Purification and Properties of D-Xylose Isomerase from Alkalophilic *Bacillus* No. KX-6

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An alkalophilic *Bacillus* No. KX-6 isolated from soil produced a D-xylose isomerase in alkaline media. The striking characteristic of this bacterium was its especially good growth in alkaline media. The D-xylose isomerase of this bacterium was purified by ammonium sulfate fractionation, DEAE-Sepharose ion exchange column chromatography and G-200 gel filtration. The molecular weight and sedimentation constant were approximately 120,000 and 9.35S, respectively. The enzyme was most active at pH 7~10 and was stable at pH 6.0 to 11.0. Enzyme activity was stimulated by cobalt ion but inhibited by Hg^{2+} , Ag^{2+} , and Cu^{2+} . Substrate specificity studies showed that this enzyme was active on D-xylose, D-glucose, D-ribose, and D-arabinose. The smaller K_m value and larger $V_{\max}$ value for D-xylose indicated that this enzyme is essentially D-xylose isomerase.

D-Xylose isomerase [D-xylose ketol isomerase, EC 5.3.1.5] occurs in various microorganisms, as have been reviewed by Bern J. Schnyder *et al.*¹⁾ Generally this enzyme is very heat-stable, active in the range 45~65°C, and the reaction does not require any regenerating cofactor such as NAD^+ or ATP. Therefore it is important commercially for the isomerization of glucose to fructose.

Horikoshi reported that alkalophilic microorganisms produced alkaline enzymes.^{2~5)} However, there have been no reports concerning D-xylose isomerases from alkalophilic microorganisms. In this study, we attempted to isolate some alkalophilic microorganisms that produced D-xylose isomerases, and as a result of screening we obtained one strain of bacteria, No. KX-6, which produces an isomerase active in a broad pH range. This paper deals with the isolation and characterization of this bacterium, and some properties of its D-xylose isomerase.

MATERIALS AND METHODS

Culture conditions. A D-xylose medium (AX-medium) was used for the isolation of D-xylose isomerase pro-

ducing bacteria: D-xylose, 5g; polypeptone, 5g; Difco yeast extract, 5g; K_2HPO_4 , 1g; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.1g; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05g; Na_2CO_3 , 10g; and 1 l of water. Sodium carbonate was sterilized separately and added to the medium.

Isolation and selection of D-xylose isomerase producing alkalophilic bacteria. A small amount of soil was suspended in sterilized water and spread on agar plates of AX-medium. After incubation for 2~3 days at 37°C, colonies which produced D-xylose isomerase were inoculated into AX-medium and incubated at 37°C overnight with shaking. The D-xylose isomerase activity of the culture fluid was measured at pH 7.5. The alkalophilic *Bacillus* No. KX-6 was selected from about 150 colonies.

Characterization and identification of bacteria. Microbiological properties were investigated by the methods described in "Aerobic Sporeforming Bacteria"⁶⁾ and "Bergey's Manual of Determinative Bacteriology."⁷⁾ Unless otherwise stated, media used for the identification were supplemented with 1% Na_2CO_3 .

Preparation of crude enzyme solution. *Bacillus* No. KX-6 was grown with shaking on a rotary shaker at 37°C overnight in AX-medium. Cells were harvested at 4°C for 10 min at $8,000 \times g$, and washed twice with 0.05M phosphate buffer (pH 7.5). The washed cells were suspended in a 0.05M phosphate buffer (pH 7.5) and then sonicated (20 kHz, 200 Watts) for 3 min in an ice bath. Cell debris was removed by centrifugation at $8,000 \times g$ for 10 min at 4°C. The supernatant fluid was used as a crude prepara-

tion of D-xylose isomerase.

Enzyme assays. D-Xylose isomerase was assayed with the standard assay mixture described previously.^{8,9} The pH of the mixture was 7.5 in 0.05 M phosphate buffer. The xylulose formed was measured by the cysteine-carbazole method.¹⁰ One unit of enzyme is defined as the amount of enzyme producing 1.0 μ mol of xylulose per min in the standard reaction mixture. Specific activity was expressed as units of the enzyme per mg of protein.

Protein measurement. Protein was measured by the Lowry method¹¹ with bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis. To examine the homogeneity of the purified enzyme preparation, PAGE was done on 10% (w/v) polyacrylamide gels with a Tris-HCl buffer system (pH 8.3). About 5 μ g purified enzyme was put on each gel and run at a constant current of 2 mA per tube until the tracing dye, BPB, reached the bottom of the tube. The protein was stained with 2.5% Coomassie blue. The D-xylose isomerase was actively stained by the method of Yamanaka *et al.*⁹

Molecular weight measured. The molecular weight of the enzyme was estimated by the SDS-PAGE method of Laemmli,¹² gel filtration chromatography, and ultracentrifugal analysis. Molecular weight markers were obtained from BIO-RAD and a Hitachi Analytical Ultracentrifuge UCA-1 was used for ultracentrifugal analysis. The sedimentation constant was calculated by the method of Schachman.¹³

Isoelectric focusing. Isoelectric focusing was done on Servalyt Procotes, pH range 3-6 (Serva). Sample (5 μ l) containing 5 μ g of protein were put on the sample applicator. The gel was prefocused at 5.5 W for 90 min and the proteins were stained with Coomassie blue.

Amino acid analysis. A Hitachi 835 amino acid analyzer was used. The samples were hydrolyzed in 6N HCl at 110°C for 24 hr. The tryptophan content was estimated by 4N methanesulfonic acid hydrolysis at 110°C for 24 hr.

Reagents. D-Xylose was obtained from the Tokyo Kasei Chemical Co. All other chemicals used were of the highest quality available commercially.

RESULTS

Characteristics of the isolate

The isolated strain No. KX-6 was an aerobic, sporeforming, gram positive, motile, and rod-shaped bacterium, clearly in the genus *Bacillus*. The striking characteristic of the

TABLE I. MORPHOLOGICAL, CULTURAL, AND BIOCHEMICAL CHARACTERISTICS OF STRAIN KX-6

Morphological characteristics		
Form, Rods; Size, 0.5~0.6 $\times$ 1.5~2.5 μ m;		
Motility, Motile; Gram stain, Positive;		
Spores, Ellipsoidal, Central;		
Cultural characteristics		
	Growth at	
	pH 7	pH 10
Nutrient broth	—	+
Nutrient agar slant	—	+
Glucose nutrient broth	—	+
Glucose nutrient agar slant	—	+
Peptone water	—	+
Biochemical characteristics		
Hydrolysis of starch	Positive	
Hydrolysis of gelatine and casein	Positive	
Use of citrate	Positive	
Reduction of nitrate to nitrite	Reduced	
Voges-Proskauer test	Negative	
Catalase	Positive	
Growth in NaCl	7%	
G/C content	43.9%	
pH for growth	pH 7.5~11	
Temperature for growth	Up to 42°C	

microorganism was that its growth was very good in alkaline media, and the optimal pH for growth was about 10. Table I summarizes the morphological and cultural characteristics of strain KX-6. It was similar to *Bacillus circulans* in taxonomic characteristics, except for growth pH.

Culture conditions for production of the enzyme

The production of D-xylose isomerase was examined in media containing sodium carbonate or various carbonate salts. The effects of the addition of sodium chloride or potassium chloride were also tested. D-Xylose isomerase activity after overnight cultivation was measured. The results are shown in Table II, which indicate the addition of carbonate or bicarbonate salts is important for the production of D-xylose isomerase.

Purification of the enzyme

Unless otherwise stated, all purification was done at 4°C. The washed *Bacillus* No. KX-6 cells (104 g wet weight from 7 l) were suspended in 0.05 M phosphate buffer (pH 7.5). Each

50-ml portion of cell suspension was treated by a French press at $1,100 \text{ kg/cm}^2$. Cell-free extracts were obtained by centrifugation at $8,000 \times g$ for 10 min. Unbroken cells were re-suspended in the same buffer and the French press treatment repeated in the same manner. The first and second supernatants were combined and centrifuged at $25,000 \times g$ for 30 min. This supernatant (4 u/ml) was fractionated with ammonium sulfate and the precipitate formed between 45%~90% saturation was collected, dissolved in 0.05 M phosphate buffer, pH 7.5, and then dialyzed overnight against the buffer used for the dissolution. The dialyzed solution was put on a DEAE-Sepharose CL-6B column ($1.5 \times 40 \text{ cm}$) that was equilibrated with 0.05 M Tris-HCl, pH 7.5. The column was then washed with 300 ml each of the same buffer containing 0.0 M, 0.1 M and 0.2 M NaCl. These washes removed a substantial amount of protein and no enzyme activity was found in the eluates. A fraction containing the

enzyme was eluted with 200 ml of buffer containing 0.3 M NaCl. This fraction was dialyzed against 0.05 M Tris-HCl buffer, pH 7.5, for 3 hr and then put on another DEAE-Sepharose CL-6B column ($1.6 \times 30 \text{ cm}$) that had been equilibrated with the same buffer. After the column was washed with 200 ml of equilibration buffer containing 0.1 M NaCl, a linear gradient (300 ml total) from 0.1 M to 0.4 M NaCl in the equilibration buffer was used to elute the D-xylose isomerase. The enzyme protein was eluted with 0.25 to 0.3 M NaCl at pH 7.5 and the active fractions (No. 27~33, 5 ml each) were pooled. In the final step, the collected fractions containing D-xylose isomerase were put on a S-200 column ($2.2 \times 90 \text{ cm}$) equilibrated with 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl. The D-xylose isomerase thus obtained was purified about 10-fold, and designated as the purified enzyme in this paper. The results are summarized in Table III.

TABLE II. D-XYLOSE ISOMERASE PRODUCTION IN VARIOUS MEDIA

Salts added to a basal medium	Initial pH	D-Xylose isomerase (units/ml)
None	7.2	ND ^b
NaCl ^a 1.0%	10.5	ND
KCl ^a 1.0%	10.5	ND
NaCO ₃ 0.5%	9.6	1.0
NaCO ₃ 1.0%	10.1	1.3
NaCO ₃ 1.5%	10.5	0.5
NaHCO ₃ 1.0%	8.9	0.7
NaHCO ₃ 2.0%	9.3	1.2

^a The pH was adjusted with NaOH. Basal medium is the AX-medium minus NaCO₃.

^b ND, undetectable.

Physical and chemical properties

PAGE and activity staining of the purified D-xylose isomerase (Fig. 1) showed that the enzyme was homogeneous, and ultracentrifugal analysis showed only one peak throughout the duration of a run of 120 min at 60,000 rpm. The sedimentation constant of the enzyme at pH 8.0 (0.05 M Tris-HCl containing 0.1 M NaCl) was about 9.35 S. The molecular weight of the enzyme estimated by gel filtration through a Toyopearl HW 55 column ($2.2 \times 90 \text{ cm}$) was about 120,000 by comparison with standard proteins (Fig. 2). Electrophoresis of the SDS-treated enzyme preparations on SDS-PAGE (10%) revealed

TABLE III. PURIFICATION OF XYLOSE ISOMERASE OF *Bacillus* KX-6

Steps	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units/mg protein)	Purification
Crude extract	210	850	1176	0.72	1
(NH ₄) ₂ SO ₄ ppt 45~90%	50	785	595	1.32	1.8
1st DEAE-Sepharose	20	560	320	1.75	2.4
2nd DEAE-Sepharose	30	478	95	5.03	7.0
Sephacryl S-200	15	194	26	7.50	10.4

FIG. 1. Electrophoretic Profile of Xylose Isomerase in Polyacrylamide Gel.

(A), active staining by triphenyltetrazolium; (B), stained by Coomassie brilliant blue.

FIG. 2. Estimation of Molecular Weight of D-Xylose Isomerase by Gel Filtration Chromatography on Toyopearl HW 55.

Standard proteins: 1, β -amylase (200,000); 2, alcohol dehydrogenase (150,000); 3, bovine serum albumin (66,000); 4, carbonic anhydrase (29,000); 5, cytochrome (12,400). Sample: ▲, KX-6 xylose isomerase.

one band with a molecular weight of 58,000. Therefore, we concluded that the D-xylose isomerase of *Bacillus* KX-6 has two identical subunits of 58,000 molecular weight. The isoelectric point of the enzyme measured using

TABLE IV. AMINO ACID COMPOSITION OF D-XYLOSE ISOMERASE

Amino acids	Number of residues/molecule		
	No. KX-6	<i>Streptomyces</i> ⁽¹⁹⁾	<i>Bacillus</i> ⁽¹⁵⁾
Aspartic acid	121	180	182
Threonine	58	56	87
Serine	51	36	70
Glutamic acid	105	152	136
Proline	27	72	44
Glycine	95	140	106
Alanine	84	180	140
Half-cysteine	0	4	0
Valine	55	72	53
Methionine	25	32	27
Isoleucine	45	40	51
Leucine	86	144	135
Tyrosine	97	36	58
Phenylalanine	63	92	96
Histidine	34	40	49
Lysine	67	40	120
Arginine	34	132	61
Tryptophan	8	32	13

Servalyt Precote was pH 4.5 and no other peak of the activity was detected. The amino acid composition of D-xylose isomerase is shown in Table IV together with those of other bacterial D-xylose isomerases. The tyrosine content is higher than that of other D-xylose isomerases; no cysteine residues were detected.

Effects of pH on activity and stability

The pH optimum of D-xylose isomerase was found using 0.05 M acetate buffer (pH 3~5); 0.05 M sodium phosphate buffer (pH 6~8); 0.05 M-glycine-NaCl-NaOH buffer (pH 8.5~10.5) and 0.05 M-Na₂CO₃-NaOH buffer (pH 11~12.5). The other conditions were the same as for the standard assay method. The D-xylose isomerase was most active at pH 7~9, and still retained activity at pH 10 (Fig. 3). Stability of the enzyme was investigated in buffer solutions of various pHs. The mixture was incubated for 30 min at 40°C and residual activity was measured at pH 7.5. The following buffer systems were used; 0.05 M acetate buffer (pH 3~5); 0.05 M sodium phosphate buffer (pH 6~8); 0.05 M glycine-NaCl-NaOH buffer

FIG. 3. Effects of pH on Enzyme Activity.

The following buffer systems were used: 0.05 M acetate buffer (pH 3~5); 0.05 M sodium phosphate buffer (pH 6~8); 0.05 M glycine-NaCl-NaOH buffer (pH 8.5~10.5), and 0.05 M Na_2CO_3 -NaOH buffer (pH 11~12.5). Other conditions were the same as for the standard assay method.

FIG. 4. Effect of pH on Stability.

Buffer systems used were acetate (0.05 M, pH 3~5); sodium phosphate (0.05 M, pH 6~8); glycine-NaCl-NaOH (0.05 M, pH 8.5~10.5), and Na_2CO_3 -NaOH (0.05 M, pH 11~12.5). The reaction mixtures were incubated at 40°C for 30 min and residual activity was measured at pH 7.5.

(pH 8.5~10.5) and 0.05 M- Na_2CO_3 -NaOH buffer (pH 11~12.5). As shown in Fig. 4, the enzyme was stable at pH 6.0~11.0.

Temperature optimum for the enzyme action and thermal stability

The optimum temperature was found under the usual assay conditions while the temperature was varied over a range of 40°C to 80°C. Figure 5 shows that the optimum temperature of the enzyme is 60°C. The thermostability of

FIG. 5. Optimum Temperature.

The reaction was done at pH 7.5 for 10 min at each temperature.

FIG. 6. Thermal Stability of Enzyme.

The reaction was done incubating the enzyme solution (1 unit/ml, 1 mM CoCl_2 pH 7.5) at each temperature for 30 min, and residual activity was measured at pH 7.5.

the enzyme was examined by incubating enzyme solutions (1 unit/ml, 1 mM CoCl_2 pH 7.5) at temperatures of 40°C to 80°C for 30 min, and residual activity was measured at pH 7.5. As shown in Fig. 6, the residual activity was 100% when heated at 40~55°C, and the enzyme gradually lost its activity above 60°C.

Substrate specificity

0.1 ml of the enzyme solution (5 units/ml) and 0.1 ml of one of 1% solutions of various sugars were mixed and incubated at 50°C overnight. The mixture was put on an Avicel SF plate and developed with a solvent system of butanol-pyridine-water (6:4:3, v/v). The sugars were detected using naphthoresorcinol

TABLE V. SUBSTRATE SPECIFICITY

Substrate	Degree of ketose formation
D-Xylose	++
L-Xylose	—
D-Glucose	+
D-Mannose	—
D-Galactose	—
D-Ribose	+
D-Sorbose	—
D-Lactose	—
D-Arabinose	+

—, negative; +, positive; ++, strongly positive.

Substrate specificity was measured by thin-layer chromatography using 1% sugar solution and enzyme solution mixture incubated at 50°C overnight.

TABLE VI. EFFECTS OF SOME METALS AND INHIBITORS ON XYLOSE ISOMERASE ACTIVITY

Reagents	Concentration (mM)	Relative activity (%)
None		100
HgCl ₂	1	8
CuSO ₄	1	33
AgNO ₃	1	27
PbCl	1	67
FeSO ₄	1	72
ZnSO ₄	1	61
MgSO ₄	1	100
BaSO ₄	1	97
MnCl ₂	1	114
CoCl ₂	1	131
CaCl ₂	1	100
NiSO ₄	1	92
PCMB	0.1	103
EDTA	10	98

reagent. As shown in Table V, the enzyme was specially active on D-xylose, but showed some affinity for D-glucose. The Michaelis constant and $V_{\max}$ value for D-xylose were 0.076 M and 50 $\mu\text{mol}/\text{min}/\text{mg}$, respectively.

Effects of metals and inhibitors

The enzyme solution (0.02 ml) was mixed with 0.2 ml of 0.05 M phosphate buffer (pH 7.5) containing a test reagent and incubated at 37°C for 10 min. Then 0.2 ml of the substrate (50 mM xylose) was added. As shown in Table VI, Co^{2+} and Mn^{2+} activated the enzyme

reaction, but Hg^{2+} , Ag^{2+} , and Cu^{2+} inhibited the enzyme.

DISCUSSION

A strain of *Bacillus* No. KX-6, a newly isolated soil bacterium, produced a D-xylose isomerase in a highly alkaline medium containing Na_2CO_3 . The microbiological properties of the isolate were very similar to those of *B. circulans*. However, the isolate was distinguished from typical strains of *B. circulans* in the growth pH; the isolate could grow and produce enzyme better in alkaline media rather than in neutral ones. It is noteworthy that D-xylose isomerase was only produced in alkaline media containing carbonate or bicarbonate salts. The homogeneity of the purified enzyme was supported by PAGE, ultracentrifugal analysis, gel filtration, and isoelectric focusing. The enzyme was most active at pH 7~9 which is a much broader range than that of other D-xylose isomerases.^{14~18)} The D-xylose isomerases of *Streptomyces*^{18,19)} were activated by Co^{2+} and Mg^{2+} . However, the enzyme of *Bacillus* No. KX-6 was activated strongly by Co^{2+} and Mn^{2+} . No activation by Mg^{2+} and Ca^{2+} were observed. This enzyme was inhibited by Ag^{2+} , Hg^{2+} and Cu^{2+} like other D-xylose isomerases.^{14~19)} Inactivation by Hg^{2+} suggests the presence of a thiol group, although other thiol agents such as *p*-chloromercuribenzoate were ineffective. The physical properties of the enzyme were very similar to those of *Bacillus coagulans*¹⁵⁾ and *Bacillus stearothermophilus*¹⁶⁾ except that *Bacillus* KX-6 enzyme dissociated into 2 subunits. Another distinctive feature of enzyme was its low molecular weight. The molecular weight of the enzyme estimated by the Archibald method was approximately 130,000 and the gel filtration method gave 120,000. This value is smaller than the enzymes produced by *Streptomyces albus* (165,000 with 4 subunits of 41,500),¹⁹⁾ *Streptomyces* sp. (157,000),¹⁸⁾ *Bacillus coagulans* (167,000 with 4 subunits of 49,000),¹⁵⁾ *Lactobacillus xylosus* (183,000 with 4 subunits of 45,000)¹⁴⁾ and

Bacillus stearothermophilus (130,000 with no subunits).¹⁶⁾ Some differences in amino acid composition were also found from other bacterial D-xylose isomerases.^{15,16,19)} The enzyme of *Bacillus* KX-6 had more tyrosine than those of *Streptomyces* sp. and *Bacillus* sp. These results seem to suggest some differences in their enzymatic properties. Further investigation is required. We conclude that the D-xylose isomerase of KX-6 is different from other D-xylose isomerases so far reported, especially in its broad pH stability and protein properties.

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