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# Characterization of the Purified Extracellular D-Xylose Isomerase Devoid of D-Glucose Isomerase from *Chainia* sp.

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## ABSTRACT

*Chainia* sp. (NCIM 2960), an actinomycete, produced an extracellular, specific D-xylose isomerase and a D-glucose isomerase, along with the conventional intracellular nonspecific D-glucose (xylose) isomerase. The extracellular, specific D-xylose isomerase devoid of D-glucose isomerase activity was purified 11.2 fold and found to be homogeneous, as observed on sodium dodecyl sulphate polycrylamide gel electrophoresis and disk gel electrophoresis at pH 7.5 and 8.3. The average mol wt of the enzyme, as determined by three different methods, is 71,000 dalton. Optimum temperature, pH, and p*I* of the enzyme are 60°C, 9.5, and 3.55, respectively. The enzyme, in the presence of $Mg^{2+}$ (5 m*M*), catalyzes isomerization of D-xylose to D-xylulose, but has no action either on D-glucose or D-ribose. $Hg^{2+}$, $Cu^{2+}$, $Sn^{2+}$, and EDTA, xylitol, mannitol, and sorbitol (5 m*M* each) were inhibitory to the enzyme activity. The equilibrium ratio of D-xylose and D-xylulose in the reaction mixture was influenced by $BO_3^{3-}$ and temperature. Chemical modifiers for arginine (phenylglyoxal, 2,3-butanedione) and histidine (diethylpyrocarbonate) were inhibitory to D-xylose isomerase activity, suggesting their involvement in the reaction mechanism of the enzyme.

**Index Entries:** Extracellular isomerase; xylose isomerase; *Chainia* sp.; glucose isomerase.

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## INTRODUCTION

D-glucose (xylose) isomerase (D-xylose ketol isomerase, EC 5.3.1.5) is an intracellular enzyme induced by D-xylose (*1–4*) in bacteria (particularly *Streptomyces* spp.). The enzyme is used in the production of high fructose corn syrup (HFCS) and has a possible application in the ethanologenesis from petose sugars (*5,6*). Purification of the enzyme to a homogeneous state has been reported from a few microorganisms (*1–3, 7–11*) and it was considered that the isomerization of D-glucose and D-xylose is catalyzed by a single enzyme. In earlier studies, Danno (*12*) made unsuccessful attempts to separate D-glucose isomerase, D-xylose isomerase, and D-ribose isomerase activities from *B. coagulans* HN-68. The presence of substrate-specific D-glucose and D-xylose isomerases, along with the conventional nonspecific intracellular D-glucose (xylose) isomerase from *Chainia,* was previously reported by us (*13*).

This paper describes the isolation, purification, and some properties of the extracellular specific D-xylose isomerase devoid of D-glucose isomerase from *Chainia* sp.

## MATERIALS AND METHODS

### Organism

The culture *Chainia* (NCIM 2960, India) was isolated by dilution poured plate technique from a soil sample collected near Haldighat, Rajasthan, India (*13*). Subcultures were maintained on potato dextrose agar (PDA) (*14*).

### Enzyme Production

Vegetative inoculum was developed for 48 h on a rotary shaker (220 rpm) at 28°C by transferring 1-wk-old culture from PDA slant to 100 mL modified Weber's medium (*15*) (0.05% $K_2HPO_4$; 0.005% $CoCl_2 \cdot 6H_2O$; 0.025% $MgSO_4 \cdot 7H_2O$; 0.05% yeast extract; 1% D-glucose). After 48 h, D-glucose (xylose) isomerase activity was induced by the addition of 5 mL solution containing 2 g of D-xylose and 2.5 mg of $CoCl_2 \cdot 6H_2O$. The culture was harvested after 72–96 h by centrifugation (10,000*g* for 30 min), and the clear culture filtrate was used for the enzyme purification.

### Enzyme and Protein Assay

D-xylose isomerase assays were carried out by cysteine–carbazole method (*13,16*). One unit of enzyme activity represents 1 μmol of D-xylulose formed per minute. Protein estimation in the samples were carried out according to the Bradford (*17*) and Lowry et al. (*18*) method using Bovine Serum Albumin as the standard.

## D-Xylose Isomerase Purification

All the operations with enzyme solutions were carried out at 0–5°C, unless otherwise stated. Culture filtrate containing 210 and 405 units of D-xylose isomerase and D-glucose isomerase activity was concentrated by ultrafiltration using UM-10 membrane and further concentrated with 3.5*M* ammonium sulfate. The pellet collected by centrifugation (10,000*g*, 30 min) was resuspended in 10 m*M* sodium phosphate buffer, pH 8.5, and was dialyzed for 16 h against the same buffer. The contents of the dialysis bag were clarified by centrifugation and heated at 60°C for 30 min to precipitate other thermolabile proteins that were removed by centrifugation. Further purification was carried out by PAGE in a glass column (16×5 cm). After completion of the run, two vertical strips of the gel were cut and D-xylose isomerase was visualized as a red band on polyacrylamide gel by *in situ* staining (*19*). The other gel strip was stained with Coomassie Brilliant Blue G-250 (CBB) to examine the protein pattern of the sample. The enzyme from the gel was eluted into 50 m*M* sodium phosphate buffer, pH 7.5, containing $MgSO_4$, 5 m*M*. The enzyme was further purified by batch-wise treatment with DEAE-Cellulose. The gel (240 mg) was added to 10 mL of the enzyme (containing 93 mg, 106 U D-xylose isomerase and 52 U D-glucose isomerase). The volume was made up to 25 mL with phosphate buffer (pH 7.5, final molarity 10 m*M*). D-xylose isomerase was adsorbed on DEAE-cellulose. After 2 h of occasional stirring, it was filtered through Whatman Filter No. 1. Thus, D-glucose isomerase was separated from D-xylose isomerase. The DEAE-cellulose was washed with 50 mL of 0.15*M* sodium phosphate, and the enzyme was eluted with 10 mL of 0.25*M* sodium phosphate. Finally, the enzyme preparation, obtained from DEAE-cellulose, was loaded on Sephadex G-200 column (1.2×100 cm), previously equilibrated with 50 m*M* sodium phosphate. Fractions (2 mL each) showing D-xylose isomerase activity with only one protein band on analytical PAGE were pooled and concentrated by lyophilization. The fractions showing an additional band of impurity in PAGE were discarded. As a result, the yield in this step was significantly lowered.

## Molecular Weight Determination

The molecular weight of the native extracellular D-xylose isomerase was estimated by gel chromotography (*20*) using Bio Gel P-150 and Sephadex G-200 (1.2×100 cm, elution buffer 50 m*M* phosphate, pH 7.5), as well as by the slope method (*21*).

## Isoelectric Focusing

Isoelectric focusing was performed using LKB 8101 (110 mL) electrofocusing column (*22*) (4°C, 48 h, 3 W power) over glycerol density gradient containing Ampholytes (1.25% w/v) of the pH range 3.5–10.0.

## SDS PAGE

SDS PAGE was performed using the method of Shapiro et al. (*23*) and Weber et al. (*24*).

## Amino Acid Analysis

The samples were analysed in a Spinco model 120-B automatic amino acid analyzer by the method of Spackman et al. (*25–28*).

## Active Site Group Specific Reagents

The effect of the active site group specific reagents on D-xylose isomerase was studied by incubating the enzyme (50 $\mu$g) with various concentrations of the reagents. At different time intervals, aliquots (0.1 mL) from the reaction mixture, along with enzyme and inhibitor blanks, were taken separately and assayed for the enzyme activity. Kinetic analysis was carried out by the method of Levy et al. (*29*).

## Reaction with Arginine- and Histidine-Modifying Reagents

D-xylose isomerase was incubated at 30°C with either phenylglyoxal or 2,3-butanedione in 50 m*M* carbonate-bicarbonate buffer, pH 9.5. The reaction mixture was shielded from light to avoid photochemical effects when 2,3-butanedione was used as the probe (*30*). Enzymes incubated with the buffer alone served as the control. Aliquots were removed at different time intervals for enzyme activity. Diethylpyrocarbonate was used as the histidine-modifying reagent at pH 7.5. The rest of the procedure was followed as mentioned above.

# RESULTS

## Enzyme Purification

The steps for the purification of extracellular, specific D-xylose isomerase from *Chainia* species are summarized in Table 1. A rapid *in situ* location of the enzyme on polyacrylamide gel facilitated purification of the specific D-xylose isomerase enzyme by PAGE (*19*). The purified D-xylose isomerase was homogeneous on SDS PAGE and on disc gel electrophoresis at pH 7.5 and 8.3 (Fig. 1). The hydroxyamidomethylation of the reduced form of the enzyme revealed a single protein band on SDS-polyacrylamide gel (*23,24*). The enzyme is stable for at least 6 mo when stored at −15°C at pH 7.5 in the presence of 5 m*M* $MgSO_4$. The enzyme loses its activity within 3 wk in the absence of $Mg^{2+}$ under the same conditions.

Table 1
Purification of Extracellular Specific Xylose Isomerase from *Chainia* sp[a]

| Step | Total protein, mg | Total units[b] | Specific activity | Purification, fold | Recovery, % |
|---|---|---|---|---|---|
| Fermented broth | 1700 | 210 (405) | 0.12 | - | 100 |
| Amicon filtration | 1533 | 193 | 0.13 | 1.0 | 91.9 |
| Ammonium sulfate precipitation | 920 | 178 | 0.19 | 1.6 | 84.7 |
| Heat treatment | 550 | 170 | 0.31 | 2.5 | 80.9 |
| Preparative PAGE | 93 | 106 (52) | 1.14 | 9.3 | 50.5 |
| DEAE-Cellulose | 38.4 | 48.4 (nil) | 1.26 | 10.2 | 23.0 |
| Sephadex G-200 | 17.5 | 24.2 (nil) | 1.38 | 11.2 | 11.5 |

[a]Average values of nine separate batches.
[b]D-Glucose isomerase activity is given in parenthesis.

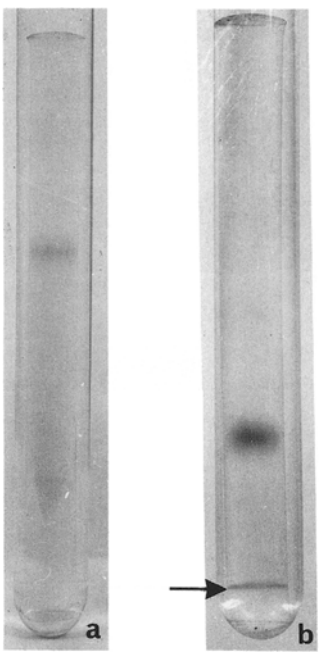


Fig. 1. Polyacrylamide gel electrophoresis and SDS-gel electrophoresis of D-xylose isomerase: (a) PAGE at pH 8.9 and (b) SDS-gel electrophoresis when the enzyme was treated with 1% 2-mercaptoethanol containing 0.5% iodoacetamide. Arrow indicates position of marker dye.

## Enzyme Properties

### *Molecular Weight, Glycoprotein Nature, and pI*

Molecular weight of the enzyme is 70,000–73,000, as estimated by gel filtration on Bio-Gel P-150 (*20*) and SDS-PAGE (*23*) and also by the slope method (*21*). Gels were stained for the detection of glycoprotein nature of the enzyme (*31*). No purple band of glycoprotein corresponding to protein band stained by CBB G-250 dye was observed, indicating an absence of carbohydrate moiety in the protein. The inability of the enzyme to get adsorbed on concanavalin A Sepharose 4B column also supported this observation (*32*). The enzyme has a p*I* of 3.55 (*22*).

### *Influence of pH and Temperature*

The effect of pH on catalytic activity was tested with 50 m*M* sodium phosphate and carbonate-bicarbonate buffers. The enzyme was most stable at pH 8 and showed optimum activity at pH 9.5 with D-xylose as the substrate (Fig. 2a and b). Optimum temperature of the enzyme is 60°C (Fig. 2c). A straight line was obtained when the data was plotted according to Arrhenius (Fig. 2d). From this, the energy of activation was calculated to be 173 kJ/mol.

The thermal stability of the enzyme (dialyzed against 50 m*M* sodium phosphate, pH 7.5, containing 5 m*M* EDTA was monitored by measuring its activity after incubating at various temperatures (35–80°C) for 10 min (Fig. 2e). The enzyme was stable up to 70°C. At 80°C, only 20% of the original activity was observed. In the presence of $Mg^{2+}$ (1 m*M*), the enzyme retained 50% of its activity when heated at 80°C for 10 min, whereas in the presence of $Co^{2+}$ (1 m*M*) under similar conditions, no loss in activity was observed (Fig. 2f).

### *Influence of Metal Ions*

The enzymic isomerization was not observed in the absence of metal ions. The relative enzyme activity in the presence of 5 m*M* each of $Mg^{2+}$, $Co^{2+}$, and $Mn^{2+}$ (taken separately) was found to be 100, 80, and 30%, respectively, under assay conditions. The enzyme activity was inhibited beyond a 5 m*M* concentration of $Mg^{2+}$.

The effect of other metal ions and additives on the enzyme activity was examined under standard assay conditions in which 5 m*M* $Mg^{2+}$ was incorporated. In the presence of $Co^{2+}$, three-fold increase in the activity was observed. $Mn^{2+}$, $Zn^{2+}$, and $Cu^{2+}$ did not affect enzyme activity, whereas $Sn^{2+}$ and $Ca^{2+}$ inhibited the enzyme activity by 50 and 90%, respectively. EDTA, pCMB, and 8-hydroxyquinoline above 5 m*M* concentrations were inhibitory to the enzyme.

### *Enzyme Specificity and Effect of Sugar Alcohol*

The enzyme was found to be active only with D-xylose when various substrates, such as D-glucose, D-mannose, D-galactose, D-ribose, and L-arabinose, were tested.

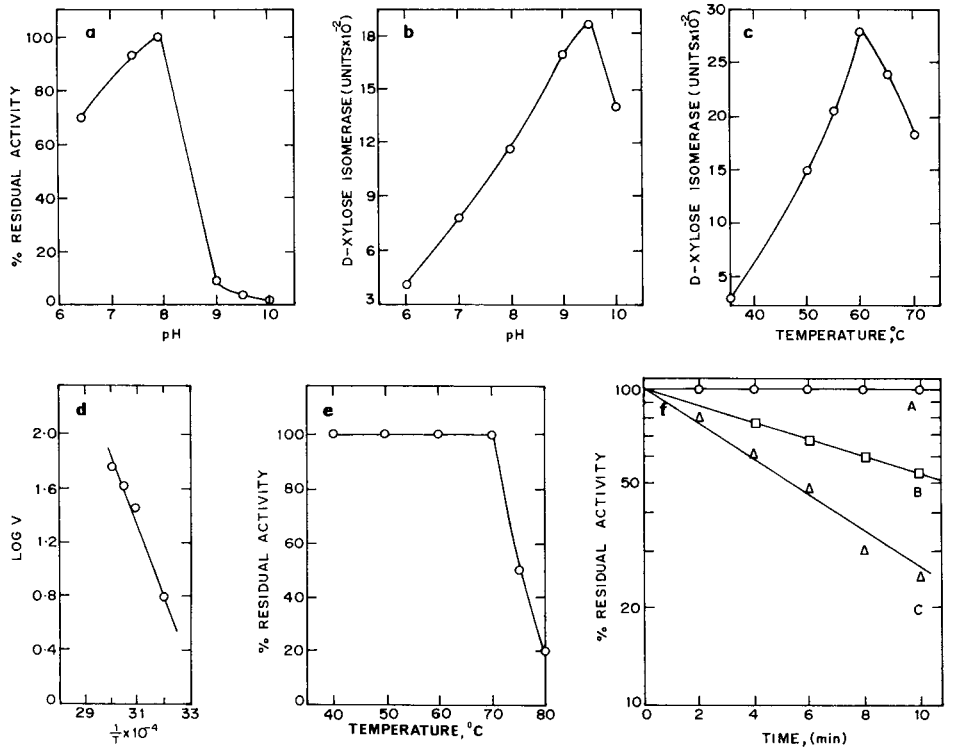


Fig. 2. (a) Stability to pH of D-xylose isomerase (5 μg) when incubated at 60°C for 30 min, (b) pH-enzyme activity profile (30 min., 50°C), and (c) temperature-enzyme activity profile. The observed activities were compared with those of reference samples maintained at 4°C at pH 7.5 for the duration of the experiment. (d) Arrhenius plot for D-xylose isomerase (30 min. assay, pH 9.5). (e) Thermal stability of D-xylose isomerase (3 μg), which was incubated (0.05*M* phosphate buffer, pH 7.5) at temperatures indicated for 10 min. The residual activity (%) in each sample was compared with control (4°C, pH 7.5). (f) Influence of metal ions on thermal stability of D-xylose isomerase (25 μg) incubated at 80°C (0.05*M* phosphate buffer, pH 7.5). A. $CoCl_2$; B. $MgSO_4$; 10 m*M*, C. without any metal ion. The activities were compared with a control sample maintained at 4°C.

Sorbitol and xylitol were found to be competitive inhibitors of the enzyme activity (Table 2). Xylitol was the most potent inhibitor than sorbitol, mannitol, or dulcitol. $K_i$ (inhibitor constant) for D-sorbitol was 9 m*M*, whereas for xylitol it was 2.1 m*M*.

## Kinetic Parameters

The $K_m$ and $V_{max}$ values using D-xylose as the substrate were calculated from Lineweaver-Burk plot and found to be 7 m*M* and 4.9 mg/min at 35°C (pH 9.5), respectively. Since the enzyme is specific for D-xylose, the $K_m$ for D-glucose could not be determined. Also, D-glucose did not inhibit enzyme up to a concentration of $5 \times 10^{-4} M$.

Table 2
Inhibition of D-Xylose Isomerase by Sugar Alcohols[a]

| Sugar alcohol Concentration, *M* | Relative activity, % | | | |
|---|---|---|---|---|
| | Sorbitol | Mannitol | Dulcitol | Xylitol |
| Control | 100 | 100 | 100 | 100 |
| $1\times10^{-2}$ | 58 | 100 | 80 | 10 |
| $2\times10^{-2}$ | 44 | 88 | 76 | 0 |
| $5\times10^{-2}$ | 30 | 69 | 65 | 0 |
| $1\times10^{-1}$ | 18 | 58 | 44 | 0 |

[a]The enzyme (3 μg) was incubated with various concentrations of sugar alcohols at 35°C for 10 min, then reaction was initiated by the addition of D-xylose.

## Equilibrium Between D-Xylose and D-Xylulose

The apparent equilibrium concentrations of D-xylose and D-xylulose in the reaction catalyzed by D-xylose isomerase varies with temperature. At 35°C, equilibrium of 79 (xylose):21 (D-xylulose) was attained, whereas at 60°C, the equilibrium was shifted to 74 (xylose):26 (D-xylulose). The addition of $BO_3^{3-}$ in the reaction mixture also shifted the equilibrium from 79:21 to 20:80 in favor of D-xylulose when 100 μg D-xylose was incubated at 35°C with 3 μg of D-xylose isomerase (Table 3).

## Amino Acid Composition

The enzyme contains higher amounts of glycine and glutamic acid, 14.9 and 11.9 on a molar percent basis, respectively, compared to other constituent amino acids (Table 4). The half cystine is absent and the molar percent of methionine, leucine, tyrosine, and phenylalanine are relatively lower than those in D-glucose (xylose) isomerases reported from other microbial sources (*12,33,35*). Partial specific volume from amino acid composition data is 0.715 mL/g (*28*).

## Action of Group-Specific Reagents

D-Xylose isomerase was rapidly inactivated on incubation with phenylglyoxal or 2,3-butanedione and diethylpyrocarbonate. The results obtained with various concentrations of phenylglyoxal, a diethylpyrocarbonate, are presented in Fig. 3a and b. The inactivation rate follows a pseudo first-order kinetics. The reaction order ($n$), with respect to the phenylglyoxal and diethylpyrocarbonate reagent, was determined from the plot of the log apparent first-order rate constant, $K_{app}$, vs the log reagent concentrations (Fig. 3a and b). The value of $n$, 0.90 and 0.95 for phenylglyoxal and diethylpyrocarbonate, respectively, suggests that the loss of activity results from reaction of one arginine and histidine residue per mol D-xylose isomerase (*30*).

Table 3
Effect of Borate on Equilibrium Concentration of D-Xylulose

| D-Xylose:Borate | | Equilibrium concentration D-Xylose:D-Xylulose | |
|---|---|---|---|
| μg | μg | μg | μg |
| 100:0 | | 79:21 | |
| 100:100 | | 33:67 | |
| 100:200 | | 20:80 | |

Table 4
Amino Acid Composition of Extracellular, D-Xylose Isomerase from *Chainia* sp[a]

| Amino acid | Number of residue mol | Molar percent of amino acid |
|---|---|---|
| Aspartic acid | 44 | 8.2 |
| Threonine[b] | 34 | 6.3 |
| Serine[b] | 36 | 6.7 |
| Glutamic acid | 60 | 11.2 |
| Proline | 38 | 7.1 |
| Glycine | 80 | 14.9 |
| Alanine | 50 | 9.3 |
| Valine[c] | 18 | 3.3 |
| Methionine | 2 | 0.4 |
| Isoleucine[c] | 16 | 3.0 |
| Leucine | 40 | 7.5 |
| Tyrosine[d] | 4 | 0.8 |
| Phenylalanine | 8 | 1.5 |
| Histidine | 30 | 5.6 |
| Lysine | 38 | 7.1 |
| Arginine | 34 | 6.3 |
| Tryptophan[d] | 4 | 0.8 |
| Half-cystine | - | - |

[a]Conditions are described in the text.
[b]Values for threonine and serine are extrapolated to zero time.
[c]Values for valine and isoleucine are those for 72 h hydrolysis.
[d]Tryptophan and tyrosine are determined spectrophotometrically (*26*).

## DISCUSSION

The microbial D-glucose (xylose) isomerase is intracellular and acts on both D-glucose and D-xylose (*5,8*). The results presented in this paper and the earlier preliminary communication (*13*) represent the first report on the detection of extracellular D-xylose isomerase from *Chainia* sp., its separation from D-glucose isomerase, and partial characterization. The stereo-

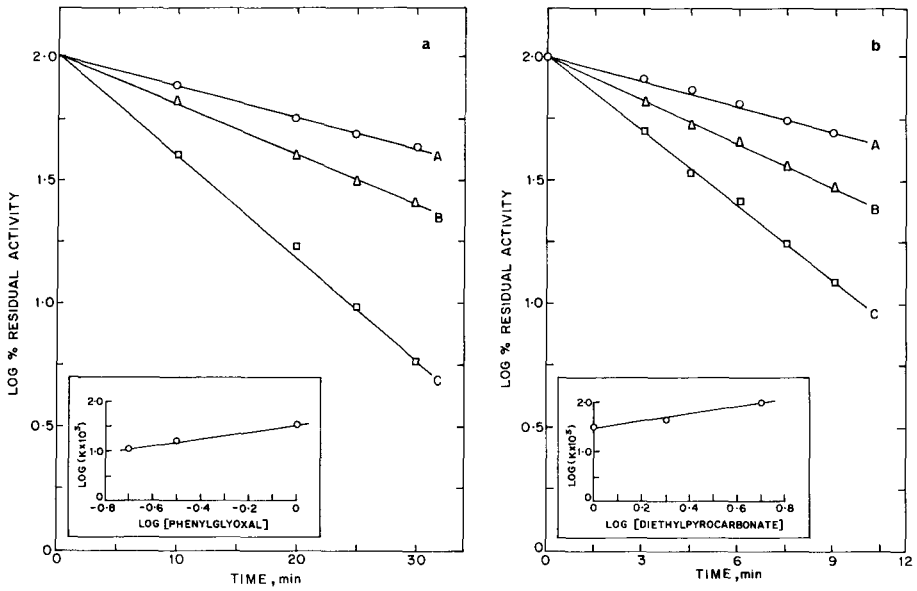


Fig. 3. (a) Kinetics of the inactivation of D-xylose isomerase (50 $\mu$g) at 28°C by phenylglyoxal (A, 0.02 m*M*; B, 0.05 m*M*; and C, 1 m*M*, in 0.02 *M* Carbonate-bicarbonate buffer, pH 9), and (b) diethylpyrocarbonate, (A, 1 m*M*; B, 2 m*M*; and C, 5 m*M*, in 0.02 *M* phosphate buffer, pH 7.5). Control was incubated under identical conditions. Inset: plot of pseudo first-order rate constant (*K*) of the inhibitor (a, phenylglyoxal and b, diethylpyrocarbonate) inactivation reactions vs log of inhibitor concentration.

specificity of D-glucose (xylose) isomerase reported from microbial sources requires that the substrates for a given isomerase should have identical configuration in the carbon atoms 2, 3, and 4 (*36*). Understandably, the D-xylose isomerase from *Chainia* sp. is inactive with D-ribose, which is a C-3 epimer of D-xylose. However, the enzyme is devoid of D-glucose isomerase activity is unusual.

The extracellular D-glucose (xylose) isomerase from a few microbial sources like *S. olivaceous* strain 13, *S. flavogriseus* (*1*) and *S. glaucescenes* ETH 22794 (*15*) have been reported. It is suggested by Gracheva et al. (*37*) that the release of D-glucose (xylose) isomerase from the cell is associated with a change in cell wall permiability and partial lysis of the cells. However, no experimental data is available. No apparent cell lysis was observed in the case of *Chainia* sp. The occurrence of extracellular specific D-glucose and D-xylose isomerase in *Chainia* sp. with the conventional intracellular nonspecific D-glucose (xylose) isomerase poses many problems both at the protein and genetic levels. At the protein level, it can be studied whether the specific D-glucose and D-xylose isomerases arise owing to the posttranslational modifications of intracellular nonspecific D-glucose (xylose) isomerase. However, this will be possible only if the intracellular

enzyme has two distinct binding sites for D-glucose and D-xylose. At the DNA level, it would be interesting to study whether the same gene codes for the intracellular D-glucose (xylose) isomerase and extracellular specific D-glucose and D-xylose isomerase.

The specific D-xylose of isomerase of *Chainia* sp. is a single polypeptide chain of mol wt 71,000. This enzyme from other sources is reported to have two (*4,10,35*) or four (*1,11,34,38*) subunits. The $K_m$ of the enzyme is relatively lower (7 m*M*) compared to commercial preparations (5–93 m*M*). The apparent equilibrium concentrations of D-xylose and D-xylulose in the reaction catalyzed by D-xylose isomerase is influenced by temperature and borate ions in a reaction mixture, as reported by Barker (*40*). Borate appears to exert its effect by trapping enzymatically-formed D-xylulose, thus displacing the equilibrium in favor of D-xylulose (*41*).

The presence of essential arginine residues has been reported in several enzymes acting upon anionic cofactors and substrates (*30*). Inactivation of D-xylose isomerase from *Chainia* sp. by phenylglyoxal and diethylpyrocarbonate may suggest the presence of arginine and histidine in the reaction mechanism followed during isomerization. In this case, comparative studies are not possible since chemical modification experiments are not reported in the case of conventional microbial D-glucose (xylose) isomerases.

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