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Purification and Characterization of the α-Glucuronidase from *Thermoanaerobacterium* sp. Strain JW/SL-YS485, an Important Enzyme for the Utilization of Substituted Xylans

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A cell-associated α-glucuronidase was purified to gel electrophoretic homogeneity from the thermophilic anaerobic bacterium *Thermoanaerobacterium* sp. strain JW/SL-YS485. This enzyme had a pI of 4.65, a molecular weight of 130,000, and two subunits; the molecular weight of each subunit was 74,000. The enzyme exhibited the highest level of activity at pH 5.4 and 60°C, as determined by a 5-min assay. The K_m and k_{cat} values of the enzyme for 4-methylglucuronosyl xylobiose were 0.76 mM and 1,083 IU/μmol, respectively. The Arrhenius energy was 26.4 kJ/mol. The specific activities of the enzyme with 4-*O*-methylglucuronosyl xylobiose, 4-*O*-methylglucuronosyl xylotriose, and 4-*O*-methylglucuronosyl xylotetraose were 8.4, 4.8, and 3.9 IU/mg, respectively. The purified α-glucuronidase and a β-xylosidase purified from the same organism interacted synergistically to hydrolyze 4-methylglucuronosyl xylotetraose.

The xylans, a group of heteropolysaccharides, are the major components of the hemicellulose fractions of terrestrial plants. These compounds account for up to 35% of the total dry weight of higher land plants (4). Xylans are abundant and are relatively easy to extract and hydrolyze. The compositions and structures of xylans vary according to their sources, but all xylans are based on a backbone chain consisting of 1,4-β-linked D-xylose residues. The backbone, however, may be branched and usually has side residues of 1,2-linked α-D-glucuronic acid or its 4-*O*-methyl ethers, 1,3-linked α-L-arabinose, and/or *O*-acetyl groups. Almost all xylans contain α-D-glucuronic acid or its methyl ethers; the only known exception is the homoxylan obtained from esparto grass (4, 19). α-Glucuronidase is the enzyme that is responsible for hydrolysis of the α-1,2-glycosidic linkage between xylose and D-glucuronic acid or its 4-*O*-methyl ether (3, 16).

α-Glucuronidase activity has been observed in culture filtrates of several fungi, including *Agaricus bisporus*, *Pleurotus ostreatus* (19), *Aspergillus niger*, *Schizophyllum commune* (8), *Dactylium dendroides* (5), and *Thermoascus aurantiacus* (9). Extracellular α-glucuronidases also occur in bacteria, including *Streptomyces* spp. (7) and *Fibrobacter succinogenes* (22). Only a few α-glucuronidases have been purified from fungi (17), and no bacterial α-glucuronidase has been purified and extensively characterized previously.

Thermoanaerobacterium sp. strain JW/SL-YS485 is a recently isolated thermophilic anaerobic bacterium that grows at pH values ranging from 3.85 to 6.35 and at temperatures ranging from 30 to 66°C (12a). Two β-xylosidases, two thermostable acetyl xylan esterases, and a cell-associated endo-xylanase have been purified from this organism and characterized (20a). In this paper we describe the purification and characterization of an α-glucuronidase from *Thermoanaerobacterium* sp. strain JW/SL-YS485.

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MATERIALS AND METHODS

Organism and growth conditions. *Thermoanaerobacterium* sp. strain JW/SL-YS485 (= DSM 8691) was recently isolated by Liu and Wiegel (12a) from a hot spring pool in the West Thumb Basin near the Vandalized Pool in Yellowstone National Park in Wyoming during a survey for anaerobic thermophiles that are able to grow with xylose and xylan as carbon sources at pH values below 4.5. This organism was identified on the basis of physiological properties, including the formation of elemental sulfur from thiosulfate, and on the basis of 16S rRNA sequence data. Strain JW/SL-YS485 clusters with *Thermoanaerobacterium saccharolyticum* and *Thermoanaerobacterium thermosulfurigenes* (19a). The medium used to cultivate the purified strain contained 0.1% yeast extract, 0.3% birch xylan, 0.1% urea, 0.018% $MgCl_2 \cdot 6H_2O$, 0.174% K_2HPO_4, 0.408% phthalic acid (monopotassium salt), 0.05% vitamin solution (6), 0.5% mineral solution (6), 0.025% $Na_2S \cdot 9H_2O$, and 0.025% cysteine $HCl \cdot H_2O$; the pH of this medium was 6.5 after it was autoclaved at 121°C for 40 to 60 min (for volumes up to 100 ml). The cells used for enzyme purification were grown in medium containing 0.3% birch xylan for 15 h in a 150-liter fermentor under anaerobic conditions at pH 6 and 60°C.

Enzyme assay. Enzyme activities were determined in international units (IU); 1 IU was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product min^{-1}. 4-*O*-Methylglucuronosyl xylobiose was used as the standard substrate to determine enzyme activity. The following compounds were used for the substrate specificity analysis: 4-*O*-methylglucuronosyl xylotriose, 4-*O*-methylglucuronosyl xylotetraose, and 4-*O*-methylglucuronosyl xylan. All substrates were prepared at the Bundesforschungsanstalt, Hamburg, Germany. The amount of the reaction product, 4-*O*-methylglucuronic acid, was determined with the copper reagent and the arsenomolybdate reagent (13, 14). The reaction was initiated by adding 5 μl (0.2 to 0.5 μg) of α-glucuronidase to 95 μl of 2 mM 4-*O*-methylglucuronosyl xylobiose in 50 mM histidine buffer (pH 5.8) at 60°C. The reaction mixture was incubated for 4 to 10 min at 60°C, and the reaction was then stopped by adding 0.3 ml of the copper reagent. The mixture was then heated in boiling water for 10 min and cooled in an ice bath. The arsenomolybdate reagent (0.2 ml) was added, and the A_{620} was determined. D-Glucuronic acid (Sigma Chemical Co., St. Louis, Mo.) was used as the standard for quantification of 4-*O*-methylglucuronic acid and to estimate the effects of different pH values, buffers, and xylose on the development of color.

Determination of reaction products. The reaction products obtained with α-glucuronidase in the presence and absence of a purified β-xylosidase were analyzed by thin-layer chromatography. The reaction mixtures and chemical markers were loaded onto a silica gel plate (20 by 20 cm; Whatman Chemical Separation, Inc., Clifton, N.J.) and partitioned, and the plate was sprayed with the reagents described by Lee and Zeikus (12). The sprayed plate was heated at 66°C for 20 min before it was carefully rinsed with water and dried at room temperature for visualization and storage.

Protein determination. To estimate the progress of purification, protein concentrations were determined by the dye-binding method of Bradford (1), using bovine serum albumin (Sigma) as the reference compound. Purified protein was quantified by determining its A_{280}, using an extinction coefficient ($\alpha_{1\%}$) of 10 (20).

Enzyme purification. Bacterial cells pelleted by centrifugation were resuspended in 50 mM phosphate buffer (pH 7.0) and disrupted with a French press at 1.25×10^5 kPa. A cell extract was obtained by centrifuging the preparation at 4°C and 100,000 × *g* for 60 min with a model L8-M ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). All purification steps were performed at room temperature in the presence of 0.02% (wt/vol) sodium azide to prevent microbial growth. Most chromatography media and prepacked columns were purchased from Pharmacia Biotech, Inc., Piscataway, N.J.; Phenyl 650M was purchased from TosoHaas, Montgomeryville, Pa.

(i) Ion-exchange chromatography on DEAE-Sepharose. A cell extract was loaded onto a DEAE-Sepharose column (2.6 by 56 cm) which had been equilibrated previously with 1 liter of 25 mM Bis-Tris propane {1,3-bis[tris(hydroxymethyl)methylamino]propane} buffer (pH 7.0). The proteins were eluted with a 0 to 1 M NaCl gradient in 1.12 liter of bis-Tris propane buffer at a flow rate of 4 ml/min; 8-ml fractions were collected.

(ii) Ion-exchange chromatography on Q-Sepharose. The active fractions from a DEAE-Sepharose column were pooled in dialysis tubing, concentrated about 20-fold by using polyethylene glycol (15), and dialyzed against 2.5 liters of 25 mM sodium citrate buffer (pH 6.4) overnight. The resulting preparation was loaded onto a Q-Sepharose column (1.6 by 30 cm) that had been equilibrated previously to pH 6.4 with 100 ml of 25 mM sodium citrate buffer. The proteins were eluted with a 360-ml pH 6.4 to pH 3 gradient formed with 25 mM sodium citrate buffer (pH 6.4) and 25 mM citric acid. A total of 60 6-ml fractions were collected at a flow rate of 1.5 ml/min.

(iii) Hydrophobic interaction chromatography. The pooled active fractions from the Q-Sepharose column were mixed with a 4 M $(NH_4)_2SO_4$ solution to give a final $(NH_4)_2SO_4$ concentration of about 1.5 M. The resulting mixture was loaded onto a Phenyl 650M column (1 by 18 cm) which had been equilibrated previously with 25 mM bis-Tris propane buffer containing 1.5 M $(NH_4)_2SO_4$. The proteins were eluted with 1.5 to 0 M $(NH_4)_2SO_4$ gradient in 240 ml of bis-Tris propane buffer at a flow rate of 1 ml/min, and 80 4-ml fractions were collected.

(iv) Ion-exchange chromatography on Mono Q. The pooled active fractions obtained from the Phenyl 650M column were dialyzed against 25 mM piperazine buffer (pH 6.3). The proteins were applied to prepacked Mono Q HR 5/5 column that had been equilibrated previously to pH 6.3 with 25 mM piperazine buffer. The proteins were eluted at a flow rate of 1 ml/min by using a 0 to 0.4 M NaCl gradient in the same buffer, and 0.5-ml fractions were collected.

Determination of molecular weight and pI. The molecular weight of purified α-glucuronidase was determined by gel filtration and gel electrophoresis. Gel filtration was performed with a prepacked Superose 6 column (10 mm by 30 cm) by using bis-Tris propane buffer containing 0.2 M NaCl as the eluent. The gel filtration molecular weight markers used (molecular weight range, 12,000 to 200,000) were obtained from Sigma Chemical Co. (catalog no. MW-GF-200). A native gradient 4 to 30% polyacrylamide gel was cast by using a Mini-PROTEAN II multicasting chamber (Bio-Rad, Richmond, Calif.) according to the instructions of the manufacturer. Gel electrophoresis was performed with a Mini-PROTEAN II dual-slab cell (Bio-Rad). The gradient gel was electrophoresed at 160 V and 4°C for 16 h with a buffer containing 90 mM Tris, 80 mM boric acid, and 2.5 mM Na_2-EDTA. Sodium dodecyl sulfate (SDS)–8 to 25% polyacrylamide gradient gel electrophoresis was performed by using a PhastSystem apparatus as described in the manual supplied by the manufacturer (Pharmacia Biotech Inc.). The molecular weight markers used in both native and SDS gel electrophoresis experiments were the markers in electrophoresis calibration kits obtained from Pharmacia Biotech, Inc. Isoelectric focusing was performed with PhastGel IEF 4-6.5 gels (Pharmacia Biotech, Inc.). The pI markers used were glucose oxidase (pI 4.2), trypsin inhibitor (pI 4.6), β-lactoglobulin A (pI 5.1), and carbonic anhydrase II (pI 5.4 and 5.9), which were obtained from Sigma Chemical Co. The proteins in the polyacrylamide gels were stained with Coomassie blue R-250.

Chemicals. All chemicals were purchased from Sigma Chemical Co. unless indicated otherwise.

RESULTS

Quantification of glucuronic acid. 4-Methyl-α-glucuronic acid, the product of enzymatic hydrolysis of 4-methyl-α-glucuronosyl xylooligomers, was not available as a purified substance. Therefore, glucuronic acid was used as the standard. The reduction of copper by glucuronic acid was affected by the buffer used in the sample solution (Fig. 1). The millimolar extinction coefficients of glucuronic acid were 30.4, 22.8, 22.8, 22, 19.5, 18, 12.3, 12.3, 4.36, and 3.47 in glutamate (pH 5), phthalate (pH 6), 50 mM acetate (pH 4.8), imidazole (pH 7.8), piperazine (pH 6), histidine (pH 6), glycylglycine (pH 7.6), bis-Tris propane (pH 6), citrate (pH 6), and phosphate (pH 6) buffers, respectively. Absorbance was also dependent on the pH value of the buffer used. When glycylglycine buffer was used at pH 7.2 and 8.8, the millimolar extinction coefficients of glucuronic acid were 12.1 and 13.4, respectively. In glycylglycine buffer (pH 7.6), the millimolar extinction coefficient of xylose was about 0.02.

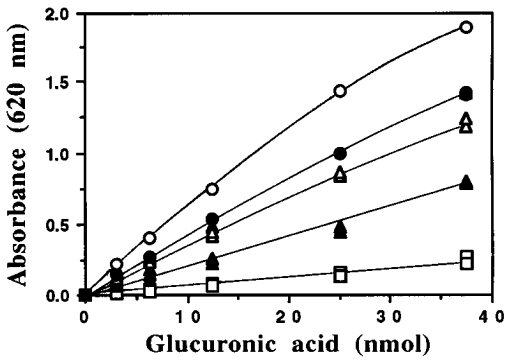

FIG. 1. Effect of buffer on color development in the quantification of glucuronic acid. A mixture containing 0.1 ml of glucuronic acid in 50 mM buffer and 0.3 ml of copper reagent was boiled for 10 min and then cooled in an ice bath. Subsequently, 0.2 ml of arsenomolybdate reagent was added to the mixture, and the A_{620} was determined. Symbols: □, citrate buffer and phosphate buffer; ▲, bis-Tris buffer and glycylglycine buffer; △, histidine buffer, imidazole buffer, and piperazine buffer; ●, acetate buffer and phthalate buffer; ○, glutamate buffer.

Induction of α-glucuronidase. When 0.6% cellobiose, 0.6% galactose, 0.6% glucose, 0.6% mannose, or 0.6% xylose was used as the sole carbon source for growth, strain JW/SL-YS 485 exhibited an α-glucuronidase activity of <0.003 μmol · min^{-1} · mg of $protein^{-1}$; however, activities of 0.013 to 0.022 μmol · min^{-1} · mg of $protein^{-1}$ were obtained when 0.4% xylan from beech wood or birch wood was used for growth, representing four- to sevenfold increases in the specific activity. The highest level of activity was observed during the exponential growth phase, and activity decreased during the late exponential phase. The majority (>85%) of the activity was cell associated.

Purification of α-glucuronidase. The method used to purify the α-glucuronidase is summarized in Table 1. The α-glucuronidase activity always eluted as a single broad peak. The α-glucuronidase eluted from the DEAE-Sepharose column together with β-xylosidases, acetyl xylan esterase, and endo-xylanase in one protein peak (fractions 58 to 74). The α-glucuronidase adsorbed to Q-Sepharose weakly; this led to the separation of α-glucuronidase from the other enzymes. The enzyme which eluted from the Mono Q column was purified to gel electrophoretic homogeneity. Only one band was observed when 2.5 μg of enzyme was applied to a native gradient gel or when 1 μg was loaded onto an SDS PhastGel gel.

Molecular weight and pI. A molecular weight of 130,000 was determined by both native gradient 4 to 30% polyacrylamide gel electrophoresis and gel filtration on Superose 6. Subunits having molecular weights of 74,000 were observed on the SDS-polyacrylamide gel. Our data suggested that the enzyme was a

TABLE 1. Purification of α-glucuronidase

Step	Total amt of protein (mg)	Total activity (IU)	Sp act (IU/mg)	Purification (fold)	% Recovery
Cell extract	3,586	393	0.11	1	100
DEAE-Sepharose	1,768	323	0.18	1.6	82
Q-Sepharose	114.4	197	1.7	15.6	50
Phenyl 650M	8.0	44.3	5.5	50.0	11
Mono Q	2.4	20.2	8.4	76.0	5

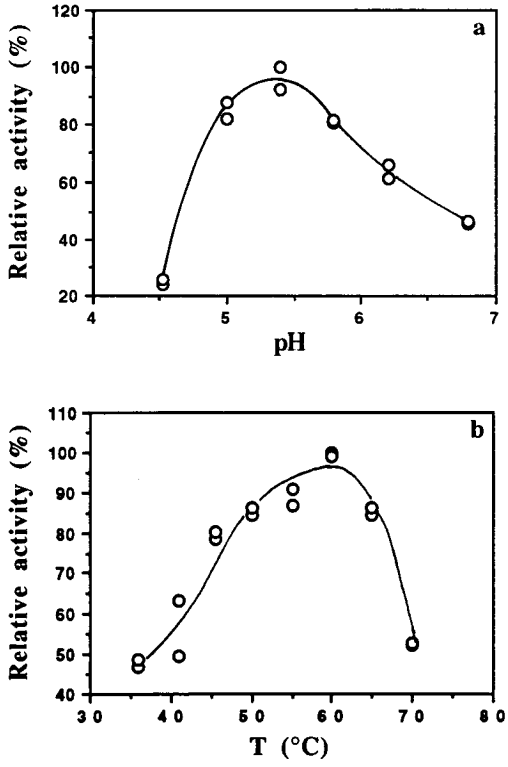

FIG. 2. Effects of pH and temperature on α-glucuronidase activity. (a) pH dependence at 60°C when a 6-min assay was used. (b) Temperature dependence at pH 5.8 when a 5-min assay was used. The highest level of activity obtained (0.04 μmol/min) was defined as 100%.

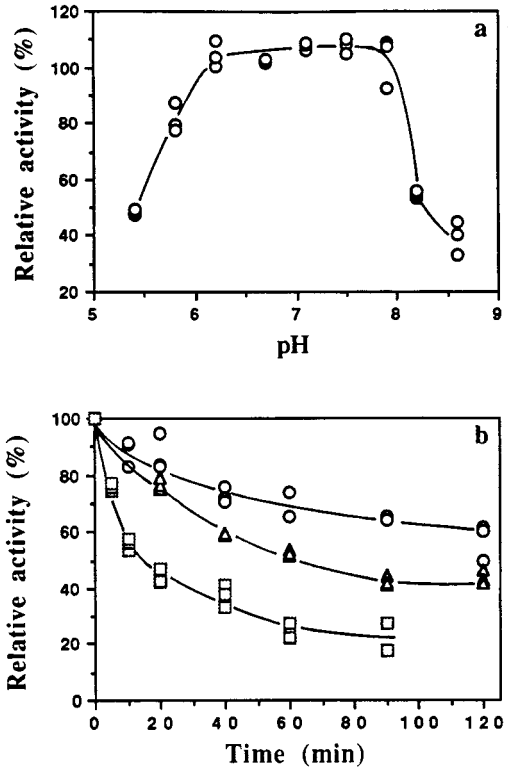

FIG. 3. Stability of α-glucuronidase at various pH values and temperatures. (a) pH stability. A 0.47-μg portion of enzyme in 50 μl of 50 mM histidine buffer (pH 5.4 to 6.2) or 50 mM glycylglycine buffer (pH 6.7 to 8.6) was preincubated for 1 h at 60°C in the absence of substrate. Activity was determined by adding 50 μl of substrate (concentration, 4 mM) in 0.1 M histidine buffer (pH 5.8) and incubating the preparation for 6 min at 60°C. Full activity (100%) was determined at each pH value. (b) Thermostability. A 0.47-μg portion of enzyme in 0.1 ml of 50 mM histidine buffer (pH 5.8) was preincubated for various times at 60°C (○), 62°C (△), and 65°C (□) in the absence of substrate. Activity was determined by adding 5 μl of substrate (concentration, 40 mM) in H_2O and incubating the preparation for 5 min. The activity of the enzyme without preincubation (0.004 μmol/min) was defined as 100%.

dimer. When the PhastSystem apparatus was used, isoelectric focusing revealed that the α-glucuronidase had a pI of 4.65.

Kinetics and enzyme stability. The highest level of α-glucuronidase activity as determined by a 5-min assay was observed at pH 5.4 and 60°C (Fig. 2). Under these conditions, the enzyme had a K_m of 0.76 mM when 4-*O*-methylglucuronosyl xylobiose was used as the substrate. The k_{cat} was 1,083 IU/μmol. The Arrhenius energy was 26.4 kJ/mol.

At 60°C, purified α-glucuronidase was stable for 1 h at pH values ranging from 6.2 to 7.9 (Fig. 3a). The purified enzyme exhibited a half-life of 1 h at 62°C in the absence of substrate when 50 mM histidine buffer (pH 5.8) was used (Fig. 3b).

Substrate specificity. The specific activities of the purified enzyme were 8.4, 4.8, 3.9, and 0.4 IU/mg when 4-*O*-methylglucuronosyl xylobiose, 4-*O*-methylglucuronosyl xylotriose, 4-*O*-methylglucuronosyl xylotetraose, and 4-*O*-methylglucuronosyl xylan, respectively, were used as the substrates. After 4-*O*-methylglucuronosyl xylotetraose was incubated with purified α-glucuronidase, the products observed on developed thin-layer chromatograms were xylotetraose and a substance which was the same color as glucuronic acid, which was a different color than the xylose and xylooligomer spots (Fig. 4, lane 5). β-Xylosidase purified from the same organism hydrolyzed only xylosidic bonds, producing 4-*O*-methylglucuronic acid-substituted xylotriose and xylobiose and unsubstituted xylose (Fig. 4, lane 3). Fastest and most extensive hydrolysis of the substrate was observed when it was incubated with both enzymes, and on the basis of the intensity of the spots, the major product was xylose (Fig. 4, lane 4).

DISCUSSION

Hydrolysis of xylans by purified endo-xylanases results in the formation of xylose and glucuronosyl xylooligomers (9, 18). Thus, α-glucuronidases are required for complete hydrolysis and utilization of xylans. α-Glucuronidase activities have been identified by various methods, including thin-layer chromatography (5), gas-liquid chromatography (5), and anion-exchange chromatography (5, 19). A colorimetric method is desirable for fast and accurate quantification of α-glucuronidase activity. Synthetic chromogenic substrates have been tried; however, microbial α-glucuronidase may not exhibit significant activities against these substrates (5). An alternative colorimetric procedure specific for uronic acids has been described by Milner and Avigad (13), and this procedure was sensitive to different buffers, pH values, and xylose residues (2, 13). Thus, we determined the millimolar extinction coefficient of glucuronic acid for the different buffers used in our investigation (Fig. 1). Acetate, phthalate, and glutamate buffers yielded the highest sensitivity values. Xylose gave only a very light color reaction compared with glucuronic acid in our experiments. Thus, the assay is useful for purification and characterization of glucuronidases.

Only a few fungal α-glucuronidases have been purified and characterized (17). Several properties of the fungal enzymes

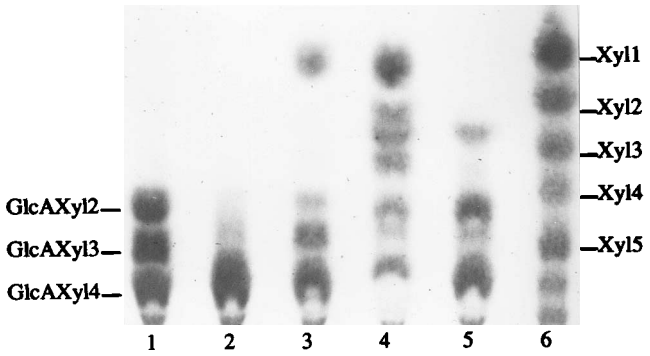

FIG. 4. Thin-layer chromatographic separation of enzymatic products from 4-*O*-methylglucuronosyl xylotetraose. Glucuronosyl xylotetraose (0.4 mg) in 50 μl of buffer (pH 5.8) was incubated for 1 h at 60°C in the presence of no enzyme (lane 2), 2.7 μg of β-xylosidase (lane 3), 1.2 μg of α-glucuronidase (lane 5), or a combination of the two enzymes (lane 4). Lanes 1 and 6 contained markers. Abbreviations: GlcA, glucuronic acid; Xyl1, xylose; Xyl2, xylobiose; Xyl3, xylotriose; Xyl4, xylotetraose; Xyl5, xylopentose.

differ from properties of the enzyme purified from the anaerobic thermophile which we studied. The pI values for the purified fungal enzymes are less than 4, while the bacterial α-glucuronidase purified in this study has a higher pI value (pI of 4.65). The molecular weights of the single polypeptides of the fungal enzymes range from 100,000 to 160,000 (17), whereas the bacterial enzyme which we examined has a molecular weight of 130,000 and consists of two 74,000-molecular-weight subunits.

α-Glucuronidases from different organisms vary in substrate specificity. Nonpurified enzyme from *F. succinogenes* was not active against 4-*O*-methylglucuronosyl xylobiose and 4-*O*-methylglucuronosyl xylan (22). The enzyme from *Agaricus bisporus* cleaves 4-*O*-methylglucuronic acid from substituted xylooligomers but not from substituted xylan. The enzymes from *Aspergillus niger*, *S. commune*, and *Thermoascus aurantiacus* exhibit activities against both 4-*O*-methylglucuronic-substituted xylooligomers and xylan, although smaller fragments are cleaved faster (2). Among the fungal α-glucuronidases, the *Thermoascus aurantiacus* enzyme has some of the highest specific activities, including up to 2.2 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ on 4-*O*-methylglucuronosyl xylobiose, 4-*O*-methylglucuronosyl xylotriose, and 4-*O*-methylglucuronosyl xylotetraose and 1.2 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ on 4-*O*-methylglucuronosyl xylan (10). Compared with this enzyme, the α-glucuronidase from *Thermoanaerobacterium* sp. strain JW/SL-YS485 is very active against 4-*O*-methylglucuronosyl xylobiose, 4-*O*-methylglucuronosyl xylotriose, and 4-*O*-methylglucuronosyl xylotetraose (specific activities, up to 8.4 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) (this study) but exhibits a low level of activity against 4-*O*-methylglucuronosyl xylan (0.4 $\mu mol \cdot min^{-1} \cdot mg^{-1}$).

The activities described above indicate that the physiological function of the *Thermoanaerobacterium* α-glucuronidase is to debranch glucuronic acid-substituted oligoxylans. This conclusion is further supported by the observed synergistic reaction between xylosidase and α-glucuronidase.

The reaction products obtained with α-glucuronidase and β-xylosidase purified from *Thermoanaerobacterium* sp. strain JW/SL-YS485 were studied by thin-layer chromatography (Fig. 4). Our results indicate that β-xylosidase and α-glucuronidase are both important for fast and complete hydrolysis of 4-*O*-methylglucuronosyl xylooligomers, the products of endoxylanase activity. β-Xylosidase cleaves xylose residues from 4-*O*-methylglucuronosyl xylotetraose and produces 4-*O*-methylglucuronosyl xylotriose or 4-*O*-methylglucuronosyl xylobiose. Since the activities of α-glucuronidase against 4-*O*-methylglucuronosyl xylobiose or 4-*O*-methylglucuronosyl xylotriose are greater than the activity against 4-*O*-methylglucuronosyl xylotetraose, the presence of the β-xylosidase enhances the release of 4-*O*-methylglucuronic acid. On the other hand, much more xylose is produced by the β-xylosidase in the presence of the α-glucuronidase, indicating that, compared with the substituted xylooligomers, unsubstituted xylooligomers (products of the α-glucuronidase) are better substrates for the β-xylosidase. Such a synergistic reaction has not been described for any of the previously reported β-xylosidases or α-glucuronidases.

The absence of an α-glucuronidase activity leads to accumulation of glucuronic acid-substituted oligomers (xylotriose and higher), as observed with *Thermoanaerobacter ethanolicus* (11, 24). Thus, transfer of this activity into organisms such as *Thermoanaerobacter ethanolicus* and *Clostridium thermocellum*, potential industrial ethanol producers, should significantly enhance hemicellulose utilization by these organisms (16, 21, 23).

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