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ORIGINAL PAPER

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Purification and biochemical properties of a thermostable xylose-tolerant β-D-xylosidase from *Scytalidium thermophilum*

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Abstract The thermophilic fungus *Scytalidium thermophilum* produced large amounts of periplasmic β-D-xylosidase activity when grown on xylan as carbon source. The presence of glucose in the fresh culture medium drastically reduced the level of β-D-xylosidase activity, while cycloheximide prevented induction of the enzyme by xylan. The mycelial β-xylosidase induced by xylan was purified using a procedure that included heating at 50°C, ammonium sulfate fractioning (30–75%), and chromatography on Sephadex G-100 and DEAE-Sephadex A-50. The purified β-D-xylosidase is a monomer with an estimated molecular mass of 45 kDa (SDS-PAGE) or 38 kDa (gel filtration). The enzyme is a neutral protein (pI 7.1), with a carbohydrate content of 12% and optima of temperature and pH of 60°C and 5.0, respectively. β-D-Xylosidase activity is strongly stimulated and protected against heat inactivation by calcium ions. In the absence of substrate, the enzyme is stable for 1 h at 60°C and has half-lives of 11 and 30 min at 65°C in the absence or presence of calcium, respectively. The purified β-D-xylosidase hydrolyzed *p*-nitrophenol-β-D-xylopyranoside and *p*-nitrophenol-β-D-glucopyranoside, exhibiting apparent K_m and V_{max} values of 1.3 mM, 88 μmol min^{-1} $protein^{-1}$ and 0.5 mM, 20 μmol min^{-1} $protein^{-1}$, respectively. The purified enzyme hydrolyzed xylobiose, xylotriose, and xylotetraose, and is therefore a true β-D-xylosidase. Enzyme activity was completely insensitive to xylose, which inhibits most β-xylosidases, at concentrations up to 200 mM. Its thermal stability and high xylose tolerance qualify this enzyme for industrial applications. The high tolerance of *S. thermophilum* β-xylosidase to xylose inhibition is a positive characteristic that distinguishes this enzyme from all others described in the literature.

Keywords β-D-Xylosidase · Xylanolytic activity · *Scytalidium thermophilum* · Thermostability

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Introduction

Xylan is, after cellulose, the most abundant renewable carbon source present in wood and agricultural residues. It is a heterogeneous molecule, consisting of a primary backbone of β-1,4-linked xylose residues that may present branches of arabinose, mannose, galactose, glucose and acidic sugars. Growing interest in the bioconversion of hemicellulose has recently arisen, as a consequence of its potential applications in several agro-industrial processes. These include the conversion of hemicellulosic materials to fuels and chemicals, paper-pulp delignification, improvement of beer consistency, improved digestibility of animal feedstocks and clarification of juices [18, 31, 33, 35–38].

In nature, the efficient hydrolysis of xylan occurs by the combined action of enzymes released by fungi and bacteria, including endo-β-1,4-xylanase, β-D-xylosidase, and several accessory enzymes, such as ∝-L-arabinofuranosidase, ∝-glucuronidase, acetylxylan esterase, ferulic acid esterase and *p*-coumaric acid esterase [31]. Among these enzymes, endo-xylanase is the major component of microbial xylanolytic systems, and its action is facilitated by accessory enzymes that remove the branches from the xylan backbone. While endo-xylanases mainly hydrolyze interior β-1,4-xylose linkages of the xylan backbone, β-D-xylosidases hydrolyze xylobiose and short xylo-oligosaccharides to xylose. Usually, the endoxylanases from different sources are inhibited by xylobiose, and β-D-xylosidase activity is required for maximal xylan hydrolysis [28, 33].

The utilization of thermostable enzymes, readily found in thermophilic microorganisms, has conferred

valuable advantages to industrial-scale processes [30, 37]. Among eukaryotic microorganisms, only a few species of fungi have the ability to live at temperatures between 45 and 55°C [21]. The fungus *Scytalidium thermophilum* is described as a good producer of thermophilic enzymes, including trehalase [16], phosphatase [11], and amylases [2, 3]. *Scytalidium thermophilum* is also described as a good cellulose decomposer [32]. Here we describe the production, purification and biochemical properties of a thermostable xylose-tolerant β-D-xylosidase from *S. thermophilum*.

Materials and methods

Microorganism and growth conditions

S. thermophilum strain 77.7.8 was a gift of Dr. G. Straatstma (Mushroom Experimental Station, The Netherlands) and was isolated from soil in India [32]. The fungus was maintained at 40°C on 4% oatmeal baby food (Quaker) medium solidified with 1.8% agar. Conidia from 7-day-old cultures were inoculated in liquid medium containing 0.2% $NaNO_3$, 0.1% $KHPO_4$, 0.1% KCl, 0.05% $MgSO_4.7\ H_2O$, 0.01% $FeSO_4.7\ H_2O$, 0.03% $ZnSO_4.7\ H_2O$, 0.8% yeast extract (Difco) and the desired carbon source at 1%; the pH was adjusted to 6.0. Solid and liquid cultures were prepared using distilled water. Erlenmeyer flasks (250 ml) containing 50 ml of medium were incubated at 40°C on a rotary shaker at 130 rpm. The mycelia formed were filtered through Whatman No. 1 paper on a Büchner funnel, rinsed with one equivalent culture volume of chilled distilled water, blotted on filter paper and divided in two equal parts. One part was used to assay β-xylosidase activity, whereas the other was heated at 90°C for 12 h and used for dry weight determinations.

Enzymatic assays

β-D-Xylosidase activity was quantified using 5.0 mM *p*-nitrophenyl-β-D-xylopyranoside (PNP-xyl) as substrate, unless otherwise indicated, in 50 mM McIlvaine buffer [23], pH 5.0. After incubation at 60°C for the desired time intervals, the reaction was stopped by the addition of two volumes of sodium-tetraborate-saturated solution and the absorbance was determined at 410 nm. The rate of *p*-nitrophenol release was linear when measured over 30 min with the concentration of enzyme used. Blank controls received distilled water instead of enzyme, and *p*-nitrophenol was used as standard. One enzyme unit was defined as the amount producing 1 μmol *p*-nitrophenol per minute. Specific activity was defined as enzyme units per milligram protein. Other aryl-β-glycosidase activities were assayed with the appropriate substrates under the same conditions described for PNP-xyl.

Effect of calcium on the enzyme activity

The influence of calcium on enzyme activity was assayed continuously in McIlvaine buffer, pH 5.0, at 50°C by following the release of p-nitrophenol at 410 nm. The assay was carried out in a final volume of 1 ml and contained 0.28 μg purified enzyme.

Cellular distribution of β-xylosidase

The total enzymatic fraction present at the mycelium surface was estimated by assaying a sample of intact mycelium rinsed with one equivalent culture volume of chilled water. A part of this enzymatic fraction (loosely bound enzyme) could be released by extensively washing the mycelium with 20 equivalent culture volumes of chilled water. The residual activity (tightly bound enzyme) was totally destroyed by treating the intact, extensively washed mycelium with HCl at 0°C, according to the procedure of Mandels [22]. The acid treatment inactivated β-xylosidase activity bound to the mycelium surface, without affecting mycelial integrity or the activity of intracellular enzymes. The intracellular β-xylosidase fraction was estimated by grinding a portion of the acid-treated mycelium with glass beads in a chilled porcelain mortar. The slurry was suspended in five volumes of 50 mM McIlvaine buffer [21], centrifuged twice, first at 2,500 *g* and then at 10,000 *g*, at 4°C, for 30 min each. The 10,000 *g* supernatant contained the soluble intracellular β-xylosidase enzymatic activity, whereas the pellets contained some residual cell-wall-bound activity.

Purification of β-D-xylosidase

β-D-Xylosidase was purified from 24-h-old xylan-grown mycelium. The mycelium (25 g wet weight), obtained by filtration, was ground in a mortar with acid-washed sand at 4°C. Disrupted cells were extracted with 100 ml of 1 mM phenylmethylsulfonyl fluoride and the slurry was centrifuged (10,000 *g*, 20 min, 4°C), saving the supernatant (crude extract). The crude extract was heated at 50°C for 20 min and then transferred to an ice bath. Denatured protein was spun down (12,000 *g*, 20 min, 4°C), and solid ammonium sulfate was added to the supernatant to achieve 30% saturation. The precipitate formed was again spun down (12,000 *g*, 20 min, 4°C) and the supernatant solid ammonium sulfate was added to achieve 75% saturation. The suspension was stored overnight at 4°C. Precipitated protein was collected by centrifugation (12,000 *g*, 20 min, 4°C), dissolved in a small volume of 50 mM sodium phosphate buffer (pH 6.8) and dialyzed against the same buffer. The dialyzed sample was applied to a Sephadex G-100 column (2×87 cm) equilibrated with the same buffer. Active fractions were pooled, dialyzed against water and lyophilized. The protein sample was dissolved in 100 mM sodium phosphate buffer (pH 6.8) and applied

to a DEAE-Sephadex A-50 column (1.8×15 cm) equilibrated with the same buffer. β-Xylosidase weakly interacted with the resin and eluted with the equilibrating buffer after the bulk of protein. Fractions containing β-D-xylosidase activity were pooled, dialyzed against water, lyophilized, and stored at −20°C until use.

Polyacrylamide gel electrophoresis

Electrophoresis under non-denaturing conditions was carried out by the method of Davis [6], using 7% acrylamide. SDS-PAGE (7%) was carried out according to the method of Laemmli [19], using phosphorylase b (94 kDa), bovine serum albumin (66 kDa), ovoalbumin (45 kDa) and carbonic anhydrase (29 kDa) as molecular mass standards (Sigma). After the runs the gels were stained with Coomassie brilliant blue.

Analytical methods

Total neutral carbohydrates were estimated by the method of Dubois et al. [9] using mannose as standard. Protein was estimated by the method of Lowry et al. [20] using bovine serum albumin as standard. Hydrolysis products resulting from the action of β-D-xylosidase on a mixture of xylo-oligosaccharides were analyzed by thin-layer chromatography on silica gel G-60, using ethyl acetate/formic acid/acetic acid/water (9:1:3:4) as the mobile phase. Mixtures of xylo-oligosaccharides containing xylobiose, xylotriose, xylotetraose and xylopentaose were obtained by the action of purified xylanase from *Humicola grisea* var. thermoidea on xylan [24]. After the chromatographic run, the hydrolysis products were stained by spraying the air-dried plate with a solution containing H_2SO_4/methanol (9:1) and 0.2% orcinol, and heating at 100°C.

Results and discussion

Optimization of culture conditions for β-D-xylosidase production

Several sugars were tested for β-D-xylosidase production, but the highest levels of enzymatic activity were obtained with xylan as the main carbon source (Table 1). Microcrystalline cellulose (avicel) and CM-cellulose were also fairly good inducers. By contrast, although high specific activities were observed with sucrose as inducer, due to the fact that *S. thermophilum* grew very poorly on this carbon source, low levels of total enzyme units were produced. Glucose, starch, maltose, xylose, cellobiose, fructose, lactose and mannitol were poor enzyme inducers. Figure 1 shows the time course of mycelial β-D-xylosidase production when *S. thermophilum* was cultivated on 1% xylan. β-D-xylosidase activity was not detectable until 18 h of growth, and the maximal enzyme level was observed at 24 h growth. The enzymatic activity decreased slowly between 24 and 48 h, while there was an abrupt decrease at 72 h. The profile of mycelial β-D-xylosidase activity nearly followed the growth curve of the fungus.

In order to assess whether xylan acted as an inducer for the enzyme production, and whether the synthesis of β-D-xylosidase was affected by catabolic repression, mycelial pads were pre-grown in 1% glucose, washed with sterile distilled water and reincubated for short periods of time in 1% glucose, 1% xylan, 1% xylan plus glucose or 1% xylan plus cycloheximide (50 μg ml^{-1}). The presence of glucose in the culture medium drastically reduced the levels of mycelial β-D-xylosidase activity (Fig. 2), suggesting that *S. thermophilum* β-D-xylosidase synthesis was indeed subject to carbon source repression. Furthermore, the presence of cycloheximide inhibited the synthesis of β-D-xylosidase, suggesting that

Table 1 Effect of carbon source on the mycelial β-D-xylosidase production by *Scytalidium thermophilum*. Data are the mean of three different cultures grown at 45°C for 24 h

Carbon source (1%)	Total protein (mg)	Total units	Specific activity [U (mg $protein^{-1}$)]
Deficient[a]	5.36	0.53	0.10
Starch	32.50	8.45	0.26
Maltose	29.28	5.27	0.18
Glucose	30.31	5.35	0.18
Sucrose	14.31	5.87	0.41
Cellobiose	29.27	2.58	0.09
Fructose	27.51	1.99	0.07
Lactose	26.13	2.87	0.11
Mannitol	28.34	2.96	0.10
Xylose	32.15	7.33	0.23
Avicel	30.30	10.90	0.36
CM-cellulose	24.60	13.80	0.56
Xylan	26.30	18.50	0.70

[a]Deficient contains only the low amount of carbohydrate present in yeast extract

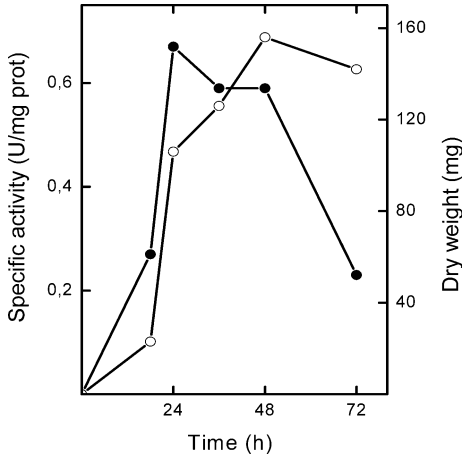

Fig. 1 β-D-Xylosidase activity by *Scytalidium thermophilum* cultures grown in 1% xylan. *Open circles* Dry weight, *filled circles* mycelial enzyme activity

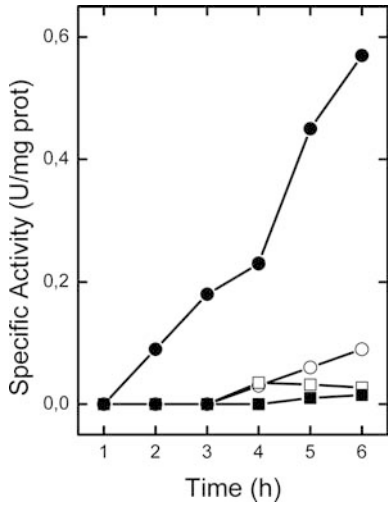

Fig. 2 Effect of glucose or cycloheximide on β-D-xylosidase activity. 1% xylan (*filled circles*), 1% xylan plus 1% glucose (*open circles*), 1% glucose (*open squares*), 1% xylan plus 50 μg ml^{-1} cycloheximide (*filled squares*)

the enzyme produced in the presence of xylan required de novo protein synthesis. β-D-Xylosidase synthesis is also affected by catabolic repression in the thermophilic fungi *H. grisea* [1], *Aspergillus phoenicis* [29] and *Aspergillus sydowii* [10].

Cellular distribution of β-D-xylosidase

A study of the cellular distribution of β-D-xylosidase in cultures grown in 1% xylan revealed that around 69% of the enzyme was surface-bound (e.g. it could be detected by assaying intact cells), and could be partially removed by successive washings with water or buffer. Twelve percent of the enzyme remained adsorbed to the cell wall, while about 19% was detected only after cell disruption. Thus, the enzymatic activity distribution suggested a physiological role for this enzyme in the final steps of xylan degradation.

Purification and molecular properties of mycelial β-D-xylosidase

The elution profiles of β-D-xylosidase from *S. thermophilum* in Sephadex G-100 and DEAE-Sephadex A-50 are shown in Fig. 3. Table 2 summarizes typical results of a purification protocol for mycelial *S. thermophilum* β-D-xylosidase. After the last chromatographic step the specific activity of the purified enzyme was 65 U per mg protein, with a purification of 125-fold. Non-denaturing 7% PAGE of the purified enzyme, run at pH 8.9, showed a single protein band after Coomassie brilliant blue staining (Fig. 4a). Slicing of a duplicate gel revealed that the enzymatic activity was coincident with the protein band (data not shown). Analysis of purified β-D-xylosidase in SDS-PAGE also showed a single band

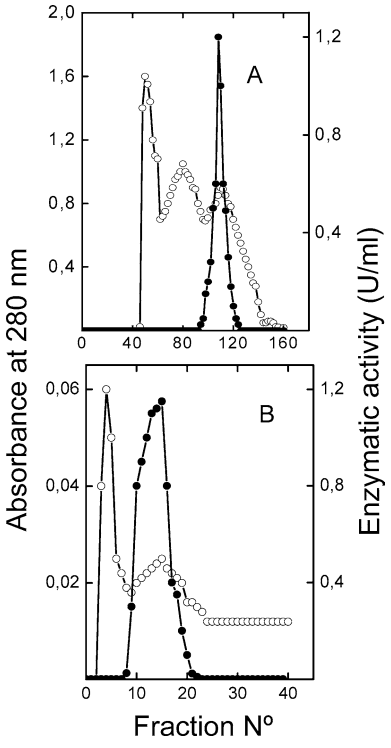

Fig. 3a, b Sephadex G-100 (**a**) and DEAE-Sephadex A-50 (**b**) chromatographic profiles of *S. thermophilum* mycelial β-D-xylosidase. β-D-Xylosidase activity (*filled circles*), absorbance at 280 nm (*open circles*). Other details given in "Materials and methods"

corresponding to a molecular mass of about 45 kDa (Fig. 4b). Molecular sieving of the purified enzyme in Bio-Sil Sec-400 produced an apparent molecular mass of 38 kDa, which was in good agreement with the value found using SDS-PAGE, suggesting that the enzyme was a monomer. Most of the purified β-D-xylosidases exhibit molecular masses above 100 kDa [33], although some exhibit lower molecular masses [1, 7, 15, 25, 33]. In addition, dimeric and monomeric forms of β-D-xylosidases are widespread among the enzymes purified from filamentous fungi [1, 15, 29, 34]. The carbohydrate content of the purified β-D-xylosidase was estimated to be 12%, similar to that reported for other β-D-xylosidases purified from fungi, which are also glycoproteins [4, 13]. However, *H. grisea* var. thermoidea mycelial β-D-xylosidase, sometimes described as a *S. thermophilum* synonymous, is not glycosylated [1]. Interestingly, isoelectric focusing of the purified β-D-xylosidase on PAGE yielded a single band with a pI value of 7.1, while most of the β-D-xylosidases studied so far exhibited acidic isolectric points, in the range of 4.0–5.0 [33].

Effect of temperature and pH

The purified β-xylosidase showed maximal activity at 60°C (Fig. 5a). In addition, the optimal pH was in the range of 5.0–6.0 (Fig. 5b).

Table 2 Purification of the mycelial β-D-xylosidase from *S. thermophilum*

Step	Total protein (mg)	Total activity (U)	Specific activity [U mg protein^{-1})]	Yield (%)	Purification (-fold)
Crude extract	261	135	0.52	100	1.0
Heat shock (50°)	208	266	1.28	197	2.5
$(NH_4)_2SO_4$ (30–75%)	174	216	1.24	160	2.4
Sephadex G-100	2	57	28.50	42	55.0
DEAE-Sephadex A-50	0.4	26	65.00	19	125.0

Data are the mean of six different preparations

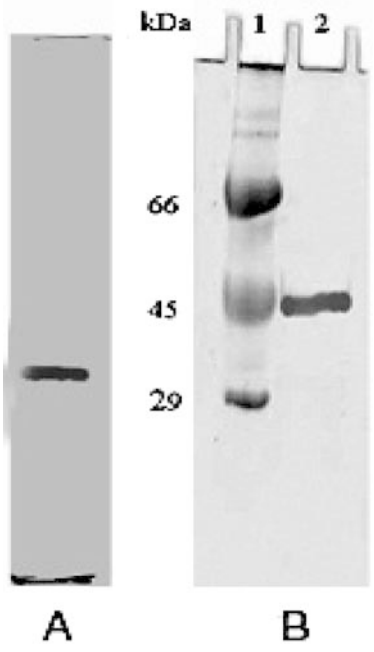

Fig. 4a, b PAGE (**a**) and SDS-PAGE (**b**) of purified *S. thermophilum* β-D-xylosidase. Fifteen μg of purified enzyme were applied to each gel. *Lane 1* Molecular mass standards, *lane 2* purified enzyme

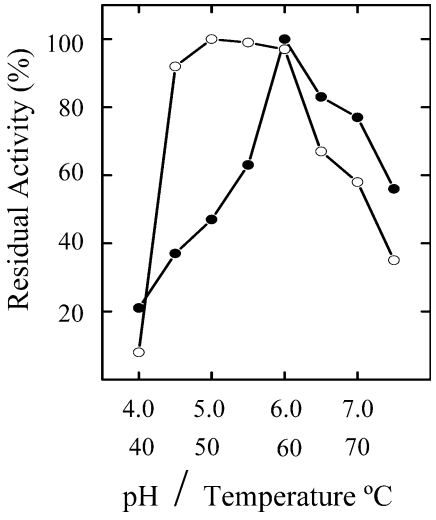

Fig. 5 Determination of optimal temperature (*filled circles*) and pH (*open circles*) for purified β-D-xylosidase. The enzyme activity was assayed with *p*-nitrophenyl glucopyranoside (PNP-glu) in McIlvaine buffer at pH 4.0–7.5. The temperature for maximal activity was determined using McIlvaine buffer at pH 5.0

Effect of potential inhibitors, activators and thermal protectors

The cations Mg^{2+}, Al^{3+}, Co^{2+}, Mn^{2+}, Cu^{2+}, K^{+}, Ba^{2+}, Fe^{2+} or Zn^{2+} at 1.0 mM did not affect enzymatic activity, while 22, 100 and 91% inhibition was observed for Zn^{2+}, Ag^{3+} and Hg^{2}, respectively (data not shown). Although 1 mM EDTA inhibited enzymatic activity by 20%, 1 mM β-mercaptoethanol had no effect. Surprisingly, calcium chloride strongly stimulated the activity of the purified enzyme (Fig. 6a). Calcium sulfate or calcium carbonate elicited identical stimulation (data not shown). Most of the β-D-xylosidases studied so far are not significantly activated by calcium. Strong activation by calcium ions has only been reported for enzymes purified from *Neocallimastix frontalis* [12] and *A. sydowii* [10]. In contrast, *H. grisea* var. thermoidea β-D-xylosidase activity was only increased by 30% with calcium [1].

The action of calcium ions as thermal stabilizers of the purified enzyme was tested by incubating the purified enzyme at different temperatures, with or without

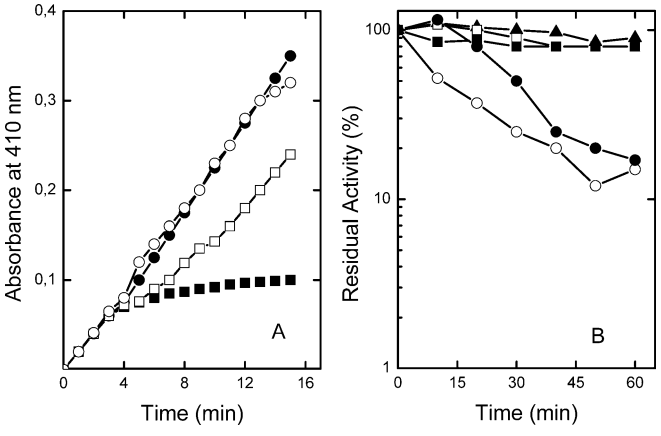

Fig. 6 a Effect of calcium on the purified β-D-xylosidase activity. *Filled squares*, *open squares*, *filled circles*, *open circles* represent activity determined at 60°C with 0, 1, 10 and 20 mM $CaCl_2$, respectively. One unit of absorbance at 410 nm is equivalent to 0.63 μmol. Other details given in "Materials and methods". **b** Effect of calcium on thermal inactivation of β-D-xylosidase. 50°C (*filled triangles*), 55°C (*open squares*), 60°C (*filled squares*), 65°C without calcium (*open circles*), 65°C with 10 mM calcium (*filled circles*). 100% activity of β-xylosidase is equivalent to 63.5 U mg protein^{-1}

10 mM calcium chloride (Fig. 6b). From 50 to 60°C, the enzyme was almost fully stable up to 60 min incubation, either in the presence or absence of calcium. At 65°C, the purified enzyme exhibited half-lives of about 11 and 30 min, in the absence and presence of calcium, respectively, suggesting that these ions acted both to activate and to protect the enzyme against thermal denaturation. Furthermore, studies of thermal stability indicated that the purified *S. thermophilum* β-D-xylosidase was more thermostable than most of the β-D-xylosidases purified from other thermophilic fungi [1, 10, 29].

Enzyme specificity

The purified enzyme hydrolyzed PNP-xyl and to some extent also *p*-nitrophenyl glucopyranoside (PNP-glu), but not *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-∝-L-arabinopyranoside, *o*-nitrophenyl-β-D-galactopyranoside, viscous CM-cellulose, avicel or xylan. Experiments with mixtures of PNP-xyl and PNP-glu revealed a hydrolysis velocity lower than that expected for the sum of the velocities measured in the presence of each substrate alone (Fig. 7). These data strongly suggested that the two substrates were hydrolyzed by the same enzyme, and thus β-aryl-glucosidase activity could not be attributed to the presence of a contaminant. This result is in contrast to the usual characteristics of β-D-xylosidases, which are very specific for xylopyranosides, while the more unspecific β-D-glucosidases act both on PNP-glu and PNP-xyl [5, 14, 17, 26]. Thin layer chromatography of the products resulting from the action of purified *S. thermophilum* β-D-xylosidase on mixtures of xylo-oligosaccharides clearly showed that the enzyme hydrolyzed xylobiose, xylotriose and xylotetraose (Fig. 8), suggesting that it is a true β-D-xylosidase. Compared to β-xylosidase purified from *A. phoenicis*, the β-xylosidase from *S. thermophilum* seems to be more applicable to xylan saccharification. The *S. thermophilum* enzyme hydrolyzed up to xylotetraose, while the *A. phoenicis* enzyme hydrolyzes only up to xylotriose [29].

Kinetic parameters

The purified enzyme exhibited a typical Michaelis-Menten kinetics, with K_m and V_{max} values for PNP-xyl of 1.3 ± 0.1 mM and 88 ± 8 U (mg protein)$^{-1}$ ($n = 6$), respectively. When PNP-glu was used as substrate, the values of K_m and V_{max} were 0.5 ± 0.03 mM and 20 ± 3 U (mg protein)$^{-1}$, respectively. The catalytic efficiency, calculated by the relation V_{max}/K_m, showed that the *S. thermophilum* enzyme hydrolyzed PNP-xyl more efficiently than PNP-glu, in spite of its higher apparent affinity for PNP-glu. In the presence of 10 mM calcium and utilizing PNP-xyl as substrate, V_{max} was increased 3.2-fold, while the K_m was not significantly altered.

Xylose up to 200 mM did not affect *S. thermophilum* β-D-xylosidase activity, suggesting a very high tolerance to inhibition by its hydrolysis product. To our knowledge, of the β-xylosidases studied thus far, only the enzyme produced by *S. thermophilum* exhibits a very high tolerance to xylose inhibition. By contrast, the thermophilic fungus *H. grisea* β-D-xylosidase was insensitive only up to 10 mM xylose [1]. Most of the β-D-xylosidases studied exhibited a K_i for xylose ranging from 2 to 10 mM [4, 8, 27, 33]. In the saccharification process,

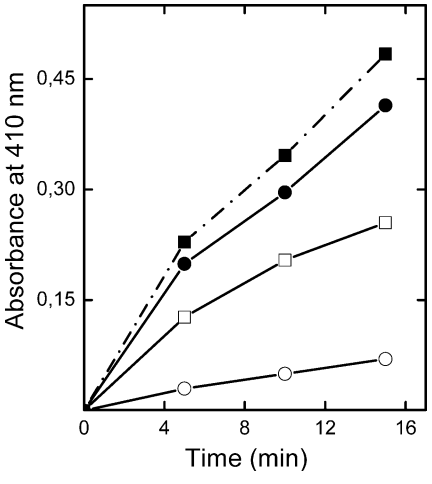

Fig. 7 Hydrolysis of PNP-xyl (*filled circles*), PNP-gluc (*open circles*) and a mixture of substrates (*filled squares*) as function of time. The theoretical rate of hydrolysis of a mixture of two substrate was based on the presence of separate enzymes. Under the standard conditions for the assay, one unit of absorbance at 410 nm is equivalent to 0.09 μmol. In the assay, 0.04 μg of purified protein in a final volume of reaction equal to 1.0 ml was used

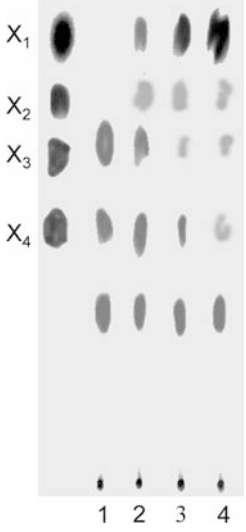

Fig. 8 Thin layer chromatography of the products of xylo-oligosaccharides hydrolysis by the purified β-D-xylosidase. Standards were a mixture of 1 mg xylose ml^{-1} (X_1), xylobiose (X_2), xylotriose (X_3) and xylotetraose (X_4). *Lanes 1, 2, 3* and *4*| are from 0, 5, 10 and 30-min samples. Details are described in "Materials and methods"

β-xylosidase activity seems to play an important role by relieving the end-product inhibition of endoxylanase [33]. This high xylose tolerance makes the *S. thermophilum* β-D-xylosidase particularly interesting for improving the saccharification of xylan.

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