0.05 M Tris buffer for 90 hours. D-Xylose isomerase was less stable in the phosphate buffer than in the Tris buffer, and the addition of cysteine could not prevent this inactivation.

FIG. 1. Temperature-Activity Curve.

A. D-Xylose isomerase. B. L-Arabinose isomerase.
 —○— Without Mn⁺⁺, —●— With Mn⁺⁺ (10⁻³ M)
 Reaction for 10 min.

FIG. 2. pH-Activity Curve.

- A. D-Xylose isomerase (Specific activity 52.5, protein 22 $\mu\text{g}/3\text{ ml}$).
 B. L-Arabinose isomerase (Specific activity 42.0, protein 25 $\mu\text{g}/3\text{ ml}$).
 —○— Tris buffer, —●— Mcllvain buffer
 Reaction at 37°C for 10 min.

FIG. 3. pH-Stability Curve.

- A. D-Xylose isomerase (Specific activity 58.4, protein 16.5 $\mu\text{g}/3\text{ ml}$).
 B. L-Arabinose isomerase (Specific activity 42.0, protein 25 $\mu\text{g}/3\text{ ml}$).
 —○— Tris buffer, —●— Mcllvain buffer.
 Incubated at 20°C for 150 min.

TABLE III. EFFECTS ON STABILITY OF ISOMERASE

Incubation (0°C, hr.)	Activity remained							
	D-Xylose isomerase Xylulose formed, $\mu\text{g}/1.0\text{ ml}$ enzyme solution				L-Arabinose isomerase Ribulose formed, $\mu\text{g}/1.0\text{ ml}$ enzyme solution			
	0	2	18	44	0	18	42	90
0.1 M $\text{NaH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ 4.0 ml	121.5	112.5	87	57.6	253	201.5	203	173
// +cysteine 5×10^{-4} M		75.5	67.8	55.1		255	205	173
0.05 M Tris 4.0 ml		97.2	99.2	105.5		205	202	176
// + MnSO_4 10^{-3} M		108.8	129	147		240	208	168
// +cysteine 5×10^{-4} M		92.8	111.4	125		221	200	192

Compositions for incubation at 0°C were as follows: Enzyme (acetone fraction, D-xylose isomerase, specific activity 58.4, protein 1.65 mg/ml, 96.2 u/ml; L-arabinose isomerase, specific activity 47.4, protein 4.35 mg/ml, 206 u/ml). 0.25 ml of diluted enzyme solution (4 times with water), buffer 4.0 ml and other ingredients. Total volume was 5.0 ml.

pH of buffer, 7.0 for D-xylose isomerase; 8.0 for L-arabinose isomerase. At intervals, 1.0 ml of this incubation mixture was pipetted out, and remaining enzyme activity was determined.

Adsorption of Isomerases on Ion Exchange Resins.

As shown in Table IV, D-xylose isomerase was found to be adsorbed on Amberlite IRC-50 at pH 4.0. But column chromatography of the isomerase by this resin at pH 4.0 was unsuccessful because the enzyme was unstable at such an acidic pH. In one ex-

periment, D-xylose isomerase (specific activity 51.3, 1250 u) was adsorbed completely on a column (0.8×11 cm) of this resin at pH 4.0 and then the enzyme was eluted with 300 ml of 0.2 M $\text{NaH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ buffer (pH 7.0) at a flow rate of 1.0 ml per ten minutes. Most of the protein was recovered between 14th to

TABLE IV. ADSORPTION ON ION EXCHANGE RESINS

A. D-Xylose isomerase

Enzyme	Exchanger (500 mg)	Activity recovered (%)				
		pH	Passed solution	1st eluate	2nd eluate	Total
Total 103 u	Amberlite IRC-50 (200~400 mesh)	4.0	0.4	49.8	25.4	75.6
Protein 2.3 mg		5.0	76.0	7.4	2.4	85.8
Specific activity 55.7		6.0	82.0	6.7	1.9	90.6
		7.0	84.7	10.5	3.0	98.2
Total 103 u	Dowex-50 (200~400 mesh)	4.0	69	4.0	0	73
Protein 2.1 mg		6.0	95	5	0	100
Specific activity 49.1	Amberlite IRA-411 (100~200 mesh)	4.0	43.4	4.9	2.0	50.3
Total 103 u		5.0	72.2	10.6	3.2	86.0
Protein 2.3 mg		6.0	76.6	11.0	3.7	91.2
Specific activity 55.7		7.0	83.2	10.0	4.1	97.3
		8.0	51.5	9.1	4.7	65.3
Total 103 u	Dowex-2 (100~200 mesh)	4.0	41.5	9.0	3.3	53.8
Protein 2.1 mg		5.0	69	19.6	6.4	95.0
Specific activity 49.1		6.0	70.5	17.4	5.8	93.7
Total 96.2 u	CM-cellulose (50 mg)	4.0	15.3	26.6	—	42.9
Protein 1.65 mg		5.0	64	6.5	—	70.5
Specific activity 58.4		6.0	81.8	5.7	—	87.5
		7.0	77.4	5.0	—	82.4

B. L-Arabinose isomerase

Total 66.2 u	Amberlite IRC-50 (200~400 mesh)	4.0	0.2	35.7	14.4	50.3
Protein 1.7 mg		5.0	29.1	8.5	3.1	40.7
Specific activity 40.3		6.0	33.4	4.2	1.3	38.9
		7.0	42.5	5.0	1.9	49.4

20th tubes, but yield of the enzyme activity was only 2.5 per cent of the original activity, and the further elution was useless.

Column Chromatography on DEAE-Cellulose.

From the above results, it was thought to be desirable to run chromatography at a neutral or slightly alkaline pH for pentose isomerases. For this purpose, DEAE-cellulose seemed to be the most useful. D-Xylose isomerase (specific activity 32.5, 745 u) was adsorbed completely on DEAE-cellulose which was previously equilibrated with 0.01 M NaH_2PO_4 - Na_2HPO_4 (pH 7.0). When a column was washed successively with each 50 ml of 0.01 M, 0.02 M and 0.05 M, and each 100 ml of 0.1 M and 0.2 M of the same buffer (pH 7.0), the enzyme was eluted with 0.1 M buffer, but recovery of the enzyme was still only 22.1 per cent owing to the unstable nature of enzyme in phosphate buffer. Elution of the enzyme with Tris buffer by stepwise elution method with increased KCl molarity recovered 75 per cent of the activity, but the enzyme activity appeared in two or more peaks. Similarly, L-arabinose isomerase was also found in two peaks by stepwise elution. Therefore the gradient elution

FIG. 4. Column Chromatography of D-Xylose Isomerase on DEAE-Cellulose.

—●— D-Xylose isomerase, - - - - - Protein

FIG. 5. Column Chromatography of L-Arabinose Isomerase on DEAE-Cellulose.

—○— L-Arabinose isomerase, - - - - - Protein

method was applied. Typical elution patterns are shown in Figs. 4 and 5. D-Xylose isomerase (1600 u) and L-arabinose isomerase (910 u) were applied and 88 and 85 per cents respectively of the original activities were recovered. Eluted fractions were collected and dialyzed against 0.025 M Tris buffer (pH 7.4) containing 5×10^{-3} M MnSO_4 . However, dialysis against the same buffer without addition of MnSO_4 caused the inactivation of the enzyme, i.e. the activity was diminished to about one half to one third. The addition of pyridoxol, pyridoxal, pyridoxamine, nicotinamide, *p*-aminobenzoate, biotin, FMN or FAD (each 400 μg) with 10^{-3} M of MnSO_4 to the inactivated enzyme preparations did not reactivate the enzyme.

Substrate Specificity and Metal Requirement.

Purified enzyme preparations exhibited a maximal absorption spectrum at 280 $\text{m}\mu$ (Fig. 6), and showed high specificity on the respective substrates. Except corresponding aldopentoses and their ketoses, no other ketoses were produced from the corresponding pentoses, hexoses or sugar alcohols. So it was assumed that the two pentose isomerases were different enzymes. L-Arabinose isomerase was activated by Mn^{++} or $\text{Co}^{++2,12}$. Further

FIG. 6. UV-Spectrum of Purified Pentose Isomerases.

X. D-Xylose isomerase, A. L-Arabinose isomerase.

TABLE V. SUBSTRATE SPECIFICITY ON PENTOSE ISOMERASE

Isomerase	D-Xylose isomerase	L-Arabinose isomerase
Specific activity	78.2	132.0
Protein used ($\mu\text{g}/\text{tube}$)	15.5	13.0
Substrate (20 μmoles)	Ketose formed (or decreased) in 10 min. at 37°C (μg)	
D-Xylose	156	0
D-Xylulose	-165	0
L-Xylose	0	0
L-Arabinose	0	205
L-Ribulose	0	-250
D-Arabinose	0	0
D-Ribose	0	0
L-Rhamnose	0	0
Glucose	0	0
Mannose	0	0
Galactose	0	0
Mannitol	0	0
Sorbitol	0	0
Dulcitol	0	0

observations were carried out on these purified enzyme preparations after EDTA-treatment as described in the previous paper¹²). When the enzyme was treated with more concentrated solutions of EDTA (over 5×10^{-3} M) or after prolonged dialysis (20 hours), it was inactivated irreversibly.

After dialysis of the enzyme in the presence of $2.5 \sim 5 \times 10^{-3}$ M EDTA against 0.025 M Tris buffer at 5°C for 20 hours, the enzyme was almost completely inactivated, but the activity was fully restored after addition of MnSO_4 . Addition of cobaltous, magnesium, strontium, calcium, barium, ferric or zinc ions showed only little recovery of activities. So it was concluded that both pentose isomerases are Mn^{++} -catalyzed enzymes.

SUMMARY

Properties of both pentose isomerases are:

	D-Xylose isomerase	L-Arabinose isomerase
Temperature optimum {without MnSO_4 with MnSO_4	45°C 60	45°C 60
pH optimum	5.8~7.0	7.8~8.0
pH for stability	7.0~7.4	8.2

Both isomerases were purified with DEAE-cellulose using a gradient elution technique, increasing KCl concentration at a constant pH in a Tris buffer.

Purified enzymes showed high specificity for

TABLE VI. REQUIREMENT OF METAL

Metal	D-Xylose isomerase Xylulose formed ($\mu\text{g}/3 \text{ ml}$)				L-Arabinose isomerase Ribulose formed ($\mu\text{g}/3 \text{ ml}$)					
	(Final, M)	0	10^{-5}	10^{-4}	10^{-3}	0	10^{-5}	10^{-4}	10^{-3}	5×10^{-3}
MnSO ₄		0	4.5	25.6	250.0	11.8	16.6	26.9	83.2	117
CoCl ₂			0	2.6	37.1		13.4	21.8	25.6	25.6
MgSO ₄			3.8	7.7	15.4		14.7	15.7	28.8	33.3
SrCl ₂			0	0	0		14.1	20.5	30.7	25.0
CaCl ₂			2.6	0	7.7		14.7	14.1	26.3	23.7
BaCl ₂			0	0	2.6		16.0	16.6	32.0	33.3
FeSO ₄			0.6	3.8	10.2		17.3	16.0	26.8	—
ZnSO ₄			0	4.5	13.4		19.2	17.3	11.5	17.9
MnSO ₄ *		256			259	92.8			105.6	

* Before dialysis.

their substrates and metal requirements.

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