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Purification and properties of an aryl β-xylosidase from a cellulolytic extreme thermophile expressed in *Escherichia coli*

Rochelle C. HUDSON, Linley R. SCHOFIELD, Tim COOLBEAR,* Roy M. DANIEL† and Hugh W. MORGAN
Microbial Biochemistry and Biotechnology Research Unit, University of Waikato, Hamilton, New Zealand

An aryl β-xylosidase was purified to homogeneity from an *Escherichia coli* strain containing a recombinant plasmid carrying a β-xylosidase (EC 3.2.1.37) gene from the extremely thermophilic anaerobic bacterium isolate Tp8T6.3.3.1 ('*Caldocellum saccharolyticum*'). It has a pI of 4.3 and shows optimal activity at pH 5.7. The enzyme is highly specific, acting on *o*- and *p*-nitrophenyl β-D-xylopyranosides and minimally on *p*-nitrophenyl α-L-arabinopyranoside. It does not act on xylobiose. The K_m for *p*-nitrophenyl β-D-xylopyranoside at the optimum pH for activity is 10 mM, and at pH 7.0 is 6.7 mM. Xylose is a competitive inhibitor with K_i 40 mM. Thermal inactivation follows first-order kinetics at 65 and 70 °C with $t_{\frac{1}{2}}$ values of 4.85 h and 40 min respectively. The $t_{\frac{1}{2}}$ at 70 °C is increased 3-fold and 4-fold by the addition of 0.5 mg of BSA/ml and 2 mM-dithiothreitol respectively.

INTRODUCTION

Microbial β-xylosidases occur in both bacteria and fungi, with the latter group being the most studied. Relatively little work has been done on β-xylosidases from bacteria, and even less on those from extreme thermophiles. Sung *et al.* (1987) and Bachmann & McCarthy (1989) have investigated the properties of β-xylosidases from the thermophilic bacteria *Bacillus* sp. K-17 and *Thermonospora fusca* respectively. β-Xylosidase is also reported to be present in the thermophile *Clostridium thermocopriae* (Jin & Toda, 1988; Jin *et al.*, 1988).

β-Xylosidase genes from the mesophilic bacilli *Bacillus pumilus* (Panbangred *et al.*, 1984), *Bacillus polymyxa* (Sandhu & Kennedy, 1986) and *Bacillus subtilus* (Bernier *et al.*, 1987) have been cloned into *Escherichia coli*. The genes were expressed but products were not secreted by the host organism.

Strain Tp8T6.3.3.1, provisionally named '*Caldocellum saccharolyticum*', is an extremely thermophilic cellulolytic anaerobic bacterium isolated from a geothermal spring in Taupo, New Zealand (Sissons *et al.*, 1987). A number of genes for cellulose and hemicellulose degradation have been cloned from '*C. saccharolyticum*' into *E. coli* (Bergquist *et al.*, 1987) including a β-xylosidase (Lüthi *et al.*, 1990). The expression of thermostable enzymes such as these in mesophilic hosts facilitates their purification by a heat-treatment process that results in precipitation of labile host proteins and inactivation of mesophilic enzyme activity (Patchett *et al.*, 1989). In the present paper we describe the purification of cloned aryl β-xylosidase by a protocol including such a process, and report on its properties.

MATERIALS AND METHODS

Organism

The bacterium used for gene bank construction, '*Caldocellum saccharolyticum*', strain Tp8T6.3.3.1, has been described elsewhere (Sissons *et al.*, 1987; Donnison *et al.*, 1989). The β-xylosidase gene was isolated from a λ200 genomic library and subcloned into plasmid pBR322. After deletion of DNA by restriction-enzyme digestion and ligation, the thermophilic DNA containing the β-xylosidase was recloned in the vector pCGN566 (Stalker *et al.*, 1988) and transformed into strain JM83 to give strain PB4855 carrying plasmid pNZ1405 (Yaniv-Perron *et al.*, 1985). These constructions were performed by Dr. P. Caughey, Department of Cellular and Molecular Biology, University of Auckland. The recombinant strain was grown on Luria medium [trypticase peptone (5.0 g/l), yeast extract (2.5 g/l), NaCl (5.0 g/l), pH 7.0] containing 100 μg of chloramphenicol/ml. The cell pellet was stored at −70 °C.

Chemicals and details of substrate

Avicel was purchased from the Asahi Chemical Industry Co. (Osaka, Japan). All other substrates and buffering agents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), as were lysozyme (L-6876), DNAase (D-0876) and BSA (A-2153; 3.4% water content). Standards for determination of M_r by molecular exclusion chromatography were obtained from Sigma Chemical Co., and those used in determination of M_r by SDS/PAGE were Rainbow Markers from Amersham (Australia Pty. Ltd.). Triton X-100 was obtained from Serva (Heidelberg, Germany). Coomassie Brilliant Blue G-250 was from Pierce Chemical Co. (Rockford, IL, U.S.A.). All other chemicals were of analytical grade.

Aqueous solutions were made up to volume with deionized reagent-grade water from an R.O. (reverse-osmosis) water-purification system (Millipore). Unless otherwise stated, buffers were adjusted to the appropriate pH at the temperature of use, using a combination electrode calibrated at this temperature. Buffers used in enzyme assays were 0.1 M-Bistris/HCl (pH_{70} 5.73) or 0.1 M-Mops/NaOH (pH_{70} 5.96) unless specified otherwise.

Preparation of a cell-free extract

The thawed cloned *E. coli* cells (771 g wet wt.) were stirred in the presence of 25% (w/w) glycerol and 1% Triton X-100 for 1.25 h at 7 °C. Then 4.5 litres of 50 mM-Mops/NaOH (pH_{20} 6.75), containing 7.5 mM-EDTA, 0.02% NaN_3, 0.02% lysozyme, 77 mg of DNAase and 460 mg of phenylmethanesulphonyl fluoride were added to the cells. Lysis was carried out in a covered plastic container, placed in a water bath set at 35 °C,

Abbreviations used: *p*NPX, *p*-nitrophenyl β-D-xylopyranoside; *o*NPX, *o*-nitrophenyl β-D-xylopyranoside.

* Present address: Dairy Research Institute, Palmerston North, New Zealand.
† To whom correspondence should be addressed.

with stirring. The lysis mixture reached a temperature of 35 °C after 2 h, and was maintained at this temperature for a further 1 h. Cell debris was then removed by centrifugation in an MSE centrifuge at 85000 *g* for 90 min at 4 °C. The supernatant was concentrated by ultrafiltration (Amicon S1YM10 membrane).

Purification of the aryl β-xylosidase

All purification steps were carried out at room temperature (approximately 20 °C), unless otherwise stated. Active material was stored at −70 °C if not taken immediately on to the subsequent purification step. All ultrafiltration was performed using YM10 membranes (Amicon).

Concentrated cell-free extract (2 litres; 16.4 mg of protein/ml) was heat-treated by stirring it in a covered stainless-steel vessel, placed in a thermostatically controlled water bath. The internal temperature reached 70 °C in 1.5 h, and was maintained at 70 °C for a further 1 h. The extract was cooled on ice then left to stand overnight at 7 °C before being centrifuged in a Sorvall centrifuge at 13000 *g* for 30 min.

The supernatant (1.6 litres) was loaded on to a DEAE-Sepharose column (30 cm × 7.5 cm) equilibrated with 20 mM-Tris/Mes buffer pH 8.0. Aryl β-xylosidase activity was eluted with an 8-litre (i.e. 6 bed volumes) linear salt gradient, increasing to 1.0 M-NaCl. Active fractions were concentrated by ultrafiltration, then dialysed against R.O. water (Spectra/Por 3, nominal M_r cut-off 3500000). The non-diffusible material was loaded on to a CM-Sepharose column (25 cm × 5 cm) equilibrated with 20 mM-Mes/Tris buffer, pH 6.5. The aryl β-xylosidase activity did not bind to the matrix and was eluted with this buffer, active fractions being concentrated by ultrafiltration. Of this material, 18% was further purified. The material was treated in 16 ml batches on a TSK Fractogel HW.55 (F) column (41 cm × 5 cm) (Toyo Soda Manufacturing Co., Tokyo, Japan) equilibrated with 50 mM-Mops buffer, pH 6.75. Active fractions were pooled, concentrated by ultrafiltration and equilibrated with the preparative PAGE sample buffer.

Discontinuous preparative PAGE using 3 mm-thick gels (LKB 2001 vertical electrophoresis unit) was by the Laemmli (1970) system with modifications as described elsewhere (Eames, 1985), with the exception, in this case, that SDS was not included. Aryl β-xylosidase activity was located by incubating fragments cut from the gel in *p*-nitrophenyl β-D-xylopyranoside at 70 °C. The active bands were cut from the gels, macerated by being forced through syringes, then soaked for 20 h in 0.1 M-Mops/NaOH buffer, pH 6.75, containing 0.15 M-NaCl. Gel fragments were removed from the elution buffer by filtration through a 3 μm-pore-size filter.

The filtrate, concentrated by ultrafiltration, was loaded on to a Bio-Gel HTP hydroxyapatite column (16 cm × 2.5 cm) equilibrated with 20 mM-K_2HPO_4/KH_2PO_4, pH 6.6. Aryl β-xylosidase activity was eluted with a 300 ml linear gradient of 0.02–0.5 M-K_2HPO_4/KH_2PO_4, pH 6.6. Active fractions were pooled and used for most of the characterization of the aryl β-xylosidase (substrate specificities for cellobiose and xylobiose used TSK Fractogel-purified enzyme).

Activity assessments during purification were by assays with *p*-nitrophenyl β-D-xylopyranoside as substrate (10 mM in 0.1 M-Mops/NaOH buffer, pH_{70} 6.3). The assay procedure was as described for the purified enzyme.

Protein determination

Protein determinations were by the dye-binding method of Bradford (1976). Both the standard protein assay and the micro protein assay were used, with BSA as a standard.

Enzyme assays of the purified aryl β-xylosidase

In all assays, 1 unit of aryl β-xylosidase activity was defined as the amount of enzyme required to produce 1 μmol of nitrophenol or xylose in 1 min. In the standard assay (performed in triplicate), 0.24–0.3 μg of aryl β-xylosidase/ml in 0.5 ml of a buffered (see the text) *o*-nitrophenyl β-D-xylopyranoside (*o*NPX) or *p*-nitrophenyl β-D-xylopyranoside (*p*NPX) solution at a concentration of at least 25 mM (unless otherwise stated) was incubated in a capped 1.5 ml Eppendorf tube at 70 °C for up to 5 min. After the appropriate time, the reaction was stopped by rapid cooling in a cold water bath, and then 0.5 ml of 1 M-Na_2CO_3 was added. The absorbances of *o*- or *p*-nitrophenoxide ions were measured at 420 and 400 nm respectively. Molar absorption coefficients of 4200 $M^{-1} \cdot cm^{-1}$ and 20500 $M^{-1} \cdot cm^{-1}$ were determined for *o*- and *p*-nitrophenol respectively, under stopped assay conditions. *p*NPX was used for most assays.

Assays with other (potentially poor) substrates used a lower incubation temperature in order to minimize thermal denaturation of the enzyme, thus allowing a longer incubation period. The sensitivity of such assays was increased by using 0.6–1.2 μg of aryl β-xylosidase/ml.

Assays with other nitrophenyl-linked substrates used a substrate concentration of 10 mM and an incubation period of 3 h at 65 °C, but were otherwise as described above. For assays using polymeric substrates aryl β-xylosidase was incubated at 65 °C with 0.2–0.4 μl of buffered substrate for 80–180 min as specified in the text. Reducing sugars liberated from oatspelts xylan, CM-cellulose, Avicel and β-glucan (all 0.25%, w/v) were determined by the *p*-hydroxybenzoic acid hydrazide method of Lever (1973). The effects of this enzyme treatment on larchwood and oatspelts xylan (1%, w/v) were analysed by h.p.l.c. on an Aminex HPX-42A column (30 cm × 7.8 mm) (Bio-Rad Laboratories, Richmond, CA, U.S.A.). A 0.1 ml portion of 20 mM-cellobiose, 18 mM-xylobiose or 20 mM-*o*NPX, all in 50 mM-Mes/NaOH, pH_{70} 5.7, was incubated with 0.1 ml of aryl β-xylosidase for 40 min at 70 °C, and samples were analysed for the presence of saccharides by h.p.l.c. using the Aminex HPX-87H column. Standards were xylose, xylobiose, glucose and cellobiose.

Synergism of aryl β-xylosidase with xylanase

E. coli 600 strain JM83 (xylanase$^+$/xylosidase$^-$) contained a xylanase gene from '*C. saccharolyticum*'. A portion (0.71 g wet wt.) of these cells was mixed with 3 vol. of 0.1 M-Mops buffer, pH 6.4, and sonicated [Dynatech sonic dismembrator, ½ in (13 mm) titanium tip] on ice at 20% relative output for 2 × 3 min. The sonicate was centrifuged at 6500 *g* in a Runne–Heidelberg model RS 85-1 bench centrifuge for 15 min and the supernatant, diluted 100-fold, was used in the assays.

Aryl β-xylosidase (0.3 μg/ml) and xylanase (10 μl) were incubated with 0.4 ml of 0.25% (w/v) oatspelts xylan (suspended in 0.1 M-Mops buffer, pH_{65} 6.0) for 10 min at 65 °C. Reducing sugars liberated were determined by the method of Lever (1973), with xylose as a standard.

Temperature effects and thermostabilities

Phosphate buffers (K_2HPO_4/KH_2PO_4) were used in these experiments as they have a very low ΔpH/°C (−0.003) (Dawson *et al.*, 1986). K_2HPO_4/KH_2PO_4 buffer, pH_{20} 6.1, at a concentration of 0.1 M was used for determination of Arrhenius plots.

For the thermostability experiments, 50–200 μl portions of purified aryl β-xylosidase solution were heated in capped 1.5 ml Eppendorf tubes at the appropriate temperature. The concentration of enzyme was 0.01 or 0.005 μg/ml in 0.5 M- or 0.25 M- (respectively) phosphate buffer, pH_{20} 6.6. After being heated for

the appropriate time interval, samples were cooled in ice/water then stored at 4 °C for up to 3 h before assay.

Determination of M_r

Discontinuous SDS/PAGE was by the Laemmli (1970) system with the modifications described elsewhere (Eames, 1985).

Determination of pI

Narrow-range isoelectric focusing, pH 4.0–6.5, was performed at 4 °C on a 20 cm × 11.5 cm × 0.2 mm thick layer of 6% (w/v) Sephadex and 5% (v/v) Ampholines (Pharmacia). The pH gradient was determined by extracting 5 mm sections in reverse-osmosis-purified water for 24 h, centrifuging out the Sephadex particles, then measuring the pH of the resulting solutions at 4 °C. Enzyme activity was located by incubating sections of the Sephadex slurry in buffered *o*NPX solutions.

RESULTS AND DISCUSSION

Enzyme assays

Assay with *o*NPX and *p*NPX were linear for at least 2 min with substrate concentrations of 1–20 mM, and for at least 5 min with substrate concentrations above this. The 5 min assays with 30 mM-*p*NPX as substrate were linear for aryl β-xylosidase concentrations of up to 0.7 μg/ml (which was greater than the highest enzyme concentration used).

Enzyme purification

Microscopic examination indicated that only a small percentage of cells remained intact after completion of the lysozyme treatment.

The aryl β-xylosidase was purified 553-fold in 4.3% yield (Table 1). Heat treatment was a particularly effective step, resulting in an 8-fold purification and 86% yield. The low overall yield was mainly due to the large activity losses that occurred during chromatography on TSK Fractogel HW.55 (F). Coomassie Blue staining of a 12.8 cm × 1.6 cm × 0.15 cm SDS/PAGE gel loaded with 19.2 μg of purified aryl β-xylosidase revealed a single protein band.

The specific activity is 6.9-fold higher for *o*NPX than for *p*NPX. This enzyme has a specific activity higher than that of other bacterial β-xylosidases (see Table 2).

Activity was not lost during storage at −70 °C over a 2-month period. Samples lost approx. 9% activity per h at room temperature in 0.5 M-phosphate buffer (K_2HPO_4/KH_2PO_4), pH 6.6.

Table 1. Purification of aryl β-xylosidase

*p*NPX was the substrate for activity determinations. For experimental details see the text.

Purification stage	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Cell-free extract	3000	33400	0.089	1.0	100
Heat treatment	2500	3400	0.72	8.1	86
DEAE-Sepharose	1700	1700	1.0	12	57
CM-Sepharose	2000	1100	1.8	20	66
TSK Fractogel	360	130	2.9	33	12
Preparative PAGE	140	7.6	19	210	4.8
Hydroxyapatite	130	2.6	49	550	4.3

Physical properties

The pI, determined by isoelectric focusing, was 4.3 ± 0.1. This is similar to the values of 4.4 and 4.7 found for β-xylosidases from *Bacillus pumilus* 12 (Kersters-Hilderson *et al.*, 1969) and *Bacillus circulans* WL-12 (Esteban *et al.*, 1982) respectively.

SDS/PAGE gave an M_r of 53000, which compared well with that determined from the sequence value of 56000 (Lüthi *et al.*, 1990).

pH optimum for activity and stability

The aryl β-xylosidase lost 50% of its initial activity over a 2 h period in 50 mM-Bistris buffer, pH_{20} 5.3. Its stability was higher when buffered at higher pH (see Fig. 1).

The pH optimum for activity was 5.7 at 70 °C. The enzyme was more active in Mops buffer than in Mes buffer. As these buffers were used at the extremes of their buffering capacity, the pH was checked upon completion of the assays, and was found not to have changed.

The pH optima of other bacterial β-xylosidases tend to be nearer neutrality, whereas fungal enzymes are usually more

Table 2. Properties of some bacterial β-xylosidases in relation to substrates and inhibitors

Enzyme source	K_m for *p*NPX (mM)	Specific activity on *p*NPX (μmol/min per mg)	K_i (mM) for inhibition by xylose	Reference
Bacillus pumilus 12	1.43	5.54	26.2	Kersters-Hilderson *et al.* (1969)
*Bacillus pumilus**	2.4	3.4	–	Panbangred *et al.* (1984)
(two enzymes)	84	0.032	–	
Bacillus sp. K-17	0.45	21.9	–	Sung *et al.* (1987)
Cellulomonas uda†	–	–	650	Rapp & Wagner (1986)
Clostridium acetobutylicum A.T.C.C. 824	3.7	16.1	–	Lee & Forsberg (1987)
Thermomonospora strain LL†	–	–	35–100	Ristroph & Humphrey (1985)
Thermomonospora fusca	0.89	8.00	19.1	Bachmann & McCarthy (1989)
'*Caldocellum saccharolyticum*'*	10.0	49.2	40	Present study

* Expressed in *Escherichia coli*.
† These studies were on crude enzymes.

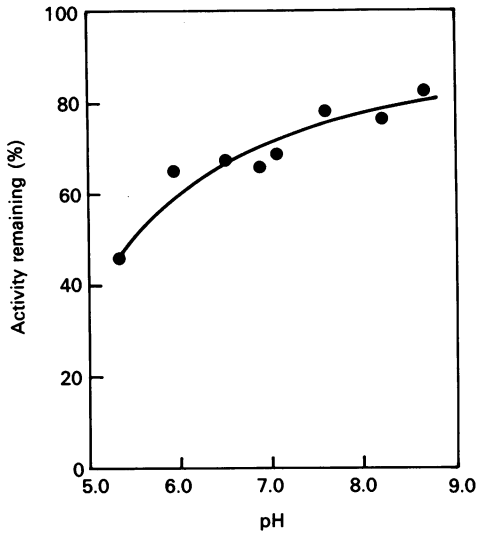

Fig. 1. pH–stability profile of the aryl β-xylosidase

The aryl β-xylosidase was kept at room temperature in 50 mM-Bistris buffer solutions, adjusted to various pH values, for a period of 2 h. Samples were then withdrawn and assayed with 30 mM-*p*NPX in 0.1 M-Bistris buffer, pH_{70} 5.73.

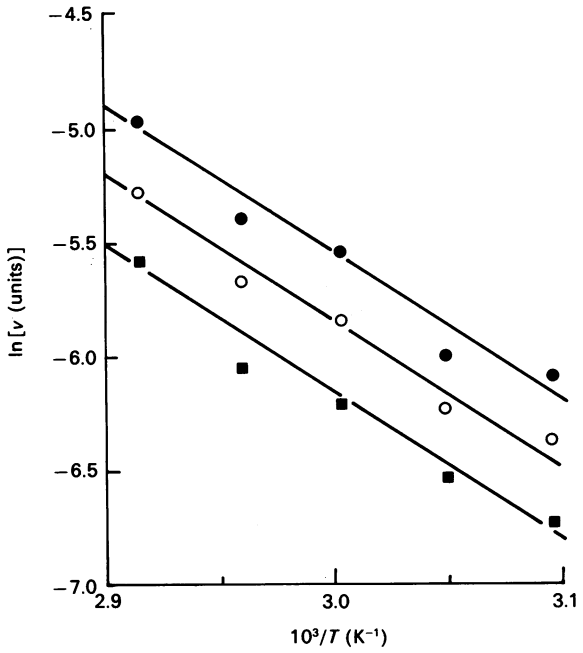

Fig. 2. Arrhenius plots for the aryl β-xylosidase

Symbols: ○, 0.1 M-xylose; ■, 0.25 M-xylose; ●, xylose not present.

acidic (< pH 5) (Lachke *et al.*, 1985; Matsuo & Yasui, 1984*a*,*b*; Matsuo *et al.*, 1987; Poutanen & Puls, 1988).

Aryl β-xylosidase thermostability

The half-lives of the enzyme at 65, 70 and 80 °C were 4.85 h, 40 min and 3 min respectively. Activity loss was first-order at 65 °C for at least 7 h and at 70 °C for at least 80 min, but at 80 °C was neither first-order nor second-order. This thermostability is not very high given the growth temperature of the source organism, but is comparable with that of the xylanase cloned from '*C. saccharolyticum*' into *E. coli*. However, it is less stable than the β-glucosidase and CM-cellulase from this organism (Patchett *et al.*, 1989; Neal, 1987). Patchett *et al.* (1989) found that the $t_{\frac{1}{2}}$ (80 °C) for '*C. saccharolyticum*' β-xylosidase expressed in *E. coli* 600 strain PB2477 was 10 min, very similar to the $t_{\frac{1}{2}}$ (80 °C) of the enzyme when expressed in the native organism (12 min). These values, for crude enzymes, are not incompatible with the $t_{\frac{1}{2}}$ (80 °C) of 3 min found for the purified aryl β-xylosidase in this study. There is no evidence that the enzyme has lost thermostability upon cloning.

The thermostability of the aryl β-xylosidase is similar to those of *Thermomonospora* strain LL β-xylosidase (Ristroph & Humphrey, 1985), which has a $t_{\frac{1}{2}}$ of 4–5 h at 65 °C, and of *Thermomonospora fusca* xylosidase, which has $t_{\frac{1}{2}}$ values of 8 h and 1.5 h at 65 °C and 70 °C respectively (Bachmann & McCarthy, 1989). It is considerably more stable than the β-xylosidase from the thermophilic alkalophilic *Bacillus* sp. K-17, which is completely inactivated in 10 min at 60 °C (Sung *et al.*, 1987).

The agents most effective in increasing aryl β-xylosidase thermostability were BSA (0.5 mg/ml) and dithiothreitol (2 mM), which increased the $t_{\frac{1}{2}}$ (70 °C) from 40 min to 120 and 160 min respectively. 2-Mercaptoethanol had little effect, possibly owing to its oxidation to form the inactive disulphide (Scopes, 1982). Exposure to 1% (w/v) Triton X-100 significantly increased enzyme activity, but not thermostability. The competitive inhibitor xylose (see below), at 0.1 M, had no influence on thermostability. Glycerol (10%, w/v), which markedly increased the thermostability of another thermophilic glycosidic enzyme, Wai21W.2 β-glucosidase (Patchett *et al.*, 1987), also had no effect.

It is clear that the enzyme is stabilized in crude extracts, since the heat treatment of 1 h at 70 °C results in less than a 15% loss of activity. The stability of the pure enzyme in dilute buffer, although interesting from a biochemical point of view, is not necessarily a good guide to the lifetime of the enzyme *in vivo*.

Effect of temperature on activity

An Arrhenius plot is shown in Fig. 2. From this an E_a (activation energy) value of 53 kJ/mol can be calculated. The presence of the competitive inhibitor xylose (see below) did not alter the slope of the Arrhenius plot (Fig. 2).

An E_a of 11.67 kJ/mol has been reported for a β-xylosidase from another thermophilic bacterium, *Thermomonospora* strain LL (Ristroph & Humphrey, 1985). β-Xylosidase from the fungi *Neurospora crassa* (Deshpande *et al.*, 1986) and *Sclerotium rolfsii* (Lachke *et al.*, 1985) were found to have E_a values of 74 and 44 kJ/mol respectively.

Effect of substrate concentration

The K_m values for both *p*NPX (Fig. 3) and *o*NPX are 10 mM. $V_{max.}$ values at the pH optimum of 5.7 are 64 units/mg and 505 units/mg for *p*NPX and *o*NPX respectively. Increasing the pH to 7.0 lowered the K_m for *p*NPX to 6.7 mM (Fig. 3). Compared with β-xylosidase from other bacterial sources, this enzyme has a relatively high K_m (Table 2).

Given the high K_m for the substrates, the concentrations used (usually 30 mM; limited by solubility) will have resulted in slightly less than maximum catalytic rates.

Inhibitors and activators

Xylose inhibited competitively (K_i 40 ± 2 mM; Fig. 4). Such inhibition is common for β-xylosidases from both bacterial and fungal sources. K_i values range from 2.3 mM for the enzyme from *Trichoderma reesei* (Poutanen & Puls, 1988) to 650 mM for that from *Cellulomonas uda* (Rapp & Wagner, 1986).

Aryl β-xylosidase was also inhibited by other monosaccharides,

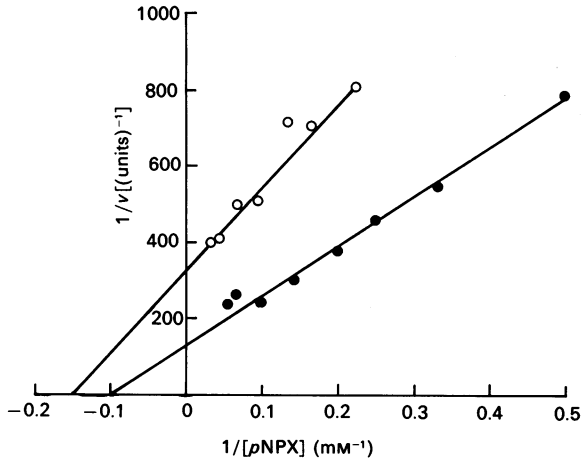

Fig. 3. Effect of pH on the K_m and $V_{max.}$ of the β-xylosidase for *p*NPX

Each assay contained 0.12 μg of aryl β-xylosidase. Symbols: ●, pH_{70} 5.73; ○, pH_{70} 7.02.

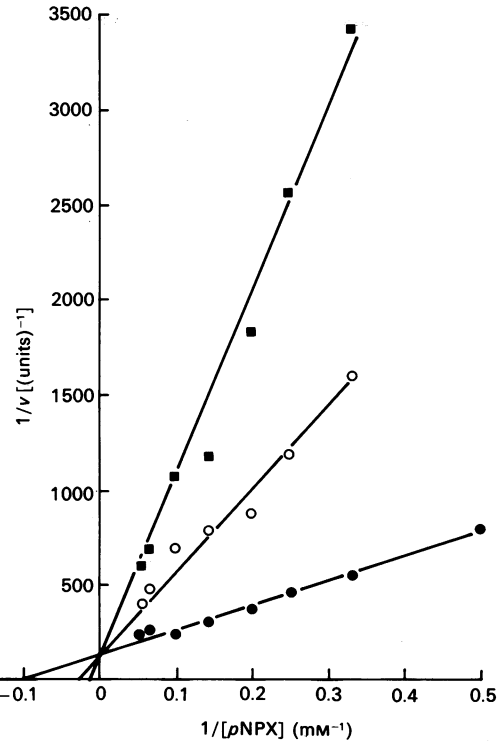

Fig. 4. Lineweaver–Burk plot for the aryl β-xylosidase with *p*NPX as substrate, and showing competitive inhibition by xylose

Each assay contained 0.12 μg of enzyme. Symbols: ○, 0.1 M-xylose; ■, 0.25 M-xylose; ●, xylose not present.

metal ions and other reagents (Table 3). Strong inhibition by *p*-chloromercuribenzoate and heavy-metal ions is a common feature of β-xylosidases from fungi (Matsuo & Yasui, 1984*b*; Kitpreechavanich *et al.*, 1986) and from bacteria (Kersters-Hilderson *et al.*, 1969; Rapp & Wagner, 1986; Sung *et al.*, 1987). Evidence for the involvement of an active-site thiol group has been found in at least one case (Kersters-Hilderson *et al.*, 1984). Inhibition by *N*-bromosuccinimide is also common, suggesting the presence of essential tryptophan residues (Lachke *et al.*, 1985).

Table 3. Effects of ions and other modifiers on aryl β-xylosidase activity

Agents that had no effect (⩽ 10% difference from the control) were: $FeCl_3$ and NaI (1 mM); dithiothreitol (1.5 mM); $CaCl_2$, LiCl and $MgCl_2$ (3 mM); NaCN, NaN_3, $NaNO_3$, NaBr, NaF, EDTA and 2-mercaptoethanol (5 mM); NaCl (9 mM); glucose and xylitol (50 mM).

Modifier	Concentration	Activity (%)
$AgNO_3$	1 mM	0
$HgCl_2$	1 mM	1
$CdCl_2$	1 mM	5
$CuCl_2$	1 mM	19
$MnCl_2$	1 mM	34
$ZnCl_2$	1 mM	43
VCl_3	1 mM	54
$CoCl_2$	1 mM	73
KCl	1 mM	87
$NiCl_2$	1 mM	88
Na_2SO_4	5 mM	113
Arabinose	50 mM	85
Galactose	50 mM	84
Xylose	50 mM	78
Xylose	250 mM	54
N-Bromosuccinimide	2 mM	45
p-Chloromercuribenzoate	0.05 mM	57
Glycerol	50 mM	84
Glycerol	1.1 M	72
Triton X-100	1% (w/v)*	120
SDS	1% (w/v)	18

* Above the critical micelle concentration.

Substrate-specificity

The aryl β-xylosidase is a highly specific enzyme. Besides *p*NPX and *o*NPX, only *p*-nitrophenyl α-L-arabinopyranoside was hydrolysed from a range of nitrophenyl-linked sugars tested, using a 3 h incubation period (those giving negative results were *p*-nitrophenyl α-L-arabinofuranoside, *p*-nitrophenyl β-D-fucopyranoside, *p*-nitrophenyl β-D-galacturonide, *p*-nitrophenyl β-D-galactopyranoside, *p*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl β-D-lactopyranoside, *p*-nitrophenyl β-D-maltoside and *p*-nitrophenyl β-D-mannopyranoside). *p*-Nitrophenyl α-L-arabinopyranoside hydrolysis was 5% of *p*NPX hydrolysis over the 3 h incubation period.

p-Nitrophenyl α-L-arabinopyranosidase activity has been reported for a *Penicillium wortmanni* β-xylosidase (Deleyn *et al.*, 1982), and *p*-nitrophenyl α-L-arabinofuranosidase activity has been reported for a *Trichoderma reesei* β-xylosidase (Poutanen & Puls, 1988).

A 2 μmol portion of *o*NPX was fully hydrolysed within 40 min to give 1.6 μmol of xylose and 0.2 μmol of xylobiose; the production of xylobiose indicates transferase activity.

The enzyme did not release reducing sugars from Avicel, CM-cellulose or β-glucan within a 3 h incubation period. Xylobiose and cellobiose were not hydrolysed within a 40 min period, although the (h.p.l.c.) assay system used would not have detected hydrolysis products representing less than 0.25% of the initial amount of these substrates.

In the absence of xylanase, xylan hydrolysis was not detectable by h.p.l.c., although the detection limits of the assay system used were such that hydrolysis products representing less than 5% of the initial amount of this substrate would not have been detected. However, assaying for the production of reducing sugars from oatspelts xylan by the *p*-hydroxybenzoic acid hydrazide method of Lever (1973) gave an activity corresponding to 2% of that

found against *p*NPX. Most other β-xylosidases have little or no activity against xylan (Deleyn *et al.*, 1982; Lee & Forsberg, 1987; Nakanishi *et al.*, 1987; Lachke, 1988).

Although the aryl β-xylosidase does not hydrolyse xylobiose, and has only very low activity against xylan, when added to a crude xylanase preparation, the amount of reducing sugars liberated from xylan was 30% more than that produced by the action of the xylanase alone. It may be that the β-xylosidase is degrading xylose oligomers (trimers or larger) to small fragments.

The above results are not inconsistent with the role of the enzyme as a β-xylosidase. According to Reilly (1981), β-xylosidases break down short xylo-oligosaccharides to xylose and have substantial transferase activity. However, since our enzyme does not degrade xylobiose, which is defined as a natural substrate of β-xylosidase (EC 3.2.1.37), we refer to it as an aryl β-xylosidase.

Exo-xylanases produce xylose at high rates from xylan, but only at low rates from short oligosaccharides, and have little or no transferase activity (Reilly, 1981). Since the enzyme of this study had only very low activity on xylan, and did display transferase activity, it is unlikely to be an exo-xylanase.

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