Purification and Some Properties of an α -D-Xylosidase from *Bacillus* sp. No. 693-1

Ning ZONG and Tsuneo YASUI

*Institute of Applied Biochemistry, University of Tsukuba,
Tsukuba 305, Japan*

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An α -D-xylosidase from *Bacillus* sp. No. 693-1 was purified by precipitation with ammonium sulfate and successive chromatography on DEAE-Toyopearl 650 and hydroxyapatite. The purified enzyme, with its isoelectric point at pH 4.25, had an apparent molecular weight of 82,000 in SDS-polyacrylamide gel electrophoresis, and 400,000 in gel filtration chromatography on Toyopearl HW-60. The optimum pH for enzyme action was 7.5 and the optimum temperature was 45°C. The enzyme was stable from pH 6.0~8.5 and up to 45°C. The activity was inhibited by monoiodoacetic acid, *p*-CNB, and metal ions such as Zn^{2+} , Cu^{2+} , and Hg^{2+} . The α -D-xylosidase was highly specific for α -xylosidic linkages, and hydrolyzed isoprimeverose [α -D-xylopyranosyl-(1 $\rightarrow$ 6)-D-glucopyranose] to produce xylose and glucose, and hydrolyzed tamarind seed polysaccharide (soluble fraction) to release xylose as well.

In nature, there are various forms of polysaccharides in which xylose residues are connected with β -D-xylosidic linkages; xylan is typical. For this reason, β -D-xylanase [EC 3.2.1.8] and β -D-xylosidase [EC 3.2.1.37] which are able to hydrolyze β -D-xylosidic linkages, have been extensively studied.^{1~3)} In contrast, there are very few reported works on the occurrence of α -D-xylosidic linkages.^{4~7)} However, in recent studies on the plant cell wall, the wide distribution of α -xylosidic linkages has been confirmed in the cell wall polysaccharides called xyloglucan.^{8~19)} Moreover, it has been found that enzymatically modified xyloglucan derived from the cell wall is an important regulator in plant cell wall elongation.^{20,21)} Hence, it is unquestionable that the study of α -xylosidase is very interesting and important. In our previous paper,¹⁵⁾ we reported the isolation of α -D-xylosidase-producing microorganisms by a novel method, their taxonomic characterization, and the cultural conditions for enzyme production of *Bacillus* sp. No. 693-1. In this paper, the

purification and some characteristics of α -D-xylosidase from *Bacillus* sp. No. 693-1 are described.

Materials and Methods

Microorganism and preparation of crude enzyme. *Bacillus* sp. No. 693-1 was used for the production of enzyme. The bacterium was grown in a medium described in a previous paper²⁵⁾ in Erlenmeyer flasks at 30°C for 7 days on a shaker. Culture broth was centrifuged at 5000 $\times$ *g* for 15 min to remove cells. The supernatant was used as an enzyme source.

Enzyme assay. α -D-Xylosidase activity was measured by the amount of *p*-nitrophenol liberated from *p*-nitrophenyl α -D-xylopyranoside (α -*p*-NPX) when incubated at 45°C for 10 min (pH 7.5) as described in our previous paper.²⁵⁾ α -D-Xylosidase activity is expressed as the amount of enzyme which releases 1 μ mol of *p*-nitrophenol per min under the above experimental conditions. The activities of other α - and β -glycosidases were measured and expressed as mentioned in our previous paper.²⁵⁾

Protein assay. Protein was measured by the method of Lowry *et al.*²⁶⁾ as modified by Hartree.²⁷⁾ Bovine serum albumin was used as a standard. In monitoring effluents of

column chromatography, protein was measured by the absorbance at 280 nm.

General methods. Paper chromatography (PPC) was done on filter paper No. 51C (Toyoroshi Co., Ltd.) and also on the No. 527 (Toyoroshi Co., Ltd.) for the preparative PPC, with the solvent system of *n*-butanol–pyridine–water (6:4:3, v/v) by the ascending method. Sugars were detected with *p*-anisidine hydrochloride. Thin-layer chromatography (TLC) was done on a Merck TLC plate of Silica Gel 60 with a solvent system of 1-propanol–water (85:15, v/v). The sugars were detected by heating at 140°C for 3 min after spraying with 20% sulfuric acid in methanol. Enzyme solutions were concentrated by an ultrafiltration apparatus (Toyoroshi Co., Ltd.) using UK-10 membranes (Toyoroshi Co., Ltd.).

Preparation of isoprimeverose. Isoprimeverose [α -D-xylopyranosyl-(1 $\rightarrow$ 6)-D-glucopyranose] was prepared by a modification of the method of Kato *et al.*²³⁾ as follows: tamarind seed polysaccharide (10 g/1.0 l of water, pH 6.0), Kokulase (3.0 g, enzyme preparation from *Aspergillus oryzae*), Meicelase p-1 (2.0 g, from *Trichoderma viride*), and NaN₃ (0.2 g) were mixed and incubated at 40°C for 26 hr. After inactivation of the enzyme by heating, the reaction solution was concentrated to a small volume and then centrifuged to remove insoluble materials. The clear supernatant was fractionated by charcoal chromatography (on a column: 6 $\times$ 45 cm) and sugar fraction was further purified by preparative PPC. The prepared material was identified by methylation analysis.

Preparation of soluble fraction of tamarind seed polysaccharide. Five gram of dry tamarind seed powder was dissolved in 100 ml of distilled water and stirred for 4 hr at room temperature, and then centrifuged (10,000 $\times g$, 1.5 hr) to remove insoluble materials. The supernatant, the soluble fraction, was dialysed against distilled water using a Cellotube (1,000 cut) and concentrated to 100 ml. The concentration of prepared solution was measured by the phenol-sulfuric acid method²⁸⁾ with glucose as a standard.

Chemicals. The Kokulase was purchased from Sankyo Co., Ltd. Meicelase P-1 was purchased from Meiji Seika Kaisha, Ltd. Glucose C-Test was purchased from Wako Pure Chemical Industries, Ltd. Tamarind seed polysaccharide was purchased from Tokyo Kasei Industries, larchwood xylan from Sigma Co., Ltd., and all of the various *p*-nitrophenyl glycosides from Nakarai Chemicals Ltd. The xylooligosaccharides²⁹⁾ (xylotriase to xylopentaose) and cellooligosaccharides³⁰⁾ (cellotriase and cellotetraose) were prepared in our laboratory. Standard monosaccharides and disaccharides were obtained from commercial sources. The trimethylsilylimidazole-C (TMSI-C) was from Gasukuro Kogyo Inc.

Purification of α -D-xylosidase. All steps of purification

were done at 4°C or 0°C.

Step 1. Ammonium sulfate precipitation. The supernatant of the cultural fluid was saturated to 65% with ammonium sulfate, and its pH was kept at 6.0 overnight; the proteinaceous precipitates were sedimented by centrifugation (10,000 $\times g$, 30 min). The precipitate was dissolved in a small amount of 0.02 M phosphate buffer (KH₂PO₄–Na₂HPO₄, pH 7.0) and then desalted by dialysis against the same buffer using a Cellotube. The dialysed solution was centrifuged again to remove insoluble materials.

Step 2. DEAE-Toyopearl 650 M ion-exchange chromatography (I). The dialysed solution from step 1 was put on a column (2.6 $\times$ 45 cm, bed volume 220 ml) of DEAE-Toyopearl 650 M (Toyosoda Mfg. Co., Ltd.) ion exchanger pre-equilibrated with 0.02 M phosphate buffer. The column was washed with the same buffer containing 0.15 M sodium chloride until the O.D. value of the effluent washing buffer was reduced to below 0.1, and then a sodium chloride linear gradient was established by mixing 1000 ml each of 0.15 M and 0.5 M NaCl in the buffer. At an flow rate of 25 ml/hr, 8- ml fractions were collected, and the protein content, and α - and β -D-xylosidase as well as α - and β -D-glucosidase activities were measured. Fractions containing α -D-xylosidase activity were pooled and concentrated by ultrafiltration using a UK-10 membrane.

Step 3. DEAE-Toyopearl 650 M ion-exchange chromatography (II). The concentrate from step 2 was put on another column (1.6 $\times$ 45 cm, bed volume 110 ml) of DEAE-Toyopearl 650 M and chromatographed in the same way as mentioned in step 2 except for an elution rate, of 20 ml/hr and 5.0-ml fractions.

Step 4. Hydroxyapatite chromatography. A column (2.2 $\times$ 45 cm, bed volume 150 ml) packed with Hydroxyapatite (Seikagaku Kogyo Inc.) was equilibrated with 0.007 M KH₂PO₄–K₂HPO₄ buffer (pH 6.8). The active fraction obtained from step 3 was put onto the hydroxyapatite column and washed with three times the column volume of starting buffer. Then, a 0.007~0.4 M linear gradient of the same buffer was applied to elute the adsorbed proteins. Fractions of 3 ml were collected at a rate of 15 ml/hr and analyzed for proteins and enzyme activities. Fractions showing only α -D-xylosidase activity were pooled and desalted by ultrafiltration as described previously.

Electrophoresis. Polyacrylamide gel electrophoresis (Disc-PAGE) was done by the methods of Ornstein *et al.*³¹⁾ and Davis *et al.*³²⁾ The enzyme preparation was put on a 5.0% polyacrylamide gel at pH 8.3. After electrophoresis at 4 mA/tube at 4°C, the protein band was stained with 0.25% of Coomassie Brilliant Blue R-250 solution (methanol–acetic acid–water=5:1:5, v/v) and destained by diffusion (methanol–acetic acid–water=30:10:60, v/v). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was done on a 7.5% polyacrylamide gel by the method of Weber *et al.*³³⁾

The enzyme preparation was treated at 100°C for 3 min in 1% of SDS, 2% 2-mercapto-ethanol, 10 mM sodium phosphate buffer (pH 7.2), and 20% of glycerol in a total volume of 0.2 ml. A sample of the mixture was then placed on each gel. After electrophoresis at 6 mA/tube for 4 hr at 20°C, the bands of proteins were detected as described above. Ovalbumin (45 K), bovine serum albumin (66 K), phosphorylase B (92.5 K), β -galactosidase (116 K), and myosin (200 K) (all from Bio-Rad Co., Ltd.) were used as molecular weight standards.

Isoelectric focusing. To investigate the homogeneity and isoelectric point of the α -D-xylosidase, analytical isoelectric focusing was done in a column (110 ml, Ampholine 8101, LKB, Sweden). The pH and density gradient were formed by mixing sucrose (0~40%) and ampholite solution (pH 3~10 or 2.5~5.0). The voltage of 800 V run for 48 hr at 4°C. Two-ml fractions were collected, and their pHs were measured. Enzyme activity as well as absorption at 280 nm were measured after dialysis.

Gel filtration chromatography. A*column (1.9 $\times$ 100 cm, bed volume 270 ml) packed with Toyopearl HW-60 gel was used to measure the molecular weight of α -D-xylosidase. The column was equilibrated with 0.02 M KH_2PO_4 - Na_2HPO_4 buffer containing 0.3 M sodium chloride (pH 7.0). At an elution rate of 8 ml/hr, 1.5-ml fractions were collected, and assayed for protein and α -D-xylosidase activity. The column was calibrated with the following proteins of known molecular weights: α -chymotrypsinogen A (25 K), egg albumin (45 K), bovine serum albumin (68 K), aldolase (158 K), katalase (240 K), ferritin (450 K), and thyroglobulin (669 K), all of which were purchased from Boehringer Mannheim Co., Ltd.

pH optimum and stability. To find the optimum pH, the α -D-xylosidase activity was measured under the standard assay conditions by using 0.1 M KH_2PO_4 - Na_2HPO_4 buffer (pH 5.0~8.0) as well as glycine-NaOH buffer (pH 8.0~9.0), instead of McIlvaine buffer (pH 7.5). To measure its pH stability, 0.01 M of NaOAc-HCl buffer, KH_2PO_4 - Na_2HPO_4 buffer, or glycine-NaOH buffer in the pH ranges of 2.5~5.0, 5.0~8.0, and 8.0~11.0, respectively, were used. After the enzyme (0.009 units) was treated with each buffer for 15 min at 45°C, remaining α -D-xylosidase activity was assayed under the standard assay conditions.

Temperature optimum and stability. The α -D-xylosidase activity was measured under standard conditions from 30~60°C to find the temperature optimum. After the enzyme was incubated at pH 7.5 at various temperatures for 15 min, and immediately cooled, its residual activity was measured under the standard assay conditions.

Effects of metal ions and inhibitors. The purified α -D-xylosidase (0.009 units) was incubated for 15 min at 45°C

in the presence of 2 mM of various metal ions and inhibitors except for *p*-chloromercuribenzoic acid (*p*-CMB) with 0.2 mM concentration. The residual activity was measured under the standard assay conditions and the relative activity was expressed as a percentage of the enzyme activity in the absence of metal ions or inhibitors.

Apparent K_m and V_{max} values. The Michaelis constants and the apparent V_{max} of purified enzyme for both α -*p*-NPX and isoprimeverose were calculated by the Lineweaver-Burk reciprocal-plots. When α -*p*-NPX was used as a substrate, the activity of α -D-xylosidase was measured under the standard assay conditions with various final concentrations of α -*p*-NPX from 0.2 mM to 10 mM, but in the case of isoprimeverose, the substrate concentration was varied from 10 mM to 100 mM, and the enzyme activity was measured by the glucose oxidase method to find the amount of glucose released from isoprimeverose.

Substrate specificity. The reaction mixture, containing 0.5 ml of 5 mM glycoside, 0.5 ml of McIlvaine buffer (pH 7.5), and 0.5 ml of purified enzyme solution (0.045 units) was incubated and the activities were measured under the standard assay conditions. In the case of poly or oligosaccharide, 2% larchwood xylan, 1.0% tamarind seed polysaccharide (soluble), or 0.7% or 5.0% of isoprimeverose, were incubated with the enzyme (0.045~1.0 units) and 2 drops of toluene under the standard conditions. At intervals, samples of the reaction mixture were taken out, inactivated by heating, concentrated, and then chromatographed to PPC or TLC.

Anomer analysis. The identification of anomeric forms of xylose initially released from α -*p*-NPX by the α -D-xylosidase was done by gas-liquid chromatography (GLC) by the trimethylsilylimidazole-C (TMSI-C) method. The enzyme reaction was done as follows: two hundred μ l of α -*p*-NPX (20 mM, in 0.02 M McIlvaine buffer, pH 7.5) and 200 μ l of enzyme solution (0.08 units) were mixed. After being incubated at 45°C for 2 min, a sample of the reaction mixture was promptly taken out, cooled in a cold ethanol bath (-45°C), and freeze-dried. Then TMSI-C (200 μ l) was added to the dry sample. The mixture was incubated at 60°C for 15 min to complete silylation. A control experiment (0 min) was done by directly adding TMSI-C to a crystal D-xylose sample for silylation. The ratio of α - and β -D-xylose in water solution at equilibrium was also measured. Twenty mM xylose solution (in 0.02 M McIlvaine buffer, pH 7.5) was incubated at 45°C for 2.5 hr. After freeze-drying, silylation was done as described above. For measuring the silylants, a Shimadzu Gas Chromatography Model GC-7A as well as a column packed with 5% OV-17 (3 mm $\times$ 2 m) were used in analysis: 8 min isothermal hold at 165°C followed by 1°C/min increase to 200°C. Peak areas were measured with a Chromatopac C-E1B (Shimadzu).

Results and Discussion

Purification of α -D-xylosidase

The purification of α -D-xylosidase are summarized in Table I.

First, a supernatant of cultural fluid was precipitated with $(\text{NH}_4)_2\text{SO}_4$ (65% saturation). After dialysis and concentration, the recovery of α -D-xylosidase activity in this step was 86% and a sixfold purification was achieved. The α -D-xylosidase fraction from step 1 was then chromatographed on an ion exchange column of Toyopearl 650 M. α -D-Xylosidase as well as other contaminating enzymes such as β -D-xylosidase and β -D-glucosidase were adsorbed to the ion exchanger. As shown in Fig. 1, α -D-xylosidase activity peak appeared at the 0.23 M NaCl eluate with a shoulder peak, but β -D-xylosidase and β -D-glucosidase activities were observed in the eluate of about 0.17 and 0.20 M NaCl. Thus, these enzymes were effectively separated and the α -D-xylosidase fraction was also separated from pigments at this step.

To separate these enzymes completely, they

were chromatographed on Toyopearl 650 M again, and a similar elution pattern was obtained. Though the fractions containing α -D-xylosidase activity did not show any β -D-xylosidase or β -D-glucosidase activities, the α -D-xylosidase fraction was heterogeneous on disc-PAGE.

The final purification step involved hydroxyapatite chromatography (Fig. 2). At this step, in spite of the fact that the specific activity of α -D-xylosidase did not increase contrary to expectation, the α -D-xylosidase preparation gave a single and sharp protein band on disc-PAGE (Fig. 3, A), indicating homogeneity.

Our methods for isolation and purification have allowed us to obtain a homogeneous α -xylosidase from *Bacillus* sp. No. 693-1. The purified α -D-xylosidase had an overall activity yield of 26% and a 23-fold increase in specific activity (Table I).

Homogeneity and molecular weight of purified α -D-xylosidase

Upon disc-PAGE (5% gel), the purified α -D-

Table I. SUMMARY OF PURIFICATION OF α -D-XYLOSIDASE FROM *Bacillus* sp. No. 693-1

Purification step	Total protein (mg)	Total α -xylo-sidase activity (U)	Specific α -xylo-sidase activity (U/mg)	α -Xylo-sidase activity yield (%)	β -Xylo-sidase activity	β -Gluco-sidase activity	Homo-geneity
Culture supernatant	24,000	232.5	0.01	100	++	++	no
1. $(\text{NH}_4)_2\text{SO}_4$ precipitation (65%-satd.)	3,056	200.5	0.065	86	++	++	no
2. DEAE-Toyopearl 650 M column chromatography (I)	688	175.5	0.25	76	+	+	no
3. DEAE-Toyopearl 650 M column chromatography (II)	374	105.5	0.28	45	—	—	no
4. Hydroxyapatite column chromatography	265	61.2	0.23	26.3	—	—	yes

Fig. 1. The First Ion Exchange Chromatogram on a DEAE-Toyopearl 650 M Column of α -D-Xylosidase from *Bacillus* sp. No. 693-1.

The α -D-xylosidase fraction from the $(\text{NH}_4)_2\text{SO}_4$ precipitation was put on a column of DEAE-Toyopearl 650 M (2.6×45 cm) equilibrated with 0.02 M KH_2PO_4 - Na_2HPO_4 buffer (pH 7.0), and then eluted by a sodium chloride linear gradient (0.15 ~ 0.5 M). The size of each fraction was 8 ml and the flow rate of eluent was 25 ml per hour.

Symbols: ●—●, enzyme activity; ----, protein; ---, molar concentration of NaCl.

Fig. 2. Chromatogram on a Hydroxyapatite Column of α -D-Xylosidase from *Bacillus* sp. No. 693-1.

The α -D-xylosidase fractions from the second DEAE-Toyopearl 650 M chromatography were adsorbed on a hydroxyapatite column (2.2×45 cm) equilibrated with 0.007 M KH_2PO_4 - K_2HPO_4 buffer (pH 6.8), and then eluted by a 0.007 ~ 0.4 M linear gradient of the same buffer. Fractions of 3 ml were collected at a flow rate of 15 ml per hour. The symbols are the same as in Fig. 1.

xylosidase gave a single, sharp protein band (Fig. 3, A), and SDS-PAGE (7.5% gel) also proved the homogeneity of the enzyme (Fig. 3, B).

The molecular weight of the purified enzyme was 40×10^4 by gel filtration. The molecular

weight of the subunit was also about 8.2×10^4 by SDS-PAGE. It was supposed that the enzyme was of an oligomeric structure. On the other hand, the enzyme may be a component bound to the cell wall or cell membrane and released into the culture broth with the au-

Fig. 3. Polyacrylamide Gel Electrophoresis of the Purified α -D-Xylosidase from *Bacillus* sp. No. 693-1.

Gel 3A: Electrophoresis of the native enzyme, on a 5% gel column, run at pH 8.3 and 4 mA per column.

Gel 3B: Electrophoresis in the presence of SDS. The purified enzyme treated with SDS at 100°C for 3 min was applied on a 7.5% gel containing 0.1% SDS and run at 6 mA per column.

Arrows indicate the top of resolving gel.

Fig. 4. Isoelectric Focusing of the Purified α -D-Xylosidase from *Bacillus* sp. No. 693-1.

Isoelectric focusing was done in a 110-ml column (LKB) at 4°C. Carrier Ampholine (pH 2.5~5.0) was used in a sucrose density gradient. A voltage of 800 V was applied for 48 hr. The volume of each fraction collected was 2 ml. Symbols: ●—●, enzyme activity; ———, absorbance at 280 nm; -----, pH.

tolysis of the cells, because the α -D-xylosidase activity in the culture broth increased with decrease of cell weight.²⁵⁾ Similarly, α -D-xylosidases I and II, from *A. niger*, had high molecular weights as well, 49×10^4 for the α -xylosidase I and 80×10^4 for the α -xylosidase II, which it was suggested were released with the autolysis of mycelia.^{22,23)}

Isoelectric point

First, isoelectric focusing was done using carrier Ampholine (pH 3~10) and second, it was repeated by using Ampholine with a narrow pH range (2.5~5.0). Both tests gave the same isoelectric point (pH 4.25) as shown in Fig. 4. The homogeneity of the enzyme was also confirmed by isoelectric focusing giving a single, sharp peak.

Effects of pH and temperature on activity and stability

The pH optimum and pH stability of α -D-xylosidase activity were investigated by measuring the enzyme activity toward α -p-NPX from pH 5.0~9.0 or pH 2.5~11.0. The maxi-

mal activity of the enzyme was observed at pH 7.5. α -D-Xylosidase preserved its activity at pH 5.0~9.0, at 45°C for 15 min.

The temperature dependence of α -D-xylosidase activity was also studied by measuring the enzyme activity toward α -p-NPX at 30~60°C. The enzyme hydrolyzed α -p-NPX with the highest rate at 45°C and retained almost all activity at temperatures up to 45°C, 50% activity at 50°C, and lost its activity at 55°C completely.

In comparison with the purified α -D-xylosidase I and II from *A. niger* (AMS 4111),^{22~24)} the bacterial α -D-xylosidase had a higher alkaline pH optimum, pH 7.5, vs pH 2.5~3.0. The temperature optima of bacterial and fungal enzymes were both at 45°C, but there was a difference between their thermostabilities. Bacterial enzyme had a much lower thermostability (up to 45°C) than that of fungal enzyme (up to 60°C).

Effects of metal ions and chemicals

Table II shows the effects of various metal ions and chemicals on the enzyme activity.

Table II. EFFECTS OF VARIOUS CHEMICALS ON THE ACTIVITY OF α -D-XYLOSIDASE FROM *Bacillus* sp. No. 693-1

The reaction mixture (400 μ l), containing 2 mM of reagents, 0.009 units enzyme, 0.01 M KH_2PO_4 – Na_2HPO_4 buffer (pH 7.5) was incubated at 45°C for 15 min, and then 0.5 ml of 5 mM *p*-nitrophenyl α -D-xyloside and 600 μ l of McIlvaine buffer (pH 7.5) were added. The residual activities were measured as described in the text and were expressed in terms of percentage of the control (no reagent).

Chemicals	Concn. ($\times 10^{-3}$ M)	Relative activity (%)	
		No. 693-1	<i>Asp. niger</i> (I) ⁽²⁴⁾
None	2.0	100	100
CaCl_2	2.0	104	111
MgCl_2	2.0	106	108
MnCl_2	2.0	117	106
FeCl_3	2.0	108	87
AlCl_3	2.0	96	—
ZnCl_2	2.0	17	100
CuCl_2	2.0	17	80
HgCl_2	2.0	17	74
<i>p</i> -CMB	0.2	16	100
Iodoacetic acid	2.0	19	100
SDS	2.0	63	26
EDTA	2.0	102	93
Glucono- δ -lactone	2.0	100	—
2-Mercaptoethanol	2.0	92	104

None of the chemicals tested significantly enhanced the activity of the enzyme except that Mn^{2+} ions had a slight activating effect ($\sim 17\%$). The α -D-xylosidase was markedly inhibited by metal ions of 2 mM Zn^{2+} , Cu^{2+} , and Hg^{2+} (83% each inhibition), but EDTA did not inhibit. Besides, it was noticed that the enzyme activity was inhibited 84% by 0.2 mM *p*-CMB and 81% by 2 mM iodoacetic acid, suggesting the participation of sulfhydryl groups in the active site. The bacterial enzyme was inhibited by chemicals more easily than the fungal enzymes were.^{22,24)}

Kinetic properties

Evaluated from Lineweaver–Burk plots, the Michaelis constant, K_m , and relative maximum velocity, $V_{\max}$, were 1.0 mM and 0.15 $\mu\text{mol}/\text{min}/\text{mg}$ protein for α -*p*-NPX, and 55.6 mM and 2.22 $\mu\text{mol}/\text{min}/\text{mg}$ protein for isoprime-

Fig. 5. Thin-layer Chromatogram of α -D-Xylosidase Action Products on Tamarind Seed Polysaccharide (Soluble Fraction).

The reaction mixture, containing 50 mg of tamarind seed polysaccharide and 1.0 units of the purified α -D-xylosidase in 5 ml of McIlvaine buffer with 0.02% NaN_3 (pH 7.5, 10 times of dilution), was incubated at 45°C. A sample of the reaction mixture was taken out at intervals as indicated in the figure, boiled to stop the enzyme action, and chromatographed on a TLC plate of Silica Gel 60 with a solvent system of 1-propanol–water (85:15, v/v).

S₁, arabinose and galactose; S₂, xylose and glucose; Xyl, xylose; Ara, arabinose; Glc, glucose; Gal, galactose.

verose. Contrary to results of the fungal α -D-Xylosidase,^{22,24)} the K_m value for α -*p*-NPX of the enzyme was far lower than that for isoprimeverose. This fact indicates that the bacterial enzyme has a much higher affinity for α -*p*-NPX than that for isoprimeverose.

Substrate specificity

The substrate specificity of α -xylosidase was examined by testing its ability to hydrolyze the following ten glycosides: *p*-nitrophenyl α -D-xyloside, β -D-xyloside, α -D-glucoside, β -D-glucoside, α -D-galactoside, β -D-galactoside, α -D-mannoside, β -D-mannoside, α -L-fucoside, and β -L-fucoside (5 mM each). Among them, only the *p*-nitrophenyl α -D-xyloside was hydrolyzed and the other glycosides were not found to be substrates under such experimental conditions.

Some polysaccharides such as larchwood xylan, tamarind seed polysaccharide (soluble

Fig. 6. Paper Chromatogram of the α -D-Xylosidase Action Products on Isoprimeverose (0.7%).

The reaction mixture, containing 0.7% of isoprimeverose, 0.08 U of purified enzyme, McIlvaine buffer (pH 7.5, 50 times of elution) and two drops toluene in the total volume of 600 μ l, was incubated at 45°C. A sample of the reaction mixture was taken at the times indicated in the figure; the PPC solvent system was *n*-butanol-pyridine-water (6:4:3, v/v).

S₁, xylose and glucose; S₂, cellobiose and cellotriose; Xyl, xylose; Glc, glucose; IP, isoprimeverose; C₂, cellobiose; C₃, cellotriose.

Fig. 7. Paper Chromatogram of α -D-Xylosidase Action Products on Isoprimeverose (5%).

The method and symbols are the same as in Fig. 6.

fraction), and a disaccharide, isoprimeverose, were tested as substrates as well. The α -D-xylosidase was not able to hydrolyze xylan at all, but did hydrolyze tamarind seed polysaccharide (soluble fraction) to release xylose (Fig. 5). As shown in Fig. 6, the enzyme hydrolyzed the isoprimeverose to produce xylose and glucose at 0.7% concentration of substrate. However, similar to most glycosidases,

it showed transfer activity to form some oligosaccharides at a higher concentration (5%) of isoprimeverose (Fig. 7).

These results and findings indicated that we could use the purified α -D-xylosidase preparation as an efficient specific tool for elucidating the structures of saccharides containing α -xylosidic linkages. Further studies on the substrate specificity of the enzyme will be published elsewhere.

Anomeric configuration of the reaction product D-xylose

The anomeric configuration of D-xylose released from α -p-NPX by the α -D-xylosidase was identified by GLC. The ratio of α - and β -D-xylose in equilibrium was 37.7%:62.3%, which was very close to the reported value (37%:63%).³⁴⁾ But it was found that the ratio of α - and β -D-xylose in the reaction mixture after 2 min of reaction was 75%:25%, indicating that there was the retention of configuration in D-xylose formed by the α -D-xylosidase. Glycanases and glycosidases acting with retention of configuration are regarded as enzymes probably operated by a single-step mechanism.³⁵⁾ There was some information on the anomeric configuration in the released D-xylose for β -D-xylosidase.^{35~40)} However, for α -D-xylosidase, the information on the anomeric configuration of released D-xylose is reported for the first time in this paper.

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