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Isolation and Some Properties of a β-D-Xylosidase from *Clostridium acetobutylicum* ATCC 824

SONG F. LEE AND CECIL W. FORSBERG*

Department of Microbiology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

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A β-D-xylosidase from *C. acetobutylicum* ATCC 824 was purified by column chromatography on CM-Sepharose, hydroxylapatite, Phenyl Sepharose, and Sephadex G-200. The enzyme had an apparent molecular weight of 224,000 as estimated by gel filtration. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the enzyme consisted of two subunits of 85,000 and one subunit of 63,000 daltons. It exhibited optimal activity at pH 6.0 to 6.5 and 45°C. The enzyme had an isoelectric point of 5.85. It hydrolyzed *p*-nitrophenylxyloside readily with a K_m of 3.7 mM. The enzyme hydrolyzed xylo-oligosaccharides with chain lengths of 2 to 6 units by cleaving a single xylose from the chain end. It showed little or no activity against xylan, carboxymethyl cellulose, and other *p*-nitrophenylglycosides.

Microbial depolymerization of commercial xylan is carried out by at least three enzymes, namely, endoxylanase, β-D-xylosidase, and arabinofuranosidase (2). The endoxylanase cleaves the polymer randomly, yielding fragments of xylan of various chain lengths. The xylosidase further hydrolyzes the oligosaccharides to xylose. Arabinofuranosidase cleaves off the arabinose residue, resulting in a better overall extent of degradation (6). Recently, it has been shown that another enzyme, the esterase, plays a significant role in the depolymerization of acetylated xylan (4). Cooperativity between the esterases and xylanases has been demonstrated (3). In the overall process, the breakdown of oligosaccharides was thought to be a critical step (18).

Recently, Lee et al. (12) reported that *Clostridium acetobutylicum* ATCC 824 produced extracellular xylanase, xylosidase, and arabinofuranosidase activities. Two endoxylanases from this organism had been purified, and they were shown to have different hydrolytic properties and antigenicity (13). In this paper, the isolation and characteristics of xylosidase from this organism are described.

MATERIALS AND METHODS

Source of enzymes. The growth of *C. acetobutylicum* ATCC 824 and the preparation of culture supernatant fluid as the source of enzymes for purification are described elsewhere (13).

Hydrophobic interaction chromatography. Phenyl Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.) was equilibrated with 2.0 M NaCl in 0.02 M sodium phosphate buffer (pH 6.0). Enzyme solution (15 ml) in 2.0 M NaCl plus buffer was applied to the gel (2.5 by 25 cm). Starting buffer (40 ml) and 300 ml of NaCl gradient (2.0 to 0 M) in buffer were used to elute the enzymes. Fractions of 3 ml were collected.

Gel permeation chromatography. Concentrated enzyme solution (6 ml) was applied to Sephadex G-200 (column size, 2.5 by 74 cm) (Pharmacia). A bed volume of 0.05 M sodium phosphate buffer (pH 6.0) was used as eluant.

Enzyme assay. Xylosidase activity was determined by measuring the amount of *p*-nitrophenol (pNP) released from *p*-nitrophenyl-β-D-xyloside (Sigma Chemical Co., St. Louis, Mo.). Assays for activity against other *p*-nitrophenylglycosides, xylan, and carboxymethyl cellulose were described previously (12). In all cases, 0.1 M sodium phosphate buffer (pH 6.0) was used. One unit of enzyme activity was defined as the amount of enzyme that catalyses the release of 1 μmol of pNP or reducing sugars per min at 39°C.

Hydrolysis of xylo-oligosaccharides. A 100-μg portion of oligosaccharides was incubated with 0.005 U of xylosidase in 200 μl of 0.025 M sodium phosphate buffer (pH 6.0). After 10 h of incubation at 39°C, the reaction was stopped by boiling for 5 min. The samples were analyzed by high-pressure liquid chromatography on an HPX-42A column (Bio-Rad Laboratories, Richmond, Calif.) (13). Xylo-oligosaccharides were prepared as described previously (13).

PAGE. Analytical isoelectric focusing-polyacrylamide gel electrophoresis (PAGE) was performed as described previously (13). Sodium dodecyl sulfate (SDS)-PAGE was done by the method of Laemmli (11). The Coomassie blue-stained gel was scanned on a slab gel scanning system (model DU-8; Beckman Instruments Inc., Fullerton, Calif.) at 595 nm.

Estimation of protein. Protein was measured by the method of Lowry et al. (14) with bovine serum albumin as standard.

Effect of metal ions. Enzymes were exposed to 1 mM metal ions at 35°C in 0.03 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer (pH 6.5) for 10 min. Residual activity was measured at 39°C in the same buffer. Enzyme assayed in PIPES buffer alone was taken as 100%.

RESULTS

Purification. *C. acetobutylicum* ATCC 824 was grown on 1.5% (wt/vol) oat spelt xylan. The culture supernatant fluid, which contained xylanase, xylosidase, and arabinofuranosidase activities, was chromatographed on the CM-Sepha-

* Corresponding author.

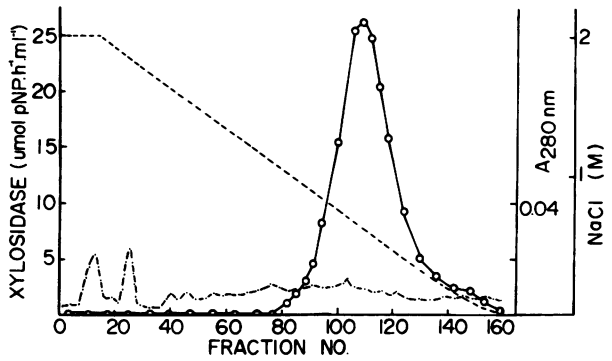

FIG. 1. Hydrophobic interaction chromatography of the xylosidase from *C. acetobutylicum* ATCC 824. Experimental details are outlined in Materials and Methods. Symbols: O, xylosidase; —, A_{280}; ----, NaCl gradient.

rose gel. The xylosidase was eluted as a single peak with contaminating xylanase and arabinofuranosidase activities (13). The xylosidase was subsequently separated from the contaminating activities by chromatography on hydroxylapatite (13). During this chromatographic step, the xylosidase was separated into a major and minor peak. Since the major peak accounted for approximately 85% of the total activity, it was selected for further studies. The major peak was pooled and chromatographed on Phenyl Sepharose (Fig. 1). The resulting enzyme, when chromatographed on Sephadex G-200, eluted as a symmetrical peak and corresponded to a size of 224,000 daltons. SDS-PAGE analysis showed two protein bands of 85,000 and 63,000 daltons (Fig. 2). Scanning of the Coomassie blue-stained protein bands by a gel scanner showed that the 85,000-dalton protein was 2.4 times denser than the 63,000-dalton protein. Isoelectric focusing-PAGE showed that the xylosidase consisted of a single protein band. The purification of this enzyme is summarized in Table 1.

TABLE 1. Summary of the purification of the xylosidase from *C. acetobutylicum* ATCC 824

Purification step	Total activity (μmol of pNP · min^{-1})	Amt of proteins (mg)	Sp act (activity · mg of $protein^{-1}$)	% Yield	Purification
Crude enzymes	30.1	280.0	0.11	100	
CM-Sepharose	25.0	59.2	0.42	83	4
Hydroxylapatite	17.1	3.5	4.89	57	44
Phenyl Sepharose	10.2	1.2	8.50	34	77
Sephadex G-200	7.0	0.4	17.50	23	159

General properties of the xylosidase. The pH activity and stability curves of the xylosidase are shown in Fig. 3. The enzyme had optimal activity at pH 6.0 to 6.5 and was stable from pH 6.0 to 8.0 (the highest pH tested). The xylosidase was most active at 45°C. It retained 100% of its activity up to 40°C and lost it rapidly above this temperature. Kinetic studies showed that the xylosidase had a V_{max} of 19.6 U/mg of protein and a K_m of 3.7 mM pNP-xyloside. Its isoelectric point was 5.85 as determined from isoelectric focusing-PAGE.

Substrate specificity. The ability of the purified xylosidase to hydrolyze various substrates was tested. The results are tabulated in Table 2. The enzyme showed high activity against pNP-xyloside but not pNP-arabinofuranoside, pNP-glucoside, pNP-mannoside, pNP-galactoside, or pNP-fucoside. It had very little activity against xylan and carboxymethyl cellulose. The ability of the xylosidase to hydrolyze xylo-oligosaccharides with a degree of polymerization of 2 to 6 units was examined. The enzyme was able to attack all these oligosaccharides (Fig. 4). However, the extent of hydrolysis varied with the chain length of the substrates. In a 10-h incubation period, the approximate amounts of substrate hydrolyzed were 29, 61, 27, 58, and 95% for xylohexaose (X_6), xylopentaose (X_5), xylotetraose (X_4), xylotriose (X_3), and xylobiose (X_2), respectively. In all cases, the major products were xylose and an oligosaccha-

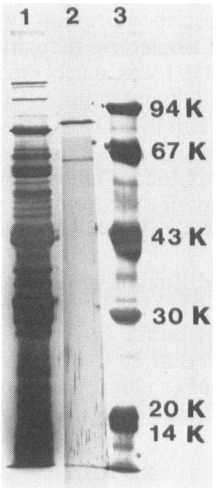

FIG. 2. SDS-PAGE of the culture supernatant fluid (lane 1) and the xylosidase (lane 2) of *C. acetobutylicum* ATCC 824. Proteins were stained with the Bio-Rad silver stain. Lane 3: protein markers (from the top), phosphorylase *b*, bovine serum albumin, ovalmin, soybean trypsin inhibitor, and α-lactalbumin.

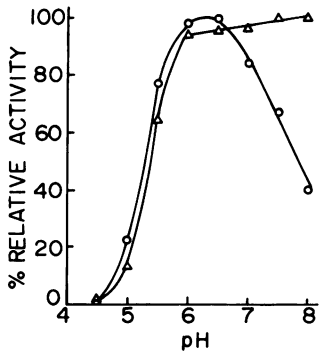

FIG. 3. pH activity (O) and stability (△) curves of the xylosidase from *C. acetobutylicum* ATCC 824. For pH activity, citrate (40 mM)–sodium phosphate (80 mM) buffer was used. For pH stability, enzymes were exposed to citrate (50 mM)–phosphate (100 mM) buffer for 30 min at 35°C. Residual activity of the enzymes (diluted sixfold before the assay) was measured in 200 mM sodium phosphate buffer (pH 6.0).

TABLE 2. Activity of the xylosidase from *C. acetobutylicum* ATCC 824 against xylan, carboxymethyl cellulose, and various aryl glycosides

Substrate	Sp act (μmol of products · min^{-1} · mg of $protein^{-1}$)
Larchwood xylan	1.3
Oat spelt xylan	0.7
Carboxymethylcellulose	1.3
pNP-β-D-xyloside	16.1
pNP-α-L-arabinofuranoside	0.1
pNP-β-D-glucoside	0.01
pNP-β-D-mannoside	0.03
pNP-β-D-galactoside	<0.01
pNP-β-D-fucoside	0.01

ride with a degree of polymerization 1 unit smaller than the substrate.

Effect of metal ions on the xylosidase activity. The xylosidase was sensitive to some bivalent cations. At 1 mM, Hg^{2+} and Cu^{2+} completely inhibited the enzyme activity. Zn^{2+}, Ni^{2+}, Co^{2+}, and Mn^{2+} showed 90, 85, 55, and 30% inhibition, respectively. The enzyme activity was not affected by Ca^{2+}, Mg^{2+}, K^+, or EDTA (5 mM).

DISCUSSION

A xylanolytic component from *C. acetobutylicum* was isolated by column chromatography. This enzyme showed hydrolytic properties typical of a β-D-xylosidase. It was able to hydrolyze xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose. High-pressure liquid chromatography analysis showed that the enzyme acted endwise on the oligosaccharides, i.e., it cleaved a single unit of xylose from the chain end. At present, it is not known whether the cleavage was from the reducing or the nonreducing end. Xylosidases from *Bacillus pumilus* (10), *Penicillium wortanni* (5), and *Aspergillus niger* (19) showed attack from the nonreducing end. Hence, it is possible that the xylosidase from *C. acetobutylicum* also acts similarly. The xylosidase from *C. acetobutylicum* lacks activity against xylan, carboxymethyl cellulose, and other aryl glycosides. Xylosidases from other sources (Table 3) also lack activity against xylan and most of the phenyl glycosides tested. However, the xylosidase from *P. wortanni* showed activity against pNP-α-L-arabinopyranoside (5), and the purified xylosidase of *Chaetomium trilaterale* showed 15-times-higher specific activity against pNP-glucoside than pNP-xyloside (20).

The extent of hydrolysis of xylo-oligosaccharides by the xylosidase from *C. acetobutylicum* varied with the chain length of the substrate. In a 10-h incubation period, approximately 95% of X_2, 60% of X_3 and X_5, and 30% of X_4 and X_6 were hydrolyzed. The orders of decreasing activity against xylo-oligosaccharides shown by *Trichoderma viride* (15) and *Emericella nidulans* (16) are $X_2 > X_3 > X_4 > X_5$ and $X_3 > X_2 > X_4 > X_5$, respectively. The *A. niger* xylosidase degraded X_3 more efficiently than X_2 (9).

C. acetobutylicum produced two endoxylanases, which were purified (13). Xylanase A, which has a molecular weight of 65,000, hydrolyzed larchwood xylan to X_2, X_3, X_4, X_5, and X_6 as end products. Xylanase B, which has a molecular weight of 29,000, degraded the xylan to X_2 and X_3. Since the xylosidase is able to hydrolyze these oliogosaccharides, one would envision that it plays a role in breaking down the oliogosaccharides which resulted from the action of the xylanases to provide xylose as nutrient during the cultivation of this organism on xylan.

The general properties exhibited by *C. acetobutylicum* xylosidase are comparable to those reported in the literature for other organisms (Table 3). One of the properties displayed by the xylosidase of *C. acetobutylicum* which has significant implications is the pH stability. For a more efficient utilization of xylan, it is desirable to grow the organism at pH 6.0 or higher, since the xylosidase (Fig. 3) and the xylanases (13) are stable under those conditions. However, it has been shown that a culture pH below 5.5, with the optimum at 4.3, was essential for solvent production by *C. acetobutylicum* (1, 8). Hence, the stability of the xylosidase may play a critical role in the direct conversion of xylan to solvents. There are two possible ways to circumvent the problem. First, Holt et al. (7) have demonstrated that *C. acetobutylicum* ATCC 824 produces solvents from glucose at pH 7.0 in the presence of exogenous butyric and acetic acids. Therefore, one can grow strain ATCC 824 in the similar way on xylan. Second, it may be possible to isolate a

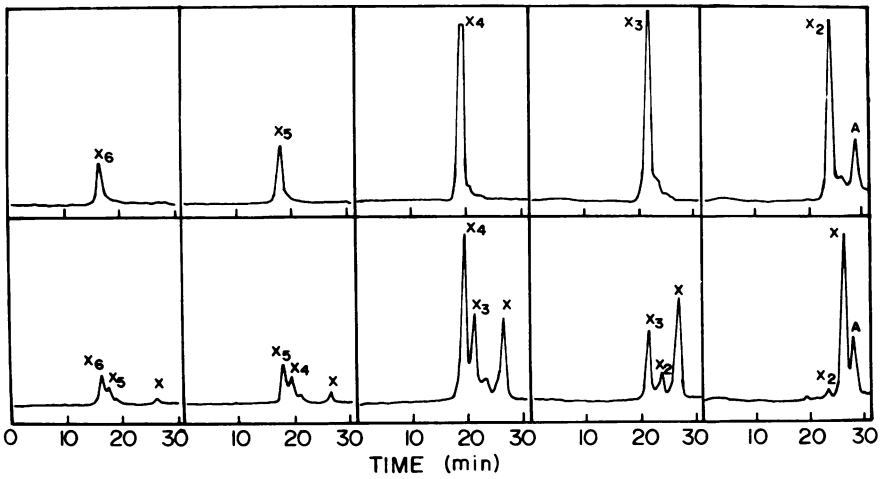

FIG. 4. HPLC analysis of the hydrolysis of xylo-oligosaccharides by the purified xylosidase of *C. acetobutylicum* ATCC 824. Oligosaccharides were incubated with heat-inactivated enzymes (top panel) and active enzymes (bottom panel). A, Unknown peak.

TABLE 3. General properties of the purified xylosidases from *C. acetobutylicum* and other organisms

Organism	pH optimum	pH stability	Temp optimum (°C)	K_m (mM pNP-X)	V_{max} (IU · mg^{-1})	Mol wt[a]	Mol wt of subunits[b]	pI	Sp act	Source or reference
C. acetobutylicum	6.0–6.5	6.0–8.0	45	3.7	19.6	224,000	85,000, 63,000	5.85	16.1	This work
B. pumilus	7.0			2.4	3.4	130,000	70,000	4.4[c]	3.4	17
Chaetomium trilaterale	4.5					240,000	118,000	4.86		20
A. niger	6.7–7.0		42	0.22[d]		78,000			5.2	9
P. wortanni	3.3–4.0	5.0–6.0		0.12		100,000		5.0	11.4	5
T. viride	4.5		55	5.8		101,000		4.45	10.8	15
E. nidulans	4.5–5.0		55	1.0[e]		240,000	116,000	3.25	62.9[f]	16

[a] Estimated by gel filtration.
[b] Estimated by SDS-PAGE.
[c] From reference 10.
[d] mM *o*-nitrophenylxyloside.
[e] mM xylobiose.
[f] Expressed as micromoles of reducing sugar per minute per milligram.

mutant which has more stable and active xylosidase at low pH.

As mentioned in the Introduction, arabinofuranosidase represents another component of the complete xylanolytic system. Work is being carried out to isolate and characterize this enzyme from *C. acetobutylicum* ATCC 824 to fully understand its contribution to the xylanolytic system.

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