Purification and Characterization of d-Xylose Isomerase from *Bifidobacterium adolescentis*

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D-Xylose isomerase was purified to homogeneity from cell-free extracts of *Bifidobacterium adolescentis* by ammonium sulfate fractionation and chromatographies on DEAE-cellulose and Butyl-Toyopearl. The molecular weight of the purified enzyme was estimated to be 168,000 by gel filtration on TSKgel G-3000SW, and 53,000 on SDS–polyacrylamide gel electrophoresis. The optimum pH was around 7 and the enzyme was stable at pH 7–8. The enzyme required bivalent cations, Mg^{2+}, Co^{2+}, or Mn^{2+} for the activity, particularly Mn^{2+} to be best. The enzyme had a pI of 4.3, and the K_m for D-xylose was 4 mM. The N-terminal amino acid sequence of the enzyme was not similar to those of D-xylose isomerases from other sources such as *Clostridium thermosulfurogenes*, *Escherichia coli*, or *Bacillus subtilis*.

Bifidobacteria are nonmotile, Gram-positive, anaerobic bacteria, which are natural inhabitants of the large intestine of humans and some animals. Bifidobacteria constitute one of the major microorganisms in the colonic flora of healthy children and adults, and it is also known that the microorganisms are necessary for maintenance of human health, and have some beneficial effects such as stimulation of immune responses and prevention of cancer.

D-Xylose isomerase (D-xylose ketol-isomerase; EC 5.3.1.5) which is also known as "glucose isomerase," catalyzes the conversion of D-xylose to D-xylulose and D-glucose to D-fructose. The enzyme is one of the most useful enzymes in food industry, because it has been used for producing fructose syrup. The enzyme is found in many kinds of microorganisms and its enzymatic properties have been studied. However, enzymatic and physicochemical properties of the D-xylose isomerase from *B. adolescentis* have not yet been reported.

Bifidobacteria also metabolize various kinds of oligosaccharides such as xylooligosaccharides and fructooligosaccharides, which are polymers of xylose, are used by bifidobacteria, but not by *E. coli* and *Clostridium* spp. Xylooligosaccharides can selectively promote the growth of bifidobacteria and help to establish favorable environmental conditions in the intestines.

In the pathway of xylooligosaccharide use in bifidobacteria, D-xylose isomerase is presumed to be one of the key enzymes. The purpose of this paper was to purify D-xylose isomerase from *B. adolescentis* and to characterize some of its enzymatic properties, which were different from those of enzymes from other sources.

**Materials and Methods**

Microorganism and culture conditions. *Bifidobacterium adolescentis* used in this experiment was kindly supplied by Dr. T. Mituoka of Nippon Veterinary and Zootechnical College. The bacterial cells were grown in 200 ml of cultivation medium containing 1.5% d-xylose, 1.3% polypeptone, 0.4% yeast extract, 0.5% NaCl, 0.5% sodium acetate · 3H₂O, 0.4% K₂HPO₄, 0.04% cysteine · 2HCl, and 0.02% MgSO₄ · 7H₂O (pH 7.2) for 20 h at 37°C in static culture. D-Xylose was autoclaved separately and added just before inoculation. Then, 150 ml of cultivated brood was inoculated into 3.0 liters of the same cultivation medium. The cultivation was done for 20 h at 37°C in static culture. Six batches of the cultivations were done.

Preparation of cell-free extracts. The cultivated cells (90 g, wet weight, obtained from 18 liters of the medium) were harvested by centrifugation at 8,000 × g for 20 min at 4°C, and washed twice with cold 20 mM potassium phosphate buffer (pH 7.2). The washed cells were suspended in the same buffer (90 ml) and then sonicated at 20 kHz for 5 min, 6 times in an ice bath. The cell debris was removed by centrifugation at 20,000 × g for 30 min at 4°C. The supernatant obtained (100 ml) was used as the cell-free extract.

Purification of D-xylose isomerase. To purify D-xylose isomerase, all purifications were done at 4°C. Solid ammonium sulfate was added to the cell-free extract (100 ml) obtained from *B. adolescentis* cells (90 g, wet weight) up to 35% saturation and the precipitate was removed by centrifugation at 20,000 × g for 30 min. Still more ammonium sulfate was added to the supernatant up to 65% saturation. The precipitate collected by centrifugation was dissolved in 20 mM potassium phosphate buffer (pH 7.2), and dialyzed against the same buffer for 10 h with several changes of fresh buffer. The precipitate formed during dialysis was discarded by centrifugation, and the supernatant was concentrated by ultrafiltration. The concentrated enzyme solution was put on a DEAE-cellulose column (3 × 16 cm) previously equilibrated with 20 mM potassium phosphate buffer (pH 7.2). The column was washed with five bed volumes of the same buffer and D-xylose isomerase was then eluted with a linear gradient from 0 to 0.5 M potassium chloride in the same buffer at a flow rate of 100 ml/h. The active fractions were collected and concentrated by ultrafiltration. The concentrated enzyme solution was put on a first Butyl-Toyopearl column (1.5 × 20 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.2) containing 35% ammonium sulfate. The column was washed with five bed volumes of the same buffer and the adsorbed enzyme was eluted with a linear gradient from 35 to 0% ammonium sulfate in the same buffer. The active fractions were collected and concentrated by ultrafiltration, and then dialyzed against the same buffer. The dialyzed solution was put on a second Butyl-Toyopearl column (1.5 × 12 cm) equilibrated with the same buffer containing 35% ammonium sulfate. The enzyme was eluted with the same conditions as the first chromatography. The active fractions were collected and concentrated by ultrafiltration to about 1 mg of protein/ml, and used as the purified enzyme solution. The preparation was stored in a freezer at −20°C until use.

Assay of D-xylose isomerase. To assay D-xylose isomerase, reaction
mixture of 0.1 ml of 50 mM D-xylose, 0.1 ml of 10 mM MnCl₂·4H₂O, the enzyme solution, and 20 mM potassium phosphate buffer (pH 7.2) was used. The total volume was made up to 1.0 ml with the phosphate buffer (pH 7.2). The reaction mixture was incubated at 37°C for 10 min and the reaction was stopped by adding 1 ml of 0.5 M perchloric acid. The D-xylose solution formed was measured by the cytochrome-c-barbazaole method. One unit of enzyme activity was defined as the amount of enzyme that produced 1.0 μmol of D-xylose per minute under the above assay conditions. The specific activity of the enzyme was expressed as units per mg of protein. Protein was measured by the Lowry method with bovine serum albumin as a standard.

**Gel electrophoresis of protein.** Polyacrylamide gel disc electrophoresis (PAGE) was done by the method of Weber using a 7.5% acrylamide gel, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemml using a 10% acrylamide gel. Proteins on the gel were stained with 0.5% Coomassie Brilliant Blue R-250 and destained with a mixture of methanol-acetic acid-water (50:75:85, by volume). Carbohydrates on the gel were detected using periodic acid Schiff staining. Isoelectric focusing was done with a 1% agarose gel containing 5% Ampholine (pH 3.0-10.0) for 3 h at a constant voltage of 500 V at 10°C, using the ATTO apparatus developed by Vesterberg. The isoelectric point of the band was calculated from a calibration curve obtained with standard pI markers (Pharmacia).

**Analysis of N-terminal amino acid sequence.** The purified enzyme was electrophoresed by SDS-PAGE, blotted onto a polyvinylidene difluoride membrane, and the N-terminal amino acid sequence of the first 39 amino acid residues was analyzed with an Applied Biosystems model 473A protein sequencer.

**Calculation of Kₘ.** The enzymatic activity was measured under the standard assay conditions except that the substrate concentration was varied over a range of 0.5-20 mM for D-xylose. The apparent Kₘ was estimated from Lineweaver-Burk plots.

**Results and Discussion**

**Purification of D-xylose isomerase**

The purification of the enzyme is summarized in Table I. The enzyme was purified to homogeneity from the cultured cells of *B. adolescente*is by ammonium sulfate fractionation, and DEAE-cellulose, the first and second Butyl-Toyopearl column chromatographies. The elution profile of the final second Butyl-Toyopearl column chromatography is shown in Fig. 1. By these procedures, the enzyme was purified 39.1-fold with a yield of 12.8%.

The homogeneity of the purified enzyme was examined by PAGE and HPLC using the TSKgel G-3000SW column. As shown in Fig. 2, the purified enzyme gave a single protein

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**Fig. 2. Electrophoretic Profile of Purified D-Xylose Isomerase in Polyacrylamide Gel.**

Lanes: (A), the purified D-xylose isomerase (about 10 μg) was stained with Coomassie Brilliant Blue R-250; (B), the purified D-xylose isomerase (about 10 μg) was stained with active staining with triphenyltetrazolium chloride.

**Fig. 3. Estimation of Molecular Weight of D-Xylose Isomerase by TSKgel G-3000SW (A) and SDS-Polyacrylamide Gel Electrophoresis (B).**

(A), (1): enolase (67,000); 2, glutathione reductase (118,000); 3, lactate dehydrogenase (142,000); 4, alcohol dehydrogenase (148,000); 5, pyruvate kinase (237,000); 6, glutamate dehydrogenase (290,000); XI, D-xylose isomerase.

(B): 1, cytochrome c dimer (24,800); 2, trimer (37,200); 3, tetramer (49,600); 4, hexamer (74,400); XI, D-xylose isomerase.

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**Table I. Purification of D-Xylose Isomerase**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (unit/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>100</td>
<td>2510</td>
<td>203</td>
<td>0.08</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitate (35 65%)</td>
<td>34</td>
<td>2116</td>
<td>187</td>
<td>0.09</td>
<td>1.1</td>
<td>92.1</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>5</td>
<td>212</td>
<td>157</td>
<td>0.74</td>
<td>9.1</td>
<td>77.3</td>
</tr>
<tr>
<td>1st Butyl-Toyopearl</td>
<td>0.5</td>
<td>17.3</td>
<td>48</td>
<td>2.77</td>
<td>34.2</td>
<td>24.0</td>
</tr>
<tr>
<td>2nd Butyl-Toyopearl</td>
<td>0.2</td>
<td>8.2</td>
<td>26</td>
<td>3.17</td>
<td>39.1</td>
<td>12.8</td>
</tr>
</tbody>
</table>
Fig. 4. Comparison of the N-Terminal Amino Acid sequence of the enzymes from *B. adolescentis* (1), *L. pentosus* (2), *B. subtilis* (3), E. coli (4), and *C. thermosulfurogenes* (5).

The conserved amino acids in all five sequences are marked with dots.

![Image of a gel with bands](image)

Fig. 5. Effects of pH on d-Xylose Isomerase Activity (A) and Stability (B).

The following buffers were used: pH 4.0-5.0, 100 mM sodium acetate buffer (●); pH 5.0-8.0, 100 mM potassium phosphate buffer (◼); pH 8.0-11.0, 100 mM glycine-NaCl-NaOH buffer (□).

(A) The enzyme activity was measured in the standard reaction mixture at indicated pH for 10 min at 37°C.

(B) The purified enzyme was incubated at 8°C for 18 h in each buffer. After the enzyme solution was adjusted to pH 7.2, the remaining activity was measured.

Estimation of molecular weight and isoelectric point of the enzyme

As shown in Fig. 3, the molecular weight of d-xylose isomerase was estimated to be about 168,000 by gel filtration on TSKgel G-3000SW. The enzyme migrated as a single band in SDS-PAGE, at a speed indicating that the molecular weight of the sub-units was about 53,000. Further, no carbohydrate was detected in the enzyme by periodic acid Schiff staining. These results indicated that the enzyme had three subunits. Most of the microbial d-xylose isomerases with molecular weights of 140,000-200,000 are composed of four identical subunits, but d-xylose isomerase of alkalophilic *Bacillus* has been reported to have three subunits. The isoelectric point of the enzyme was about 4.3 by isoelectric focusing, which is close to those of the enzyme from *Thermus aquaticus* and an alkalophilic *Bacillus*.

*N*-Terminal amino acid sequence

The N-terminal amino acid sequence of d-xylose isomerase from *B. adolescentis* is aligned in comparison with four other sequences (Fig. 4). N-Terminal amino acid sequence of the enzyme from *B. adolescentis* shows a low homology to those of the enzyme from *Lactobacillus pentosus* (42% amino acid homology), *Bacillus subtilis* (34%), *E. coli* (33%), and *Clostridium thermosulfurogenes* (25%). The homology of *B. adolescentis* d-xylose isomerase was slightly higher with *L. pentosus* than with other d-xylose isomerases.

Effects of pH and temperature

The optimum pH of the purified enzyme was around 7. As shown in Fig. 5, more than 70% of the maximal activity was observed between pH 6 and 9. The enzyme activity was stable between pH 7 and 8, but unstable under pH 4 and over pH 10.

Figure 6 shows that the optimum temperature for the activity was 60°C. The enzyme activity was kept almost fully under 50°C, and completely inactivated at 80°C. These properties of the purified enzyme were similar to those of the isomerase from *Lactobacillus xylosus*.

Substrate specificity

As shown in Table II, the purified isomerase had the
Table III. The $K_m$ of α-Xylose Isomerase

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Temperature (°C)</th>
<th>$K_m$ (mM)</th>
<th>α-Xylose</th>
<th>D-Glucose</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bifidobacterium adolescentis</td>
<td>37</td>
<td>4*</td>
<td>398*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillus brevis</td>
<td>35</td>
<td>5</td>
<td>920*</td>
<td>(21)</td>
<td></td>
</tr>
<tr>
<td>Clostridium thermosulfurogenes</td>
<td>65</td>
<td>20</td>
<td>140</td>
<td>(19)</td>
<td></td>
</tr>
<tr>
<td>Streptomyces griseofuscus</td>
<td>60</td>
<td>54</td>
<td>220</td>
<td>(22)</td>
<td></td>
</tr>
<tr>
<td>Bacillus coagulans</td>
<td>40</td>
<td>1</td>
<td>90*</td>
<td>(23)</td>
<td></td>
</tr>
<tr>
<td>Bacillus steearothermophilus</td>
<td>60</td>
<td>100</td>
<td>220</td>
<td>(24)</td>
<td></td>
</tr>
</tbody>
</table>

a The reaction was done with 5.6 μg of the purified enzyme at 37°C for 10 min.
b The reaction was done with 168 μg of the purified enzyme at 45°C for 30 min.
c The reaction was done at 50°C for 30 min.

Table IV. Effects of Metal ions on α-Xylose Isomerase Activity

The enzyme was treated with 20 mM phosphate buffer (pH 7.2) containing 20 mM EDTA for 12 h at 4°C, then dialyzed against the same buffer without EDTA for 12 h at 4°C. The metal requirement for the enzyme activity was measured in the standard reaction mixture containing metal ion for 10 min at 37°C.

<table>
<thead>
<tr>
<th>Metal ion (mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>0.01</td>
</tr>
<tr>
<td>0.1</td>
<td>82</td>
</tr>
<tr>
<td>1.0</td>
<td>100*</td>
</tr>
<tr>
<td>10.0</td>
<td>78</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>0.01</td>
</tr>
<tr>
<td>0.1</td>
<td>25</td>
</tr>
<tr>
<td>1.0</td>
<td>67</td>
</tr>
<tr>
<td>10.0</td>
<td>75</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>0.01</td>
</tr>
<tr>
<td>0.1</td>
<td>18</td>
</tr>
<tr>
<td>1.0</td>
<td>40</td>
</tr>
<tr>
<td>10.0</td>
<td>22</td>
</tr>
</tbody>
</table>

a The activity in the presence of 1.0 mM Mn$^{2+}$ was taken as 100%.

highest activity for α-xylose, but showed no affinity for α-glucose, α-arabinose, α-ribose, α-lyxose, α-galactose, or α-mannose under the standard assay conditions. However, the enzyme showed a little activity for α-glucose under unusual conditions such as a high concentration of α-glucose and high reaction temperature. Table III shows the $K_m$ of α-xylose isomerase from B. adolescentis and other microorganisms. The $K_m$ of B. adolescentis for α-xylose and α-glucose were 4 mM and 398 mM, respectively. The value of B. adolescentis for α-xylose was very close to that of Lactobacillus brevis. The $K_m$ of the enzyme from Bifidobacterium for α-xylose was different from those of other thermophilic enzymes.

Effects of metal ions

Almost all of α-xylose isomerasers require bivalent cations for the activity. In general, the enzyme from Streptomyces required Mg$^{2+}$, and that from Lactobacilli required Mn$^{2+}$. The effects of metal ions on α-xylose isomerase activity of B. adolescentis are shown in Table IV. After extensive dialysis against 20 mM potassium phosphate buffer (pH 7.2) containing EDTA, the α-xylose isomerase lost the activity and the enzyme activity was restored by the addition of bivalent cations such as Mn$^{2+}$, Co$^{2+}$, or Mg$^{2+}$. Mn$^{2+}$ was clearly preferred. The metal requirement of the enzyme was similar to that from Lactobacillus.

We purified a α-xylose isomerase having high activity for α-xylose from B. adolescentis, and studied its enzymatic and physicochemical properties. We conclude that the enzyme from B. adolescentis is different from other α-xylose isomerases so far reported, especially in its N-terminal amino acid sequence. Further experiments are under way to clarify the role of α-xylose isomerase for xylooligosaccharide metabolism of bifidobacteria.

References

11) B. S. Leach, J. F. Collawn, and W. W. Fish, Biochemistry, 19, 5734–5741 (1980).