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Purification and Characterization of a Thermostable β-Xylosidase from *Thermoanaerobacter ethanolicus*

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A highly thermostable β-xylosidase, exhibiting similarly high activities for arylxylose and arylarabinose, was purified (72-fold) to gel electrophoretic homogeneity from the ethanologenic thermophilic anaerobe *Thermoanaerobacter ethanolicus*. The isoelectric point is pH 4.6; the apparent molecular weight is around 165,000 for the native enzyme (gel filtration and gradient polyacrylamide gel electrophoresis) and 85,000 for the two subunits (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). The enzyme exhibited the highest affinity towards *p*-NO_2-phenyl xyloside (pNPX) (substrate concentration for half-maximal activity = 0.018 mM at 82°C and pH 5.0) but the highest specific activity with *p*-NO_2-phenylarabinofuranoside. $T_{opt,\ 5\ min}$, the temperature for the maximum initial activity in a 5-min assay of the purified enzyme, was observed around pH 5.9 and 93°C; however at 65 and 82°C, the pH optimum was 5.0 to 5.2, and at this pH the maximal initial activity was observed at 82°C (pH 5.0 to 5.5). The pH curves and temperature curves for arylxylosides as substrates differed significantly from those for arylarabinosides as substrates. An incubation for 3 h at 82°C in the absence of substrate reduced the activity to around 75%. At 86°C the half-life was around 15 min. With pNPX as the substrate, an Arrhenius energy of 69 kJ/mol was determined. The N-terminal sequence did not reveal a high similarity to those from other published enzyme sequences.

Hemicellulose, a variety of pentose-containing, β-glycosidic polymers, is the second most abundant renewable polysaccharide. The utilization of hemicellulosic material is becoming more and more important for an economical use of the renewable resource lignocellulose. Xylan-degrading enzymes are especially important for various applications, such as biomass conversion for chemical production, treated feed additives, and alternative pulping processes, which are under development. Glycosidases such as β-xylosidases and α-arabinosidases play a crucial role in a complete and fast degradation of branched and substituted xylans to the final products, xylose and arabinose, respectively.

Although many fungal β-xylosidases have been studied, only a few β-xylosidases from bacteria have been purified and characterized. These include the enzymes from the thermophilic aerobic bacterium *Bacillus stearothermophilus* (12), the anaerobic bacterium *Clostridium acetobutylicum* (9), and the "thermophilic" actinomycete *Thermomonospora fusca* (1). More recently, the β-xylosidase from the anaerobic thermophilic bacterium *Caldocellum saccharolyticum*, expressed in *Escherichia coli*, was described by Hudson et al. (6). However, this enzyme is a xylose transferase rather than a true β-xylosidase, because it could use neither xylobiose nor triose as a substrate, although arylxylosides were hydrolyzed. High activity and thermostability of β-xylosidase have attracted considerable attention as characteristics of an efficient enzyme. Some thermostable β-xylosidases have been purified and characterized (1, 6, 12). Furthermore, a few β-xylosidases with an additional α-arabinosidase activity (3, 9, 14, 16) or β-glucosidase activity (17) have been reported.

In this paper we describe a highly thermostable β-xylosidase with an unusually high aryl-α-arabinosidase activity. The enzyme was isolated from the thermophilic anaerobic ethanol producer *Thermoanaerobacter ethanolicus*, which can convert xylose, xylooligomers, and xylan to ethanol (18, 19).

MATERIALS AND METHODS

Organism. *T. ethanolicus* JW 200 (ATCC 31550) was obtained from our own collection; it was subcultured from a stock culture (50% glycerol) made from the original isolate. The cells used for enzyme purification were grown in 10-liter batches (12-liter glass carboys) under an atmosphere of O_2-free N_2 at 54°C to the late logarithmic phase in a prereduced liquid medium containing 0.16% yeast extract, 0.05% NH_4Cl, 0.05% $(NH_4)_2SO_4$, 0.6% xylose, 0.34% KH_2PO_4, 0.43% K_2HPO_4, 0.018% $MgCl_2 \cdot 6H_2O$, 0.05% vitamin solution (5), 0.5% mineral solution (5), 0.025% $NaS \cdot 9H_2O$, and 0.025% cysteine $HCl \cdot H_2O$. Cells were harvested by ultrafiltration (Diaflo Ultrafilter; Amicon Corp., Lexington, Mass.).

Enzyme assay. β-Xylosidase activity was determined by assaying the amount of *p*-nitrophenol (pNP) released from the substrates *p*-nitrophenyl β-D-xylopyranoside (pNPX), *o*-nitrophenyl β-D-xylopyranoside (oNPX), *p*-nitrophenyl α-L-arabinopyranoside (pNPAP), and *p*-nitrophenyl α-L-arabinofuranoside (pNPAF). An enzyme unit is defined as the amount of enzyme producing 1 μmol of pNP/min, and specific activity is defined as units per milligram of protein. If not otherwise indicated, 0.1 ml of culture supernatant or cell-free crude extract was added to 0.3 ml of 0.1 M acetate buffer (pH 4.5) and 0.1 ml of 5 mM pNPX and the mixture was incubated for 10 min at 75°C. When the purified enzyme was assayed, 10 μl of enzyme (2 to 5 μg of protein per ml) was added to 0.38 ml of 0.1 M potassium phthalate buffer (pH 6) and 10 μl of pNPX (with a concentration up to 40 mM or a concentration of 600 mM in dimethyl sulfoxide when pNPAP or pNPAF was used). The mixture was incubated for 5 min at 82°C or as indicated. The color of pNP was

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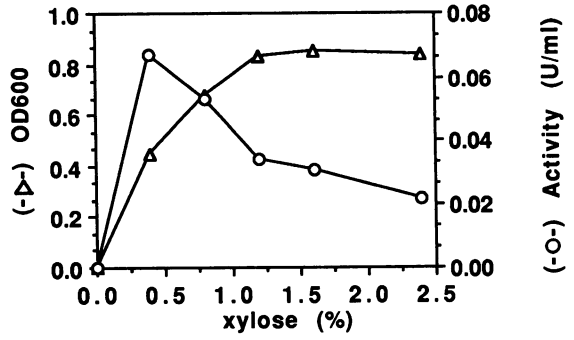

FIG. 1. Dependence of β-xylosidase activity on concentration of xylose as growth substrate. Cells were grown at 60°C in a yeast extract-containing mineral medium with a starting pH of 7.0. OD_{600}, optical density at 600 nm.

developed by adding 1 ml (assay using extracts) or 0.6 ml (assay with purified enzyme) of 1 M Na_2CO_3, and the A_{405} was read. A standard curve was prepared by using *p*-NO_2-phenol. Comparison of enzyme activities in cultures grown under various conditions were made using cell extracts prepared by French press treatment of the whole cultures (>90% cell breakage, microscopic examination). Activity was reported as either units per milliliter of culture or as specific activity after determining the protein content of the extract containing the cell debris.

Protein concentrations were determined by the dye-binding method of Bradford (2) with bovine albumin as the standard.

Purification. Cells of *T. ethanolicus* were harvested from 10-liter carboy cultures and resuspended in 120 ml of water (about 1 g [wet cells] per ml). The cells were broken with a French press at 18,000 lb/in^2, and the cell extract was prepared by centrifugation at 100,000 × *g* for 90 min. The β-xylosidase was purified at room temperature; thus, all buffers contained 0.02% (wt/vol) sodium azide to prevent microbial growth. The following steps were used.

(i) First ion-exchange chromatography. The crude extract (110 ml) was applied directly onto a DEAE-cellulose (DE23; Whatman, Maidstone, England) column (2.6 by 36 cm) which was preequilibrated with 20 mM citric acid–Na-citrate buffer (pH 6.6). Proteins were eluted with 1,400 ml of 20 mM Na-citrate buffer with a pH gradient from 6.6 to 3.0. Fractions of 7.5 ml were collected every 12 min.

(ii) Hydrophobic interaction chromatography. Proteins in the fractions (DEAE-cellulose column) with β-xylosidase activity were precipitated by adding solid $(NH_4)_2SO_4$ to a concentration of 3 M and collected by centrifugation (10 min at 15,000 × *g*). The pellet was dissolved in 2 ml of water and loaded onto a phenyl-Sepharose CL-4B column (1.6 by 16 cm) which was preequilibrated with 20 mM Na-citrate buffer (pH 6.6) containing 0.8 M $(NH_4)_2SO_4$. The column was washed with 100 ml of 0.8 M $(NH_4)_2SO_4$ in Na-citrate buffer (pH 6.6), and proteins were eluted with a decreasing (0.8 to 0 M) $(NH_4)_2SO_4$ gradient at a flow rate of 30 ml/h.

(iii) Second ion-exchange chromatography. Pooled fractions with β-xylosidase activity, eluted from the phenyl-Sepharose CL-4B column, were dialyzed against three changes of 1.5 liters each of 20 mM Na-citrate buffer (pH 6.6) and loaded onto a DEAE-Sephacel (Pharmacia, Pleasant Hill, Calif.) column (1.2 by 16 cm) previously washed with the 20 mM Na-citrate buffer (pH 6). The enzyme was eluted with a linear pH gradient from 6 to 3; the fraction size was 4.8 ml, and the flow rate was 30 ml/h.

Estimation of molecular mass and pI. The molecular mass of purified β-xylosidase was determined by sodium dodecyl sulfate (SDS)- and native-gradient polyacrylamide gel electrophoresis (PAGE) and by gel filtration on a Superose 12 column (1.3 by 70 cm; Pharmacia Inc., Piscataway, N.J.). SDS-PAGE (5% gel) was done according to the instructions of Bio-Rad Laboratories with molecular mass protein standards (catalog no. 161-0303; Bio-Rad Laboratories, Richmond, Calif.) from 45 to 200 kDa. Gel filtration molecular mass markers from 12 to 200 kDa (MW-GF-200; Sigma Chemical Co., St. Louis, Mo.) were used for the gel filtration. For the native-gradient (4 to 24%) PAGE, an electrophoresis calibration kit (6K160A; Pharmacia) was used. The isoelectric point (pI) was determined by using ampholytes with a range of 2.9 to 5.0 and a horizontal polyacrylamide gel (45 by 125 mm; model 1415 electrophoresis cell; Bio-Rad) according to Bio-Rad instructions.

N-terminal sequence. The N-terminal sequence was determined by automated Edman degradation (protein sequencer model 470A or 477A; Applied Biosystems, Inc., Foster City, Calif.).

Hydrolysis of xylo and arabino oligomers. The highly purified enzyme was incubated with the xylo and arabino oligomers with between two and six sugar units (MegaZyme, North Rocks, New South Wales, Australia). At various time intervals samples were taken and analyzed with a Dionex System (Onguard-P; Dionex Corp., Sunnyvale, Calif.) as described previously (14).

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

RESULTS

Culture conditions for formation of β-xylosidase activity. The majority (60 to 80%) of the β-xylosidase activity in a culture of *T. ethanolicus* was found associated with the cells. Only one protein band exhibited β-xylosidase activity (pNPX) when a crude extract was subjected to gel isoelectrofocusing (pH 3.5 to 10). Although mannose (0.4% [wt/vol])-supplemented medium yielded the best growth, with a final optical density at 600 nm of 0.745 compared with 0.21 for sucrose-supplemented medium or xylose-supplemented medium (each at 0.4% [wt/vol]), cultures grown with man-

TABLE 1. Purification of the β-xylosidase from *T. ethanolicus*

Step	Total protein (mg)	Total activity (U)[a]	Sp act (U/mg)	Purification (fold)	Yield (%)
Crude extract	1,160	1,068	0.92	1	100
DEAE-cellulose	168	570	3.4	3.7	53
Phenyl-Sepharose	6	233	39	42	22
DEAE-Sephacel	2.3	152	66	72	14

[a] Determined at 75°C using Na acetate buffer at pH 4.5 and pNPX as the substrate.

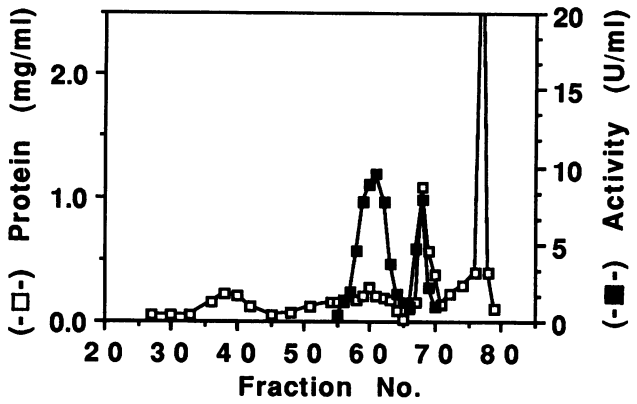

FIG. 2. Hydrophobic interaction chromatography (phenyl-Sepharose CL-4B) (for details see Materials and Methods).

nose exhibited only a low xylosidase activity of 0.005 U/ml of culture compared with 0.005 and 0.014 U of xylosidase activity per ml for cultures grown on sucrose and xylose, respectively. Within a growth cycle, the highest xylosidase activity was reached at the late logarithmic growth phase. If the pH of the culture was below pH 5.8, the activity decreased during the stationary phase. Xylose concentrations above 0.4% caused a decrease in the β-xylosidase activity (Fig. 1). The effects of various saccharides on induction of the xylosidase activity were determined in culture grown in the basic medium supplemented with 0.2% (wt/vol) mannose and 0.2% (wt/vol) of the compound to be tested. Comparable growth rates were obtained in the presence of the various compounds, and the cultures were harvested at the same time in the late logarithmic growth phase, with optical densities at 600 nm between 0.45 and 0.51. The following specific activities in units per milligram of cell protein (values in parentheses are units per liter of culture) were obtained: glucose, 0.9 (2.0); cellobiose, 1.0 (2.0); galactose and arabinose, 1.2 (2.5); mannose, 1.6 (3.6); birch xylan, 3.0 (6.5); and xylose, 38.6 (85).

Enzyme purification. The major purification steps are summarized in Table 1 and described in Materials and Methods. When eluted from the DEAE-cellulose column, β-xylosidase activity was found in two peaks. The ratio of the two peaks in the various preparations depended on the ionic strength of the eluent. Almost all of the β-xylosidase activity appeared in the second peak when a pH gradient of 20 mM Na-citrate buffer was used as the eluent, indicating that the enzyme undergoes some conformational changes, which, however, were not further investigated. During hydrophobic interaction chromatography, the β-xylosidase activity again appeared in a major and a minor peak (Fig. 2). The enzyme from the major peak was further purified by DEAE-Sephacel column chromatography. One fraction from this step showed very high β-xylosidase activity and led to a single band on native PAGE and IEF gels when 4 μg of protein was loaded on 0.8-mm-thick gels.

Physical properties. The apparent molecular mass of the β-xylosidase was estimated to be 165 kDa by gradient PAGE and gel filtration chromatography with cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), albumin (66 kDa), and β-amylase (200 kDa) as molecular mass standards. The denatured β-xylosidase showed one protein band at 85 kDa by SDS-PAGE, indicating that the native β-xylosidase is composed of two subunits with the same apparent molecular weight Isoelectric focusing (pH 2.9 to 5) yielded a single activity band at pH 4.6.

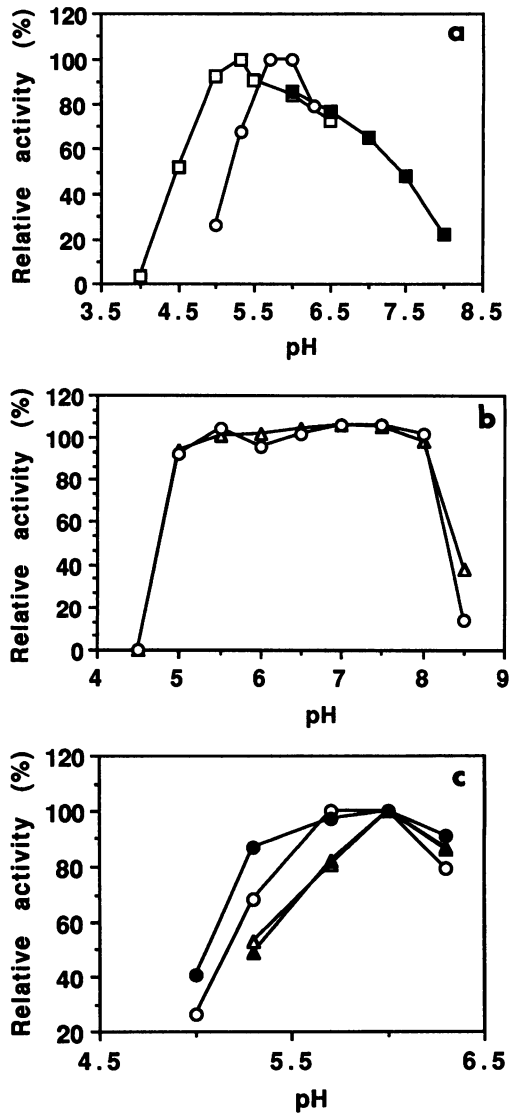

FIG. 3. pH dependence of β-xylosidase. (a) pNPX activity at 82 and 93°C. The activity was determined in a 5-min assay with 0.088 μg of purified enzyme per ml and 100 mM sodium citrate (□) or potassium phosphate (■) buffer at 82°C and 100 mM potassium phthalate buffer (○) at 93°C. (b) Stability of the purified enzyme (1.1 μg/ml of incubation mixture) at various pH values determined at 82°C, using 100 mM sodium citrate buffer (pH 4.5 to 7) and glycylglycine buffer (pH 7.0 to 9.0). Activity remaining after 20 (△) and 40 (○) min was determined with 1 mM pNPX as the substrate. (c) pNPAF (△), pNPAP (▲), oPNX (●), and pNPX (○) activities at the $T_{opt,\ 5\ min}$ values of 75, 80, 90, and 93°C, respectively, and 1 mM substrate concentration. One hundred percent activity corresponded to hydrolysis of 116 μmol of pNPX, 34 μmol of oPNX, 154 μmol of pNPAF, and 260 μmol of pNPAP min^{-1} mg of $protein^{-1}$.

Effects of temperature and pH. With pNPX as the substrate, the pH_{opt} (optimum pH) and the $T_{opt,\ 5\ min}$ (the temperature for the highest initial activity in a 5-min assay) were mutually dependent (Fig. 3 and 4). With citrate or phthalate buffer (data omitted for clarity of Fig. 3), the pH_{opt} was between 5.0 and 5.2 at 65 and 82°C, whereas at 93°C

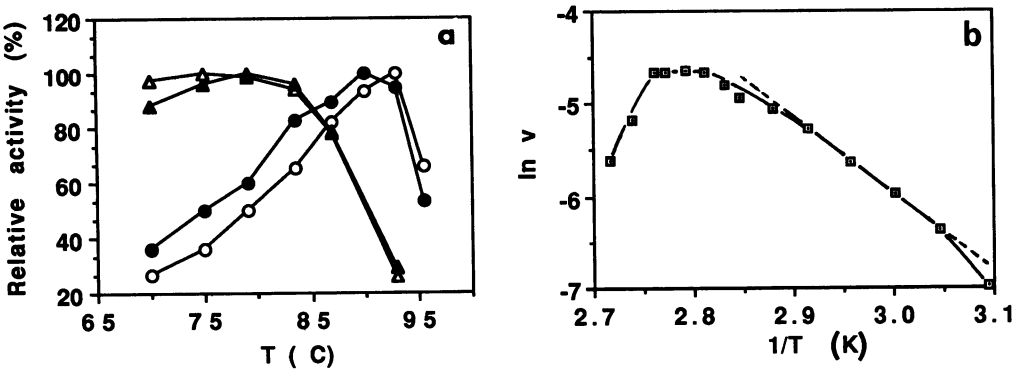

FIG. 4. (a) Temperature dependence of β-xylosidase activity with 1 mM pNPX (○), oNPX (●), pNPAF (△), and pNPAP (▲) as substrates at pH 5.7. One hundred percent activity corresponded to hydrolysis of 116 μmol of pNPX, 34 μmol of oPNX, 154 μmol of pNPAF, and 260 of μmol pNPAP min^{-1} mg of $protein^{-1}$. (b) Arrhenius plot for pNPX initial (5-min assay) activity, using 100 mM sodium citrate buffer (pH 5.0).

($T_{opt,\ 5\ min}$ at pH 6.0) the pH_{opt} was between 5.8 and 6.0. Maximal initial (5 min) activity for oNPX and pNPX occurred at 90 and 93°C, respectively, whereas for pNPAF and pNPAP the values were 75 and 80°C, respectively (Fig. 4a). However, although the temperature data differ significantly for the xylosides and arabinosides, the pH_{opt} values (determined at the corresponding T value for the maximal initial activity) were only slightly different (between 5.7 and 5.9). An Arrhenius energy (E_A) of 69 kJ/mol was calculated from the linear portion of the Arrhenius plot for the β-xylosidase pNPX activity at pH 5.0 and 82°C (Fig. 4b). In the absence of substrates, the enzyme was stable at 75°C for more than 1 h, retained 75% of its activity at 82°C after 3 h, and exhibited a half-life of around 15 min at 86°C (Fig. 5).

Substrate specificity and affinity. The purified enzyme hydrolyzed pNPX, as well as oNPX, pNPAF, and pNPAP (Table 2). No measurable activities were obtained with other arylglycosides, including the arylglucoside and arylmannoside. The substrate saturation followed a non-Michaelis-Menten saturation kinetics. The Cornish-Bowden plots yielded multiple intersections, and the double-reciprocal plots ($1/v$ versus $1/[S]$) and the Hanes plot ($[S]/v$ versus $[S]$) yielded upward-curving graphs. Thus, only $K_{0.5}$ values (substrate concentrations for half-maximal activity) are given at this time. The enzyme exhibited higher specific activities with the arylarabinosides than with the arylxylosides as substrates; however, the enzyme had a higher affinity for the arylxylosides (Table 2). The enzyme hydrolyzed xylobiose and, at a lower rate, xylopentaose, the largest oligomer we tested. Despite the high rate of hydrolysis found for the *p*-nitrophenyl arabinosides, none of the tested L-arabino oligomers (n = 2 to 5) were hydrolyzed. However, the enzyme released arabinose and xylose from wheat arabinoxylan (MegaZyme). This activity requires further quantitative analysis with purified α(1→3)- and α(1→2,3)di-arabinyl-β(1→4) xylo oligomers. These substrates are presently not commercially available.

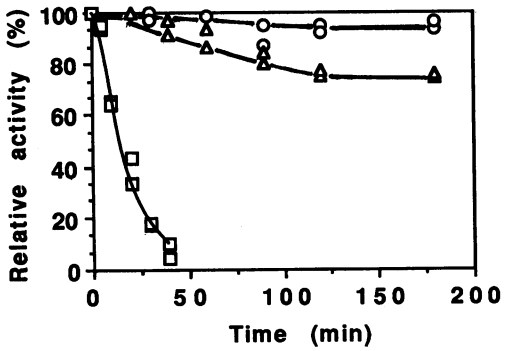

FIG. 5. Thermostability of β-xylosidase. Aliquots of purified enzyme (0.044 μg in 0.4 ml of 100 mM sodium citrate buffer [pH 6.5]) were incubated in 1.5-ml Eppendorf vials at 75 (○), 82 (△), and 86 (□)°C in the absence of substrate. At the indicated times, samples were stored on ice until the end of the experiment and then analyzed for remaining activity, using 1 mM pNPX as the substrate and a 5-min assay time.

N-terminal amino acid sequence. The following sequence of the 35 N-terminal amino acids was obtained with the purified enzyme preparation: Met-Lys-Pro-Leu-Tyr-Leu-Asp-Ser-Thr-Gln-Ser-Val-Glu-Lys-(Arg)-Val-Glu-Asp-Leu-Leu-Gln-Gln-Met-Thr-Ile-Glu-Glu-His-Val-Ala-Gln-Leu-Asn-(Ser)-(Pro or Arg).

DISCUSSION

To our knowledge, the β-xylosidase from *T. ethanolicus* described here exhibits the highest thermostability (the half-life at 82°C was longer than 3 h) and is stable over the broadest pH range (pH 5.5 to 8) of β-xylosidases described to date (1, 3, 6, 9). Only a few thermostable β-xylosidases have been purified. The enzymes from *Thermomonospora fusca* and from the thermophile *Caldocellum saccharolyticum* (which, although it exhibits arylxylosidase activity, is a transferase rather than a xylosidase) exhibited half-lives of 90 and 40 min, respectively, at 70°C. The broad pH stability of the enzyme is similar to the extreme broad pH_{opt} for growth of this organism. Furthermore, the enzyme has the highest specific activity and the highest affinity for the artificial substrate pNPX (1, 3, 6, 7, 9–12, 14, 16). The published K_m values range from 0.08 (*Trichoderma reesei* [14]) to 5.8 mM (*Trichoderma viride* [10]) for fungal enzymes and from 2.4 (*Bacillus pumilis* [12]) to 10 mM (*Caldocellum saccharolyticum* [6]) for bacterial enzymes, including those expressed in *E. coli*.

The observed dual activity of an (aryl)xylosidase and an (aryl)arabinosidase with the purified enzyme from *T. ethanolicus* is of interest for the utilization of substituted xylans. The β-xylosidases from *Penicillium wortmanni* and *Tricho-*

TABLE 2. Kinetic properties of the β-xylosidase (arabinosidase) from *T. ethanolicus* with various arylsaccharides as substrates

Substrate	$K_{0.5}$[a] (mM)	pH_{opt}	$T_{opt, 5\ min}$[b] (°C)	Sp act (U/mg of protein)[c]
pNPX[d]	0.018	5.0–5.2	82	122 (0.1)
	0.038	5.8–6.0	93	183 (0.2)
oNPX	0.06	6.0	90	50 (0.5)
pNPAF	4.6	6.0	75–80	1,073 (12)
pNPAP	2.1	6.0	75–80	758 (9)

[a] Substrate concentration observed for half-maximal activity in the v versus [S] plot at the indicated pH_{opt} and $T_{opt, 5\ min}$. Double-reciprocal plot ($1/v$ versus $1/[S]$) and Hanes plots yielded upward-curving graphs; thus, no K_m values are given.

[b] Temperature at which highest activity was observed in a 5-min assay.

[c] Calculated by using the v_{max} observed at the substrate concentration (millimolar) given in parentheses per milligram of protein. At substrate concentrations higher than indicated in the parentheses, substrate inhibition (about 10 to 20% for double the concentration) was observed.

[d] Two pH_{opt} values and corresponding $T_{opt, 5\ min}$ values were found for this enzyme with pNPX as the substrate (Fig. 3 and 4). The pH_{opt} was dependent on the incubation temperature, and $T_{opt, 5\ min}$ (the temperature for the maximal observed activity) was dependent on the pH. Thus, the affinity and maximal velocity observed were determined at both optima for the pH and temperature. The values for the other substrates were determined only at the higher pH and temperature.

derma reesei exhibited some activity with pNPAP (3) and pNPAF (14), respectively. Low levels of α-arabinosidase activity were also detected with some bacterial β-xylosidases (6, 9, 16). But to date, no other β-xylosidases have been found with such a high arylarabinosidase activity with both pNPAP and pNPAF (Table 2). Because the enzyme has a much higher affinity for pNPX than for pNPAP and pNPAF, we are still assuming that the purified enzyme is primarily a β-xylosidase, although the specific activity is about four- and sixfold lower with pNPX than with pNPAP and pNPAF, respectively. At this time, it is not clear whether the non-Michaelis-Menten kinetics are due to the use of the nonphysiological substrates or whether they are a true property for substrate binding of this enzyme. However, further kinetic studies, using oligoxylans, oligoarabinosans, and arabinosubstituted oligoxylans as natural substrates, are required for determination of K_m values for the true substrates and for a final classification of this enzyme.

The differences in the temperature and pH curves for the arylxylosides and arylarabinosides (Fig. 3 and 4) indicate the possibility that the binding sites for the two sugars are different, with either two different catalytic sites existing within the enzyme or different groups being involved in substrate binding within one catalytic center. Further research is needed with the physiological substrates.

The same is true for the two observed optimal conditions for arylxyloside activity, which suggest that the enzyme undergoes pH- and temperature-dependent conformational changes causing shifts in enzyme stability and activity, including substrate affinity. However, these effects could be also due to the use of the unphysiological substrates.

E_A values of 11.67, 44, 53, and 74 kJ/mol have been reported for the fungal and bacterial β-xylosidases from *Thermomonospora* strain LL (15), *Sclerotium rolfsii* (8), *Caldocellum saccharolyticum* (6), and *Neurospora crassa* (4), respectively. The E_A of 69 kJ/mol found for the xylosidase from *T. ethanolicus* fits into this range and is relatively close to the one found for the arylxylosidase from the thermophile *Caldocellum saccharolyticum*.

At the present time, not enough sequences of bacterial xylosidases or arabinosidases are available to make any conclusions about homologies among these glycosidases. The closest homology was found with the N-terminal amino acid sequence from the purified xylosidase from *Thermotoga* strain Fj SS3-B.1 (11a).

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