Purification and Characterization of a Thermostable β -Xylosidase from *Thermomonospora fusca*

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Xylan-degrading enzymes, including β -xylosidase (EC 3.2.1.37), were induced when *Thermomonospora fusca* was grown at 50 °C in liquid medium containing 0.2% xylan. The intracellular β -xylosidase activity was concentrated and characterized by fast protein liquid chromatography and gel electrophoresis. A zymogram technique was developed to identify β -xylosidase directly on polyacrylamide gels. A single enzyme (168 kDa; pI 4.37) was identified and purified to homogeneity. The consistent detection of a single band on denaturing SDS gels suggested that the enzyme was composed of identical subunits; since the subunit molecular mass was 56 kDa, a trimeric structure is suggested. High activity against *p*-nitrophenyl β -D-xylopyranoside (pNPX) occurred in the pH range 5.0–9.0 and temperature range 40–60 °C. The enzyme was stable at room temperature at pH 6.0–8.0; it had a half-life of 8 h at 65 °C, and of 1.5 h at 70 °C. The purified enzyme did not exhibit any detectable activity against arabinoxylan, carboxymethylcellulose or *p*-nitrophenyl β -D-glucopyranoside. The enzyme had a K_m of 0.89 mM (pNPX) and was inhibited by D-xylose (K_i 19 mM) but not D-glucose. The size of the *T. fusca* enzyme is in the range reported for the few other bacterial β -xylosidases described, but the acidic nature of the protein and its affinity for the substrate have more in common with some of the monomeric β -xylosidases described in fungi.

INTRODUCTION

Xylan is the second most abundant renewable polysaccharide in nature and comprises a β -1,4-linked D-xylose polymer with arabinofuranose, glucuronic acid and methylglucuronic acid side groups (Whistler & Richards, 1970). In addition, arabinoxylan from hardwood species is often acetylated (Chesson *et al.*, 1983; Timel, 1967). Consequently, complete hydrolysis requires the action of several different enzymes, possibly analogous to the synergistic enzyme action involved in crystalline cellulose degradation (Shewale, 1982). Endoxylanases randomly cleave the β -1,4 bonds in the polyxylose backbone to yield oligosaccharides of varying chain length. This activity has been described in a wide range of micro-organisms, including actinomycetes (Dekker & Richards, 1976; McCarthy, 1987). More recently, the involvement of arabinofuranosidase, α -glucuronidase, β -xylosidase and various esterases has been studied (Biely, 1985; MacKenzie *et al.*, 1987; Puls *et al.*, 1987) and various enzyme combinations have been shown to act synergistically (Biely *et al.*, 1986; Deshpande *et al.*, 1986). β -Xylosidase (EC 3.2.1.37; 1,4- β -D-xylan xylohydrolase) activity generates D-xylose from short-chain oligosaccharides and xylobiose and plays a crucial role in the relief of end-product inhibition of endoxylanase activity by xylobiose (Reilly, 1981).

In general, the xylan-degrading enzymes of fungi have received more attention than those of bacteria, and a number of fungal extracellular β -xylosidases have been characterized (John *et*

Abbreviations: pNP, *p*-nitrophenol; pNPX, *p*-nitrophenyl β -D-xylopyranoside.

al., 1979; Matsuo & Yasui, 1984; Takenishi *et al.*, 1973; Uziie *et al.*, 1985). The first bacterial β -xylosidase isolated was from *Bacillus pumilus* (Kerstens-Hilderson *et al.*, 1969) and more recently the properties of a β -xylosidase from *Clostridium acetobutylicum* have been reported (Lee & Forsberg, 1987). The β -xylosidase activity of a thermophilic *Thermomonospora* strain has been described as notable in that it exhibits broad pH and temperature optima (Ristroph & Humphrey, 1985), although no attempt was made to isolate or purify the enzyme from crude cell extracts. In fact, *Thermomonospora* strains have attracted considerable attention as sources of highly active and thermostable enzymes for lignocellulose saccharification in general (McCarthy, 1987). Some progress has been made towards biochemical characterization of the cellulose-degrading system in *Thermomonospora fusca* (Calza *et al.*, 1985) and we are adopting a similar approach to identification of the enzymes involved in xylan degradation. In this paper, purification and characterization of the thermostable intracellular β -xylosidase of *T. fusca* is described.

METHODS

Organism. Thirty strains of *Thermomonospora fusca* and *T. curvata* were screened for the production of extracellular xylanase and intracellular β -xylosidase activities when grown on xylan. *T. fusca* strain BD21 was selected as the most active producer of xylan-degrading enzymes under these conditions.

Culture conditions. The strain was grown in liquid medium containing 2.0 g $(\text{NH}_4)_2\text{SO}_4$, 0.2 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 11 mg CaCl_2 , 1.0 g yeast extract (Lab M), 2.0 g oat spelt xylan (Sigma) and 100 ml 1.0 M-potassium phosphate buffer (pH 8.0) in 900 ml distilled water. Cultures were grown for 72 h at 50 °C with shaking at 200 r.p.m. and harvested by centrifugation (10 min at 10000 g).

Enzyme assays. β -Xylosidase activity was measured by assaying the amount of *p*-nitrophenol (pNP) liberated from the substrate *p*-nitrophenyl β -D-xylopyranoside (pNPX, Sigma). The standard reaction mixture contained 650 μl 10 mM-pNPX dissolved in 50 mM-potassium phosphate buffer (pH 6.5) and 100 μl (40–50 μg protein) of diluted intracellular enzyme preparation. After incubation at 55 °C for 30 min, the reaction was stopped by the addition of 1.5 ml 1 M- Na_2CO_3 and the absorbance at 400 nm measured. This assay procedure was scaled down to a total volume of 200 μl , and addition of Na_2CO_3 omitted, for qualitative identification of active fractions in microtitre plates. One unit (U) of enzyme activity is defined as the amount of enzyme which produced 1 μmol pNP min^{-1} under the above conditions, and specific activity is expressed in U (mg protein) $^{-1}$. Arabinofuranosidase and β -glucosidase activities were measured by following the liberation of pNP from their respective pyranosides (Sigma) at pH 6.0 and 50 °C.

Xylanase activity was determined by measuring the release of reducing sugar from oat spelt xylan as described by McCarthy *et al.* (1985). Endoglucanase activity was measured by replacing xylan with 20 mg carboxymethyl-cellulose (low viscosity; BDH) in the xylanase assay.

Protein was measured by the Lowry method using bovine serum albumin (Sigma) as standard.

Purification of β -xylosidase. Harvested *T. fusca* cells were washed three times and resuspended in 5 ml 100 mM-Tris/HCl, pH 7.0. Intracellular β -xylosidase activity was released by sonication for 10 min at 18 μm peak-to-peak on ice. The crude lysate was cleared by centrifugation (10 min at 10000 g) and 0.03% (w/v) sodium azide was added prior to storage at –20 °C. Portions of this crude enzyme preparation were stored for up to 6 weeks, during which no significant loss in activity was detected.

The enzyme activity was concentrated and purified using fast protein liquid chromatography (FPLC). The first step involved anion-exchange chromatography using a Mono Q HR 5/5 column (Pharmacia) pre-equilibrated with 50 mM-Tris/HCl, pH 7.0 (buffer A). Crude lysate (1.5 ml; 2.5–3.0 mg protein) was loaded onto the column and eluted with 1 M-NaCl in buffer A (buffer B) at a flow rate of 1.0 ml min^{-1} with the following gradient: 0% B in 4 ml; 0–35% B in 20 ml; 35–100% B in 4 ml. Fractions of the eluate were screened for β -xylosidase activity and the most active fractions pooled and immediately applied to a Superose 12 HR 10/30 column (Pharmacia) equilibrated with buffer A and washed with 30 ml buffer A at a flow rate of 0.5 ml min^{-1} . Protein was detected at 280 nm and fractions containing β -xylosidase activity were pooled and stored at –20 °C.

Electrophoresis. Discontinuous SDS-PAGE and non-denaturing PAGE (Laemmli, 1970) were used to ascertain the degree of protein purity and confirm molecular masses derived from column chromatography data. Where appropriate, samples of protein were boiled for 5 min prior to electrophoresis on 10% (w/v) polyacrylamide resolving gels at 40 V for 16 h using a vertical gel electrophoresis unit (BRL). After electrophoresis, protein bands were visualized by silver staining (Merrill *et al.*, 1981) using a commercially available kit (Bio-Rad). Molecular mass markers (SDS 6H and SDS 7; Sigma) were used in all gels. Polyacrylamide gels were also developed as zymograms to detect the presence of enzyme activity after electrophoresis. Prior to electrophoresis, samples were incubated at 50 °C for 1 h in sample buffer, with or without SDS as appropriate. Immediately after electrophoresis, denaturing gels were soaked in 3 $\times$ 400 ml volumes of 40 mM-Tris/HCl, pH 6.5 (non-denaturing

gels were washed in only one volume of buffer), and sandwiched to a 1% (w/v) agarose gel containing 10 mM-pNPX, supported on gel bond (ICN Biomedicals). The gels were sealed in cling film and incubated at 50 °C for 1 h. Enzyme activity was detected as a yellow band resulting from the release of pNP by β -xylosidase.

Chromatofocusing. The isoelectric point (pI) of the β -xylosidase was determined by focusing enzyme activity in the narrow pH range 4.0–7.0. Samples of crude lysate (500 μ l; 0.8–1.0 mg protein) were loaded onto a Mono P HR 5/20 column (Pharmacia) pre-equilibrated with 25 mM-Bis Tris, pH 7.0, and proteins were eluted with 40 ml Polybuffer TM 65 (Pharmacia) pH 4.0, at a flow rate of 0.5 ml min⁻¹. Fractions (1.0 ml) were collected and assayed for β -xylosidase activity. Isoelectric focusing was also performed using pre-cast polyacrylamide gels, pH 3.5–9.5 (LKB). They were developed at 880 V for 3 h using Shandon gel apparatus and broad-range pI markers (Pharmacia). These gels were developed as zymograms using the method described above.

pH and temperature relationships. Portions of crude lysate or purified protein (100 μ l) were added to 650 μ l 10 mM-pNPX solution in Britton and Robinson Universal buffer (Johnson & Lindsey, 1939) at pH values from 3.0 to 11.0. These were then assayed for β -xylosidase activity as described above. Enzyme stability at pH 6.0 and 8.0 was determined by incubating enzyme preparations at room temperature and assaying samples for β -xylosidase activity at intervals up to 72 h.

The optimum temperature for assaying the β -xylosidase activity was determined by incubating assays in the range 30–80 °C in a water bath for 30 min. Thermostability was assessed by incubating enzyme preparations at 50, 65 and 70 °C and removing samples at hourly intervals. β -Xylosidase activity was determined after samples had been immediately cooled on ice.

The Michaelis–Menten kinetics of the crude and purified enzyme preparations were measured according to the method described by Ristroph & Humphrey (1985). Samples containing 0.02 mg protein ml⁻¹ were used in all assays and the substrate concentration was varied from 0.045 to 10.0 mM-pNPX. Inhibition kinetics were determined by the addition of 20, 50 and 90 mM-D-xylose or D-glucose to the reaction mixture.

RESULTS AND DISCUSSION

Enzyme purification

The β -xylosidase activity was concentrated and purified using two sequential steps: anion exchange and gel filtration chromatography. The final purification was approximately 14-fold and the mean overall yield was 95% (Table 1). In both separations, the β -xylosidase activity eluted as a single peak (Fig. 1), although the anion-exchange step resulted in only a partial purification (Fig. 2, lanes 3 and 4). The molecular mass of the enzyme was estimated at 168 kDa ($\pm$ 10 kDa) using gel filtration chromatography with aldolase and glucose oxidase as molecular mass standards. The purified protein was detected as a single band on native PAGE (Fig. 2, lane 6), and the crude estimate of molecular mass obtained by this method was in agreement with the estimate obtained by gel filtration chromatography.

Denaturing PAGE further confirmed the homogeneity of the purified protein, giving a single band of approximately 56 kDa ($\pm$ 6 kDa), (Fig. 2, lane 5). This suggests that the native protein is composed of three subunits.

Native and SDS gels were also developed as zymograms. Native electrophoresis of the crude lysate (Fig. 2, lane 7) confirmed the presence of a single active intracellular β -xylosidase in *T. fusca*. This protein band was identical to the purified enzyme (Fig. 2, lane 8). Activity was only detected in native PAGE gels; no activity was found on SDS-PAGE gels developed as zymograms, indicating that the subunits were inactive.

The estimated molecular mass of the *T. fusca* β -xylosidase (168 kDa) is well within the range reported for other bacterial β -xylosidases (Claeysens *et al.*, 1975; Lee & Forsberg, 1987; Panbangred *et al.*, 1984). Furthermore, the β -xylosidase of *Clostridium acetobutylicum* is also described as having a trimeric structure, although heterogeneous, comprising one 63 kDa and two 85 kDa subunits (Lee & Forsberg, 1987). In contrast, the β -xylosidases of fungi described to date are monomeric proteins of lower molecular masses, in the range 83–102 kDa (Deleyn *et al.*, 1978; Deshpande *et al.*, 1986; Matsuo & Yasui, 1984).

Isoelectric point

The pI of the β -xylosidase was estimated by chromatofocusing as 4.37, indicating that the enzyme is an acidic protein. This was to some extent confirmed by the presence of a single band of activity at pH 4.5 (data not shown) on an isoelectric focusing gel (developed as a zymogram)

Fig. 1. Purification of *T. fusca* β -xylosidase by FPLC. (a) Elution profile after anion-exchange chromatography on Mono Q HR5/5. (b) Elution profile after the pooled β -xylosidase activity from the Mono Q column was separated by gel filtration on Superose 12. —, A_{280} ; ---, NaCl gradient (see Methods for actual concentrations); ●····●, β -xylosidase activity.

Table 1. Purification of intracellular β -xylosidase

Step	Total protein (mg)	Total activity (U)	Specific activity [U (mg protein) ⁻¹]	Yield (%)
Cleared lysate	3.03	1.69	0.56	100
Mono Q eluate	0.41	1.15	2.81	68*
Superose 12 eluate	0.20	1.60	8.00	95

* This apparent reduction in yield is probably due to the inhibitory effect of NaCl on enzyme activity. We have shown that *T. fusca* β -xylosidase activity is sensitive to NaCl and expected an approximately 25% loss in yield (data not shown).

Fig. 2. PAGE of *T. fusca* β -xylosidase purified by FPLC. Active fractions were analysed by SDS-PAGE and proteins were detected by silver staining: lanes 3 and 4, partially purified protein after anion-exchange chromatography; lane 5, protein after further purification by gel filtration chromatography. The purified enzyme was also visualized by native PAGE and silver staining (lane 6). Lanes 7 and 8, native PAGE of the crude lysate (lane 7) and purified protein (lane 8) with gels developed as zymograms by overlaying with pNPX. Lanes 3, 4 and 6 were loaded with 25 μ g protein, lane 5 with 50 μ g protein and lane 7 with 75 μ g protein.

loaded with crude enzyme preparation and standards of known pI (Pharmacia). The β -xylosidase of *T. fusca* is therefore more acidic than the other bacterial β -xylosidases described (Kerstens-Hilderson *et al.*, 1969; Lee & Forsberg, 1987), but is similar in pI to those produced by fungi (Deshpande *et al.*, 1986; Matsuo & Yasui, 1984; Takenishi *et al.*, 1973).

pH and temperature relationships

Under the assay conditions described, the effect of pH and temperature were identical for the crude and purified enzyme preparations. In the range pH 5.0–9.0, β -xylosidase exhibited 90–100% of its maximum activity, with approximately 30% detected outside this range. In a separate experiment, the β -xylosidase activity was found to be stable at room temperature at pH 6.0 and pH 8.0, no significant loss of activity being detected after 72 h. For the 30 min assay, maximum yields of product (pNP) were recorded with incubation at 40–60 °C. As the assay temperature was increased above 60 °C, enzyme activity decreased: at 70 °C activity was approximately 50% of the maximum. These effects of assay temperature are reflected in the thermostability data for the purified β -xylosidase (Fig. 3). The enzyme lost no activity when incubated at 50 °C for 8 h prior to assay, but exhibited a half-life of 8 h at 65 °C and of 1.5 h at 70 °C. The *T. fusca* β -xylosidase appears therefore to be more thermostable than the β -xylosidases of fungi and other bacteria studied to date, and exhibits activity across a relatively broad pH range.

Kinetics of β -xylosidase activity

The affinity of the *T. fusca* β -xylosidase for the substrate pNPX was examined using a Lineweaver–Burk plot. The apparent K_m for the crude preparation was 0.97 mM (standard deviation 0.01; $n = 3$) and for the purified enzyme, 0.89 mM (standard deviation 0.01; $n = 3$).

Fig. 3. Thermostability profiles of purified *T. fusca* β -xylosidase. Purified enzyme was held at 50 °C (●), 65 °C (■) and 70 °C (○). All the results are the means of three replicates; standard deviations were within $\pm 5\%$ of the mean.

These values are in accord with the apparent K_m published for the crude β -xylosidase activity of a different *Thermomonospora* strain (Ristroph & Humphrey, 1985). The *Neurospora crassa* and *Aspergillus* β -xylosidases show a greater affinity for this substrate than the *T. fusca* enzyme, with apparent K_m values of 0.05 mM and 0.22 mM respectively (Deshpande *et al.*, 1986; John *et al.*, 1979). However, other bacterial and fungal enzymes studied show less affinity for this substrate than the *T. fusca* enzyme.

The enzyme was not inhibited by D-glucose but it was inhibited by D-xylose, with an inhibition constant of 19.1 mM (standard deviation 1.3; $n = 3$) xylose. End-product inhibition of β -xylosidase activity by xylose is important in limiting the rate of lignocellulose saccharification in any process, since it is likely that the action of β -xylosidase itself, in removing oligosaccharides inhibitory to endoxylanase activity, would be a key regulatory mechanism.

Substrate specificity

The purified β -xylosidase did not exhibit detectable endoxylanase, endoglucanase or β -glucosidase activities. However, very low levels of arabinofuranosidase activity were detected after prolonged (overnight) incubation of the reaction mixture. This is likely to be due to the presence of small amounts of contaminating α -arabinofuranosidase activity, since this activity elutes from the anion-exchange column immediately before the β -xylosidase activity (data not shown). Furthermore, the separation between arabinofuranosidase and β -xylosidase by gel filtration is incomplete, probably resulting in the presence of trace amounts of arabinofuranosidase undetectable by PAGE. However, relatively broad substrate specificities are not uncommon amongst enzymes involved in the hydrolysis of cellulosic substrates, and fungal β -xylosidases with additional arabinofuranosidase activity (Deleyn *et al.*, 1978) or β -glucosidase activity (Uziie *et al.*, 1985) have been reported. No comparable information is available for the few bacterial β -xylosidases described thus far.

In addition to the high levels of intracellular β -xylosidase activity in *T. fusca*, low levels can be detected in the culture supernatant. Preliminary analysis indicates that this activity is due to a protein which is chromatographically identical to the intracellular β -xylosidase, suggesting leakage of the intracellular protein into the surrounding medium. As in other bacteria, therefore, *T. fusca* β -xylosidase is an intracellular enzyme and apparent detection of extracellular activity should be interpreted with caution.

Although few bacterial β -xylosidases have been characterized, that produced by *T. fusca* appears to be similar in size to these enzymes, and distinct from the smaller, monomeric β -xylosidases described in fungi. The broad pH and temperature stabilities of the *T. fusca* enzyme

compare favourably with those from other micro-organisms in general, further supporting the potential of thermophilic actinomycetes as a source of enzymes for lignocellulose saccharification.

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