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Biochemical Properties of a β-Xylosidase from *Clostridium cellulolyticum*

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A 43-kDa β-xylosidase from *Clostridium cellulolyticum* was purified to homogeneity. The enzyme releases xylose from *p*-nitrophenylxylose and xylodextrins with a degree of polymerization ranging between 2 and 5. The N-terminal amino acid sequence of the enzyme showed homologies with three other bacterial β-xylosidases. By proton nuclear magnetic resonance spectroscopy, the enzyme was found to act by inverting the β-anomeric configuration.

Clostridium cellulolyticum, a mesophilic bacterium able to degrade crystalline cellulose (14), has been studied with regard to both its ability to ferment various substrates (14, 15) and its native cellulolytic system (9, 24). Several genes encoding β-1-4-endoglucanases have been cloned in *Escherichia coli* and sequenced (2, 5, 8, 10, 26, 32). Three endoglucanases, A, C, and D, expressed in *E. coli* have been characterized (4, 11, 12, 33). Little research has been done, however, on the xylanolytic activity of *C. cellulolyticum* (24). To further our knowledge about this organism, we investigated its ability to metabolize xylan. Only a few studies have dealt with the hydrolysis of xylan in various species of clostridia (21–23, 25, 28–31, 34). Complete enzymatic hydrolysis of xylan requires the cooperative action of endo-β-1-4-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), and a series of enzymes that cleave side chain groups (27). A preliminary study showed that *C. cellulolyticum* is able to grow by using xylan, and a search for the enzymes involved in xylanolysis initially pointed to a β-xylosidase. In this report, we describe the purification and biochemical characterization of this enzyme.

Bacterial strain and cultivation. *C. cellulolyticum* was grown as described previously (15). When it was grown on various substrates (cellulose-MN300, cellobiose, glucose, xylose, and xylan), production of β-xylosidase was five to eight times higher with xylan than with any of the other carbon sources.

Enzyme assay. The protein concentration was determined as described by Bradford (6) with bovine serum albumin as the standard. β-Xylosidase activity was measured by determining its A_{400} development with *p*-nitrophenyl-β-D-xylanopyranoside (PNPX) in 25 mM phosphate buffer, pH 7.0, (PB7) at 37°C. The other substrates tested were *p*-nitrophenyl-α-L-arabinopyranoside, *p*-nitrophenyl-α-L-arabinofuranoside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, and *p*-nitrophenyl-β-D-lactopyranoside. One unit of enzyme activity was defined as the release of 1 μmol of *p*-nitrophenol per min from each substrate (a *p*-nitrophenol standard solution from Sigma diluted in PB7 was used for calibration). Activities on carboxymethyl-cellulose, barley glucan, lichenan, and xylan were estimated by measuring the reducing sugars released as described previously (12). One activity unit was defined as the release of 1 μmol of xylose equivalent per min. All of the compounds used for these assays were from Sigma.

Purification. Crude enzyme was prepared from a 2-day-old culture with xylan (1 g/liter) as the carbon source. A very low level of activity was detected in the culture supernatant. The cells were harvested and resuspended in 20 mM Tris/HCl buffer, pH 8.5, broken with a French press, and centrifuged at 10,000 × *g* for 20 min. From 85 to 90% of the activity was recovered from the supernatant. Streptomycin sulfate (20 g/liter) was then added to the supernatant, and the cells were centrifuged again to precipitate the nucleic acids. Two protein fractions were separated after successive precipitations with ammonium sulfate at saturation rates of 50 and 80%. The 80% fraction was dissolved in 100 ml of 20 mM Tris/HCl buffer containing 0.75 M potassium acetate (KAB). The sample was loaded onto a phenyl Sepharose column (5 by 21 cm; IBF) preequilibrated with KAB. A decreasing KAB-to-water gradient was applied. The active fraction, concentrated in an Amicon PM 30 filtration unit and dialyzed against water, was loaded onto a TSK DEAE 5 PW column (21.5 by 150 mm; LKB) and eluted with a linear gradient of 0 to 0.5 M NaCl in 20 mM Tris/HCl buffer. After the separation, an identical chromatography was performed on the dialyzed active fraction but with a slower 0 to 0.2 M NaCl gradient. The results are summarized in Table 1. At each step, active fractions were analyzed by performing sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis with a Pharmacia Phast System. The final sample gave a single band at 43 kDa (Fig. 1). The specific activity was 15.1 IU/mg, and the enzyme was purified up to 169-fold.

N-terminal sequence analysis. The N-terminal sequence of the purified enzyme was determined with an Applied Biosystems 470A sequence analyzer. The following 28-residue sequence was established: Xaa-Xaa-Glu-Asn-Glu-Pro-Leu-Val-

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TABLE 1. Purification of β-xylosidase from xylan-grown *C. cellulolyticum*

Sample source	Total activity (IU)	Total protein (mg)	Sp act (IU/mg)	Yield (%)
French pressure cell treatment	125.0	1,400.0	0.09	100.0
Streptomycin sulfate treatment	76.0	376.0	0.20	60.0
80% ammonium sulfate precipitation	63.0	71.8	0.88	50.0
Phenyl Sepharose chromatography	26.0	11.0	2.36	20.8
DEAE column chromatography with 0–0.5 M NaCl gradient	26.0	5.8	4.48	20.8
DEAE column chromatography with 0–0.25 M NaCl gradient	5.4	0.4	15.11	4.4

Thr-His-Leu-Tyr-Thr-Ala-Asp-Pro-Ser-Alu-His-Val-Phe-Glu-Gly-Lys-Leu-Tyr-Ile-Tyr-Pro-Ser.

This sequence was compared with sequence data banks with the BLAST program (1) from the National Center of Biotechnology Information. Considerable similarities were observed with three enzymes having related functions: 67.9% with xylosidase-arabinosidase from *Bacteroides ovatus* (35) (GenBank accession no. U041057), 64.3% with XynD of *Bacillus polymyxa* (16), and 50.0% with XylA of *Clostridium stercorarium* (30).

General properties of the enzyme. The effect of temperature on the reaction was assayed by equilibrating the reaction mixtures in PB7 at various temperatures in the 20 to 55°C range, and 25 mM potassium phosphate at pHs 6.0 to 9.0 was used to test the effect of pH on the enzyme reaction. The temperature and pH optima were 35°C and 7.5, respectively. The enzyme showed no activity below pH 6.0. The effect of the substrate concentration on β-xylosidase was studied by measuring the hydrolytic activity in a mixture containing 6 μg of the enzyme and PNPX at 0.05 to 0.7 mg ml^{-1} in PB7. Incubation was performed at 37°C for 20 min. Kinetic studies showed that β-xylosidase had a maximum rate of hydrolysis of 18 IU/mg of protein and a K_m of 0.40 mM with PNPX. The enzyme showed a high level of activity against *p*-nitrophenylxyloside (15.1 U/mg), very low levels with *p*-nitrophenylarabinofuranoside (0.17 U/mg) and xylan (1.7 U/mg), and no activity with the other *p*-nitrophenylglucosides, carboxymethyl-cellulose, barley glucan, and lichenan. Activities of the enzyme on xylodextrins (ranging from xylobiose [polymerization, ×2] to xylopentaose [polymerization, ×5] [purchased from Megazyme]) were analyzed by high-pressure liquid chromatography (Varian) on a resex-oligosaccharide (2 by 1 cm; Interchim) column heated at 75°C. The eluent used was water, and the flow rate was 0.2 ml min^{-1}. The sugars were detected and quantified with a refractive index detector by means of an LC Star Workstation from Varian. A 200-μl volume containing 0.1 to 2.5 g of each xylodextrin per ml (at least in triplicate) in PB7 was incubated at 37°C with 5 μg of the enzyme. Samples were heated at 75°C for 10 min to stop the enzymatic reaction, and 50 μl was loaded for high-pressure liquid chromatography analysis. Xylose and xylodextrins were used as standards. One unit of enzyme activity was defined as the release of 1 μmol of xylose per min. The extent of hydrolysis varied with the chain length of the substrate. In all of the cases studied, the main products were xylose and an oligosaccharide with a degree of polymerization one step lower than that of the substrate. The specific activities were higher than that on PNPX, and they were almost identical on xylotriose (80 U/mg), xylotetraose (66 U/mg), and xylopentaose (72 U/mg) but significantly lower on xylobiose (46 U/mg). A similar pattern has been observed in *Aspergillus niger* (20).

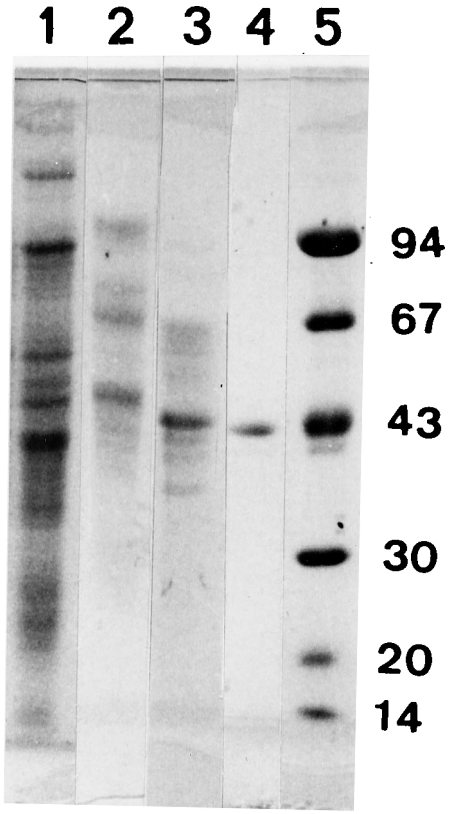

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of β-xylosidase at various purification steps. Lanes: 1, crude extract; 2, 80% ammonium sulfate precipitate; 3, result of phenyl Sepharose chromatography; 4, result of second DEAE-trisacryl chromatography; 5, Molecular mass markers. The numbers on the right are molecular masses in kilodaltons.

^{1}H nuclear magnetic resonance (NMR) analysis of the stereoselectivity of the hydrolysis. ^{1}H NMR spectroscopy has been extensively used to characterize the stereoselectivity of cellulases and xylanases (3, 7, 11). The procedure used was that described by Gebler et al. (13). The enzyme and xylotetraose (2.5 mg/ml in PB7) were prepared in D_2O. The enzyme (0.19 nmol) was added to 400 μl of deuterized substrate placed in a 5-mm-diameter NMR tube at 35°C. Proton NMR spectra were recorded every 4 min during the first 15 min and then every 15 min for 3 h on a Bruker AM 200 spectrometer. In a previous study with D-glucose and cellobiose, doublets were found to be centered at δ = 5.22 ppm (J = 3.7 Hz) and δ = 4.65 ppm (J = 8 Hz). Xylotetraose contains two doublets, one at δ = 5.17 ppm (J = 3.54 Hz) corresponding to the α anomeric proton and one at δ = 4.57 ppm (J = 7.39 Hz) corresponding to the β anomeric proton, at a ratio of 35% α to 65% β (Fig. 2). When the enzyme was added (t = 0), an increase in the α signal was observed after 4 min of reaction up to 12 min of incubation. At 16 min, the mutarotation process occurred and a new doublet centered at δ = 4.56 ppm (J = 7.93 Hz) emerged (data not

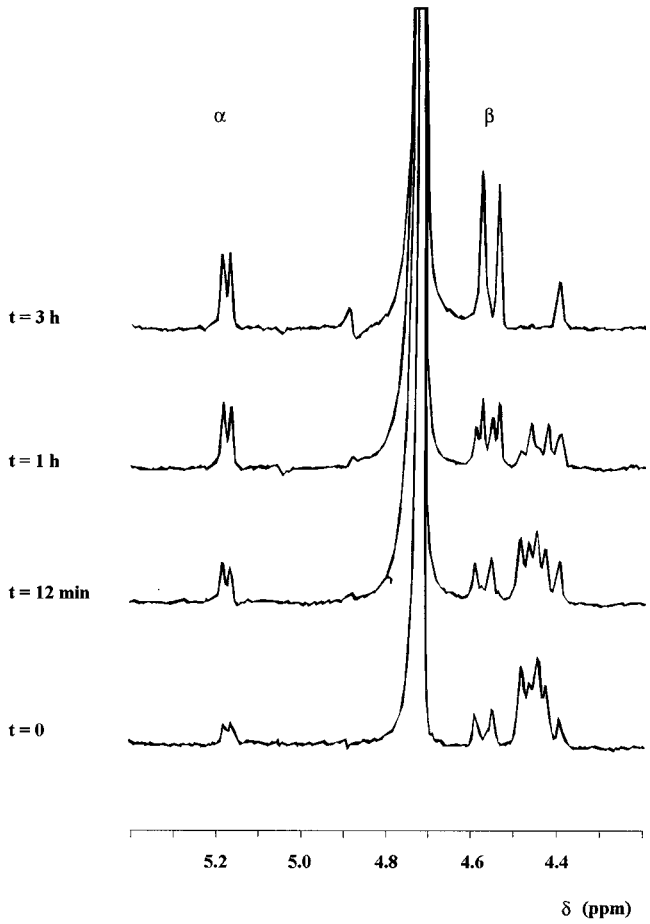

FIG. 2. ^{1}H NMR analysis of the stereoselectivity of the reaction catalyzed by the β-xylosidase from *C. cellulolyticum*. ^{1}H NMR spectra of xylotetraose during hydrolysis in the region of the anomeric proton resonance at various times (0, 12, 60, and 180 min) after addition of β-xylosidase.

shown). This doublet was assigned to the β anomeric proton of the xylose entity produced at the first step in the tetraose degradation process. After 30 min of incubation, superimposed β protons from the mono-, di-, tri-, and tetraose were observed but there was no change in the α anomeric proton doublet which occurred at δ = 5.17 ppm. Complete degradation of xylotetraose to xylose took 3 h. When the mutarotation equilibrium was reached, the two doublets taken to be the α anomeric proton (δ = 5.18 ppm, J = 3.54 Hz) and the β anomeric proton (δ = 4.56 ppm, J = 7.93 Hz) were at a ratio of 35% α to 65% β. These results indicate that the β-xylosidase of *C. cellulolyticum* acts by inverting the anomeric configuration.

In conclusion, the β-xylosidase from *C. cellulolyticum* shows a high level of hydrolytic activity on *p*-nitrophenylxyloside and xylodextrins and a very low level with *p*-nitrophenylarabinofuranoside, xylan, and lichenan. This indicates that its substrate specificity is relatively narrow and also suggests that the enzyme has the exo type of action, hydrolyzing terminal nonreducing ends. When tested with PNPX, the enzyme showed one of the highest affinities recorded so far and a fairly high maximum rate of hydrolysis in comparison with other bacterial xylosidases (19). Moreover, the enzyme is able to cleave the β-(1,4)-xylosidic linkage but no other glycosidic linkages. The N-terminal amino acid sequence of the purified enzyme showed similarities to three enzymes with related functions. These enzymes have not been classified (17, 18) and probably constitute a new family of glycosyl hydrolases. The β-xylosidase from *C. cellulolyticum* has a hydrolytic mechanism which results in inversion of the anomeric configuration, whereas the action of XynZ from *C. thermocellum* and Xyn from *Schizophyllum commune* involves retention stereoselectivity (13). This enzyme is probably part of the xylanolytic system of *C. cellulolyticum*. Work is in progress with a view to isolating other xylanolytic enzymes and cloning the corresponding genes.

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