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Catalytic properties of the endoxylanase I from *Thermoascus aurantiacus*

E. Kalogeris [a], P. Christakopoulos [a], M. Vršanská [b], D. Kekos [a], P. Biely [b], B.J. Macris [a,*]

[a] *Biosystems Technology Laboratory, Department of Chemical Engineering, National Technical University of Athens, 5 Iroon Polytechniou Avenue, Zografou Campus, 15700 Athens, Greece*

[b] *Institute of Chemistry, Slovak Academy of Sciences, 842 38 Bratislava, Slovak Republic*

Abstract

Endo-β-1,4-xylanase I previously purified from *Thermoascus aurantiacus* solid state culture was further characterized. The enzyme had a molecular weight of 33 kDa by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and 31 kDa by gel filtration. Thin layer chromatography (TLC) analysis showed that endoxylanase liberates aldotetrauronic acid MeGlcAα-1,2-Xylβ-1,4-Xylβ-1,4-Xyl as the shortest acidic fragment from glucuronoxylan and an isomeric xylotriose (Xyl_3) of the structure Xylβ1-3Xylβ1-4Xyl from rhodymenan. The enzyme performed ideally on *O*-acetyl-4-*O*-methyl-glucuronoxylan, liberating large amounts of short acetylated and non-acetylated fragments. Also, the enzyme was capable to hydrolyse arabinoxylan to arabinose (Arab), xylose (Xyl) and xylobiose (Xyl_2). The enzyme degraded pNPX (4-nitrophenyl β-D-xylopyranoside) by a complex reaction pathway that involved both hydrolysis and glycosyl transfer reactions. The enzyme tolerates the replacement of β-xylopyranosyl units in several artificial substrates by β-glucopyranosyl, α-L-arabinopyranosyl and α-L-arabinofuranosyl units and was active on pNPC (4-nitrophenyl β-D-cellobioside), pNP-Arap (4-nitrophenyl α-L-arabinopyranoside) and pNPAraf (4-nitrophenyl α-L-arabinofuranoside). The enzyme also hydrolysed the 4-methylumbelliferyl glycosides of β-D-xylobiose and β-D-xylotriose at the agluconic linkage. The results suggested that the xylanase I from *T. aurantiacus* has catalytic properties similar to those belonging to family 10.

Keywords: Endoxylanase; Family 10; *Thermoascus aurantiacus*

1. Introduction

Xylan is the second most abundant polysaccharide in nature, found in almost all parts of green plant cell walls. In recent years, microbial enzymes depolymerizing this polysaccharide found applications in food industry, animal feed, and paper and pulp industries [1–3]. The enzymes acting on the xylan backbone are classified in two groups: endo-β-1,4-xylanases (E.C. 3.2.1.8, xylan xylanohydrolase, EX) and exo-β-1,4-xylanases (E.C. 3.2.1.37, D-xylan xylanohydrolase). EXs have been classified into two groups, family 10 (formerly F) and 11 (formerly G) based on

* Corresponding author. Tel.: +30-1-7723160; fax: +30-1-7723161.

E-mail address: macris@orfeas.chemeng.ntua.gr (B.J. Macris).

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hydrophobic cluster analysis and amino acid sequence homologies [4,5]. In spite of limited knowledge on the differences in catalytic properties of EXs in the two families, it is certain that the enzymes belonging to family 10 exhibit greater catalytic versatility or lower substrate specificity than enzymes of family 11. EXs of family 10 show better capability of cleaving glycosidic linkages in the xylan main chain closer to the substituents, such as 4-*O*-methyl-D-glucuronic acid (MeGlcA), acetic acid and α-L-arabinofuranose [6]. Furthermore, the disturbance of the xylan chain by replacing β-1,4-linkages by β-1,3-linkages, like it is in rhodymenan, represents a more serious steric barrier for EXs of family 11 than for family 10 EXs [6]. In consonance with these considerations, EXs of family 10 liberate from 4-*O*-methyl-D-glucuronoxylan, rhodymenan and, with some exceptions, also from acetylxylan and arabinoxylan, smaller products than those formed with EXs of family 11 [6].

The thermophilic fungus, *Thermoascus aurantiacus* has been shown to produce high levels of highly thermostable xylanases under solid state culture (SSC) with wheat straw as carbon source. Two EXs were purified and characterized [7]. The major EX, assigned XYLI, was found to be a neutral enzyme, while the minor enzyme (XYLII) was found to be an acidic one [7].

The main purpose of the present study was to further investigate the biochemical and catalytic properties of the XYLI and to establish its relationship with the recognized endoxylanase families.

2. Experimental

2.1. Materials and methods

2.1.1. Chemicals

Sephadex G-100 was purchased from Pharmacia, Sweden. Beechwood 4-*O*-methyl-D-glucuronoxylan was isolated from sawdust as described [8]. *O*-Acetyl-4-*O*-methyl-D-glucuronoxylan was obtained by the extraction of beechwood holocellulose with dimethylsulphoxide [9]. Rhodymenan, a β-(1,3)-β-(1,4)-xylan from *Rhodymenia stenogona* was a gift from Dr. A.I. Usov (Russian Academy of Sciences, Moscow, Russia). Xylooligosaccharides and arabinoxylan were purchased from Megazyme (Ireland). Methylumbelliferyl β-glycosides of xylobiose ($MeUmb\text{-}Xyl_2$) and xylotriose ($MeUmb\text{-}Xyl_3$) were synthesized as will be described elsewhere (Vršanská, M., Nerinckx, W., Biely, P. and Claeyssens, M., unpublished data). [1-^{3}H]-Reducing-end labelled xylooligosaccharides with specific radioactivity of approximately 10 MBq/μmol, were obtained by catalytic tritiation of unlabelled compounds by the method of Evans et al. [10]. Methylumbelliferyl β-glycosides of cellobiose, cellotriose, cellotetraose and cellopentaose (abbr. $MeUmb\text{-}Glc_x$) were obtained by courtesy of Dr. M. Claeyssens (State University, Gent, Belgium). All the remaining chemicals used were analytical grade and purchased either from Sigma, or other companies.

2.1.2. Enzyme

The investigated XYLI was produced by *T. aurantiacus* Mieche, IMI 216529 under solid state culture with wheat straw as carbon source and was purified as described previously [7].

2.1.3. Determination of molecular weight

2.1.3.1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). This was determined using PhastSystem electrophoresis unit (Pharmacia) with 10–15% polyacrylamide gel (Pharmacia), according to the instructions given in the Pharmacia PhastSystem manual.

2.1.3.2. Gel filtration. A column (2.5 × 100 cm) of Sephadex G-100, pre-equilibrated with 50 mM citrate-phosphate buffer, pH 5, was used, and elution was performed with the same buffer (flow rate 60 ml/h). The molecular weight (MW) of the purified enzyme was estimated using a MW-marker kit (Sigma).

2.1.4. Protein estimation

The protein concentration was determined by the dye-binding procedure of Bradford [11] using bovine

serum albumin as a standard. A_{280} was used to monitor protein in column effluents.

2.1.5. Hydrolysis of polysaccharides and aryl glycosides

The 2.0% (w/v) solutions of polysaccharides and 150 mM aryl glycosides in 50 mM citrate-phosphate buffer, pH 5.0 were incubated with appropriately diluted enzyme at 50°C. The aliquots were analyzed at different time intervals, for hydrolysis products, by thin layer chromatography (TLC) on microcrystalline cellulose (DC-Alufolien Cellulose, Merck) in the solvent system ethylacetate–acetic acid–water (3:2:1, by vol.). Reducing sugars were visualized by the aniline–hydrogen phthalate reagent. 4-Nitrophenol (pNP) and 4-nitrophenyl-β-D-glycosides were visualized on plates under UV light.

Quantitative data were obtained by analysis of samples using an HPLC system (Waters 600E) with CarboPac PA1 column (Sodium hydroxide, 0.5 M; Sodium acetate, 1 M), and Dionex ED40 electrochemical detector (Dionex, USA).

2.1.6. Bond cleavage frequencies and kinetic parameters of [1-^{3}H]-xylooligosaccharides

The initial bond cleavage frequencies of reducing-end labelled oligosaccharides, identical with relative rates of the cleavage of individual glycosidic linkages, were determined using the method of Allen and Thoma [12] at 0.25 mM substrate concentration and appropriate enzyme dilutions. The reactions were performed in 0.02 M acetate buffer (pH 5.0) at 50°C. Aliquots of the mixtures were removed at time intervals and subjected to TLC in ethyl acetate–acetic acid–water (3:2:1, by vol.). After detection of guide strips with standards with aniline–hydrogen phthalate reagent, the radioactivity in substrate and products was measured in a liquid scintilation counter. The ratio of radioactivity in the substrate and products was determined and used for graphic determination of the initial bond cleavage frequencies, i.e. frequencies corresponding to the zero reaction time. From the same experiments, the decrease of radioactivity in the substrate as a function of time was used to calculate initial rates of hydrolysis of individual oligosaccharides.

2.1.7. Determination of kinetic parameters for the hydrolysis of artificial substrates

2.1.7.1. Determination of kinetic parameters for the hydrolysis of aryl glycosides. Hydrolysis of aryl glycosides was carried out in 50 mM citrate-phosphate buffer, pH 5.0 at 40°C using a SPECTRAmax 250 Microplate Spectrophotometer (Molecular Devices, USA). Reactions after temperature equilibration were initiated by the addition of 10 μl solution of pure xylanase in a total volume of 210 μl. The substrate concentration ranges used were: 0.1–40 mM for 4-nitrophenyl β-D-cellobioside (pNPC), 4-nitrophenyl α-L-arabinopyranoside (pNP-Ara*p*) and 4-nitrophenyl α-L-arabinofuranoside (pNP-Ara*f*); 0.1–150 mM for 4-nitrophenyl β-D-xylopyranoside (pNPX). The release of pNP was monitored spectrophotometrically at 410 nm. Kinetic constants (K_m and k_{cat}) were determined based on the initial rates of hydrolysis at 12 different substrate concentrations and calculated using Lineweaver–Burk plots.

2.1.7.2. Determination of kinetic parameters for the hydrolysis of MeUmb-glycosides. The initial hydrolysis rates of MeUmb-glycosides were determined by incubating the substrates (0.1–2 mM concentration) in 50 mM citrate phosphate buffer, pH 5.0 at 50°C. Reactions were initiated by the addition of 15 μl solution of pure xylanase to 65 μl incubation mixture. The 5-μl aliquots were removed at different time intervals and were applied to the column. All kinetic studies for fluorogenic substrates were carried out using HPLC system (Waters 600E) with NH_2-μ Bondapak column (300 × 3.9 mm) (Waters) with acetonitrile:water (92:8) as a mobile phase at a flow rate of 1.0 ml min^{-1}. 4-Methylumbelliferone (MeUmb) and MeUmb-glycosides were identified using Waters UV detector (Model 440) at 313 nm. The products were quantified on the basis of peak height using standard MeUmb$(Glc)_n$.

2.1.8. Isolation and identification of products of hydrolysis of 4-O-methyl-D-glucuronoxylan

A solution of beechwood 4-*O*-methyl-D-glucuronoxylan (0.5 g in 25 ml of distilled water, pH 5.0) was incubated for 8 days at 50°C with 5 U of *T. aurantiacus* XYLI. After this time, the polysaccha-

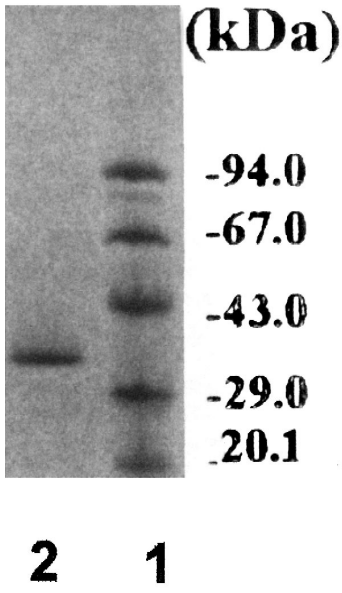


Fig. 1. Analysis of the purified XYLI from *T. aurantiacus* by SDS-PAGE. Lane 1: standard protein markers in the order of increasing molecular mass: trypsin inhibitor, carbonic anhydrase, ovalbumin, albumin and phosphorylase b; lane 2: xylanase I.

ride was found to be almost completely hydrolyzed into a mixture of D-xylose, xylobiose, small amount of xylotriose and an acidic oligosaccharide migrating on TLC identically as an aldotetrauronic acid. To isolate the acid, the mixture was passed through a column of Dowex 1 (acetate form), which was washed first with distilled water until no more neutral sugars were found in the eluate. Aldotetrauronic acid was liberated from the column by 3-M solution of acetic acid. The acetic acid was eliminated by evaporation in vacuo and the resulting syrup (75 mg) was used for ^{13}C NMR spectroscopy (Bruker AVANCE DPX 300, Germany).

3. Results

3.1. Molecular weight of xylanase

The purified major xylanase I gave a single band of protein on SDS-PAGE (Fig. 1). The molecular weight of the xylanase was estimated to be approximately 33,000 by SDS-PAGE and 31,000 by gel filtration on a Sephadex G-100 column. These results indicate that XYLI is a monomeric enzyme.

3.2. Action on polysaccharides

The hydrolysis products released from polysaccharides by *T. aurantiacus* XYLI were analyzed by TLC (Fig. 2). From beechwood 4-*O*-methyl-D-glucuronoxylan, the enzyme liberated mainly xylose, xylobiose, xylotriose and acidic oligosaccharides

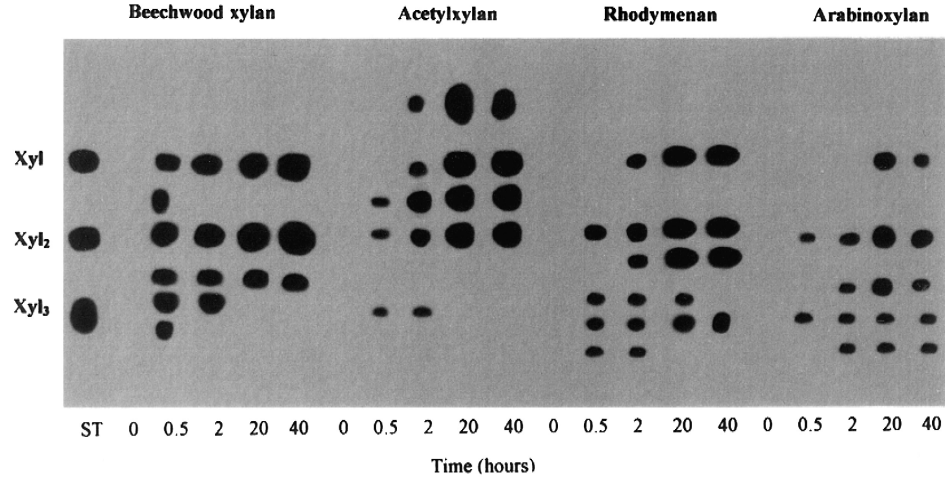


Fig. 2. TLC of the hydrolysis products of beechwood glucuronoxylan, acetylxylan, rhodymenan and arabinoxylan by *T. aurantiacus* XYLI. The position of products identified on the basis of authentic standards is marked on the margin.

which had the R_{Xyl} values 0.53 and 0.36, a chromatographic mobility compatible with aldotetrauronic and aldopentauronic acids. With longer time of the enzyme treatment, almost all xylotriose disappeared and aldopentauronic acid was converted to aldotetrauronic acid, the shortest acidic fragment (Fig. 2). Aldotetrauronic acid, isolated from such polysaccharide hydrolysate, showed ^{13}C NMR spectrum identical with the spectrum of the shortest acidic oligosaccharides liberated from glucuronoxylan by endoxylanases of family 10, which is identical with MeGlcA α-1,2-Xylβ-1,4-Xylβ-1,4-Xyl [6] (Fig. 3).

The main products of rhodymenan hydrolysis were xylose, xylobiose and an isomeric oligosaccharide with β-1,3-linkage, which had a chromatographic mobility of an isomeric xylotriose, Xyl1-3Xylβ1-4Xyl (R_{Xyl} 0.59). This isomeric xylotriose does not occur among the products of rhodymenan hydrolysis by EXs of family 11 [6].

From beechwood *O*-acetyl-4-*O*-methylglucuronoxylan XYLI liberated a large number of both acetylated and non-acetylated products which could be resolved by two-dimensional TLC, with deacetylation of compounds between the two developments (Fig. 4). This pattern of products is similar to that

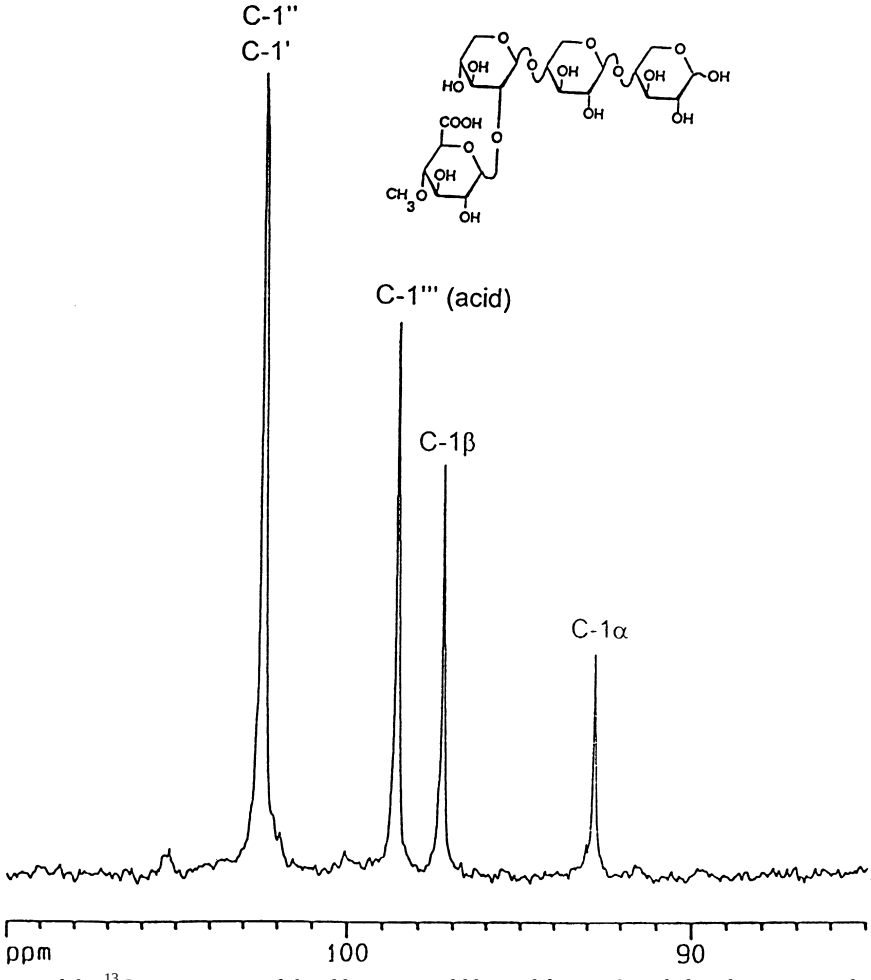


Fig. 3. Anomeric region of the ^{13}C NMR spectra of the aldouronic acid liberated from 4-*O*-methyl-D-glucurono-D-xylan by *T. aurantiacus* XYLI.

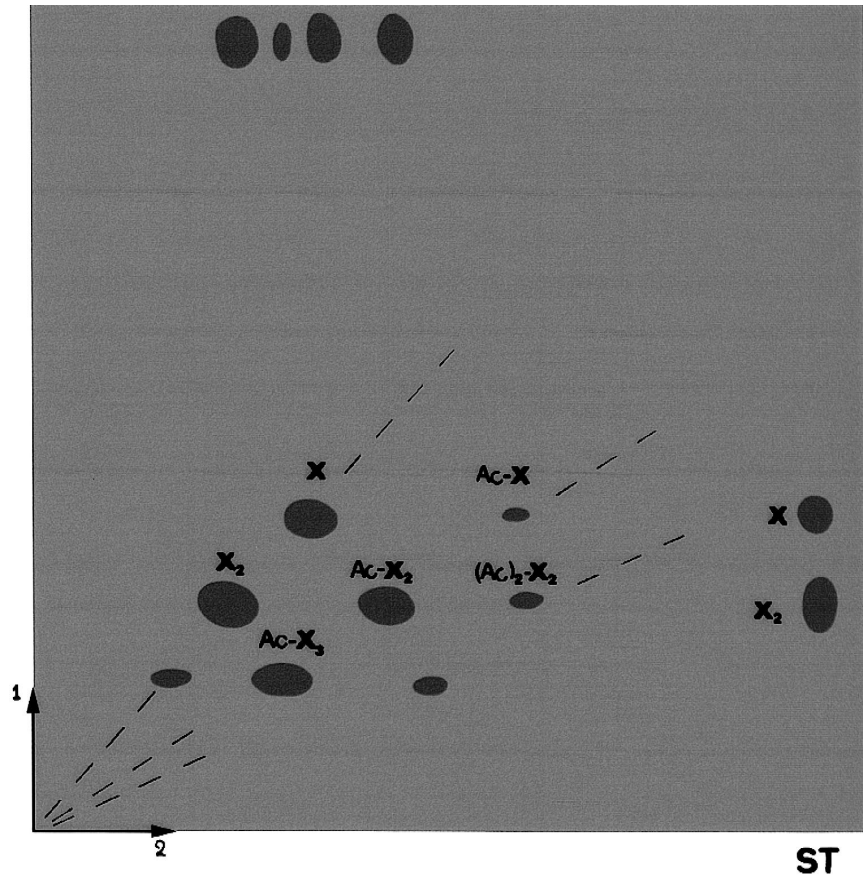


Fig. 4. Two-dimensional chromatography of products of hydrolysis of beechwood acetylxylan by *T. aurantiacus* XYLI. The hydrolysate was chromatographed first in direction 1, then the dried chromatogram was exposed to ammonium hydroxide vapours to deacetylate all acetylated products of hydrolysis, and then the compounds were chromatographed in direction 2. The products of acetylxylan hydrolysis appear to belong to three distinct series: (i) non-acetylated products, laying on the diagonal; (ii) monoacetylated fragments, laying on the line with a slope of approxim. 0.65; (iii) diacetylated products laying on the line with a slope of about 0.47.

obtain with XlnA from *Streptomyces lividans*, an EX of family 10 [13]. Non-acetylated products, mainly Xyl, Xyl_2, and Xyl_3, lay on diagonal (slope approx. 1.0). Acetylated products belong to two categories: monoacetylated lying on the second line (slope approx. 0.65) and di-*O*-acetylated on the third line (slope approx. 0.45). First time we report here the liberation of small amounts of monoacetylated xylose. This observation together with significant quantities of acetylated Xyl_2 and Xyl_3 in the hydrolysate demonstrates that the enzyme tolerates well the small substitutents on the main xylan chain. Action of the enzyme on arabinoxylan yielded arabinose, xylose, xylobiose and significant amounts of two compounds migrating behind xylobiose and xylotriose, most probably arabinoxylotriose and arabinoxylotetraose.

3.3. Action on oligosaccharides

Bond-cleavage frequencies of xylooligosaccharides determined at 0.25 mM substrate concentration are shown in Fig. 5. The enzyme cleaved [1-^{3}H]-xylotriose exclusively at the first glycosidic linkage from the reducing end, liberating [1-^{3}H]-xylose as the only radioactive product. Xylotetraose was hydrolyzed preferentially in the middle, while [1-^{3}H]-

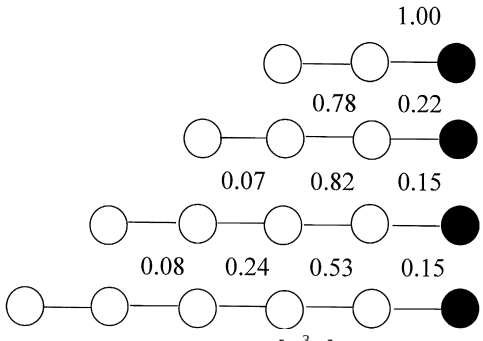


Fig. 5. Bond-cleavage frequencies of [1-^{3}H]-labelled xylooligosaccharides with XYLI from *T. aurantiacus*. (○) A non-reducing xylopyranosyl residue; (●) [1-^{3}H]-labelled-reducing-end xylopyranosyl residue.

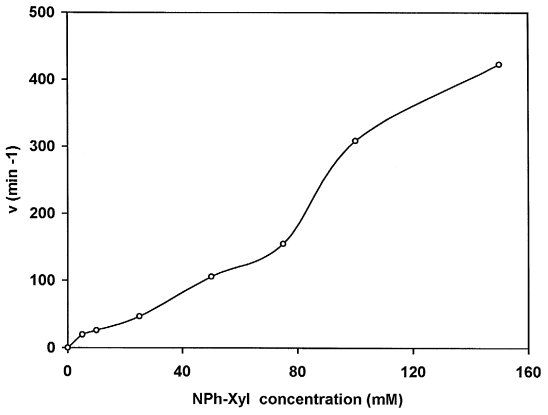


Fig. 6. The rate of liberation of PNP from PNPX by XYLI from *T. aurantiacus* as a function of substrate concentration.

xylopentaose and [1-^{3}H]-xylohexaose mainly at the second glycosidic linkage from the reducing end. The enzyme showed certain preference for attacking the linkages from the reducing end of the oligosaccharides. A similar mode of action on oligosaccharides was exhibited by *Cryptococcus albidus* EX, also a member of family 10 EXs [14]. As shown in Table 1, the difference in the initial rate of hydrolysis of oligosaccharides referred to unit xylanase concentration is about two orders in going from xylotriose to xylohexaose. There is only a two-fold increase of the rate of hydrolysis in going from xylopentaose to xylohexaose.

3.4. Action on artificial substrates

pNPX was attacked by XYLI., however, the rate of liberation of pNP as a function of pNPX concentration did not obey well the Michaelis–Menten kinetics (Fig. 6). The reaction rate increased slower at substrate concentrations below 80 mM than at higher substrate concentrations. At high substrate concentrations (150 mM), the liberation of pNP was accompanied not only by the formation of xylose but also pNP-glycosides (Fig. 7) and β-1,4-xylooligosac-

Table 1
Initial rates of hydrolysis of tritiated xylooligosaccharides (all 0.25 mM) by *T. aurantiacus* XYLI recalculated to a unit concentration of the enzyme

Oligosaccharide	[*E*] (U/ml)	Initial rate of hydrolysis (mM/min/U/ml)	Multiple
Xylotriose	2.7	1.7×10^{-3}	
Xylotetraose	1.2	5.2×10^{-3}	3
Xylopentaose	0.18	8.7×10^{-2}	17
Xylohexaose	0.06	1.6×10^{-1}	2

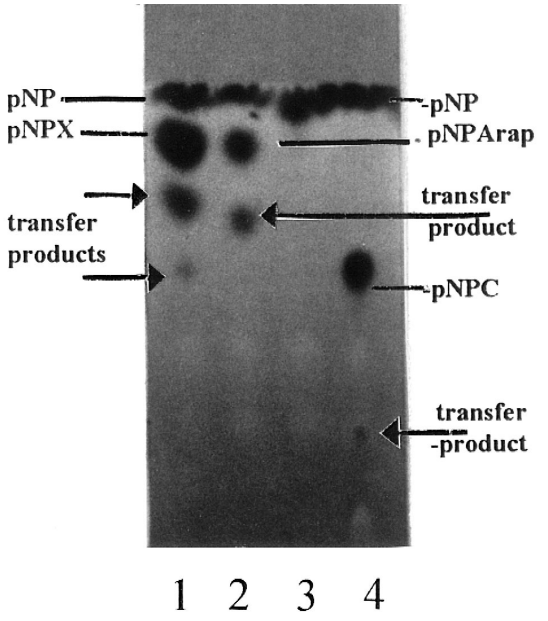


Fig. 7. TLC analysis of nitrophenol and 4-nitrophenyl-β-D-glycosides produced by the action of *T. aurantiacus* XYLI on aryl glycosides at 150 mM concentration (lane 1: pNP-xylopyranoside; lane2: pNP-arabinopyranoside; lane 3: pNP-arabinofuranoside; lane 4: pNP-cellobioside). Only compounds detectable under UV light are shown.

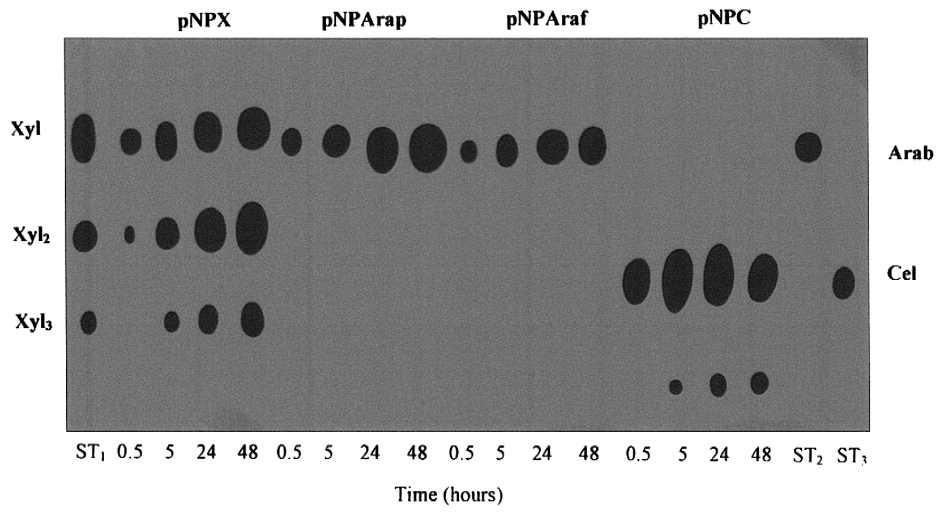
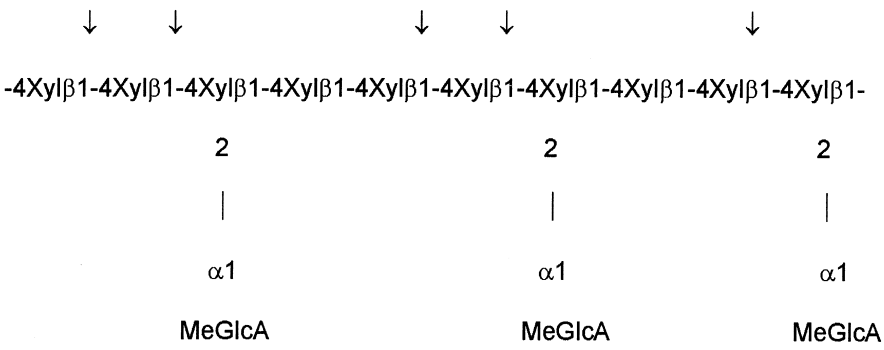


Fig. 8. TLC analysis of the sugars produced by the action of *T. aurantiacus* XYLI on 150 mM aryl glycosides (pNPX: pNP-xylopyranoside; pNPArap: pNP-arabinopyranoside; pNPAraf: pNP-arabinofuranoside; pNPC: pNP-cellobioside). Reducing sugars were detected by the aniline–phthalate reagent. ST_1–ST_3, standards.

charides (Fig. 8), as a result of glycosyl transfer reactions. A useful criterion for differentiating EXs of families 10 and 11 is their action on pNPC. XYLI from *T. aurantiacus* also hydrolyzed pNPC and TLC product analysis demonstrated that the cleavage took place exclusively at the agluconic linkage to afford pNP and cellobiose. The K_m value was found to be 82 mM. Similar high K_m values were reported for the same substrate for family 10 EXs from *S. lividans* and *C. albidus* [6]. At high substrate concentrations (150 mM), in addition to hydrolysis, cellobiosyl transfer to another substrate molecule was observed. TLC analysis of the reaction mixture showed the presence of low amounts of pNP-glycosides with mobility of pNP-glycoside of a tetrasaccharide (Fig. 7) and low amounts of a compound migrating as glucotetrasaccharide (Fig. 8). An unexpected catalytic ability of XYLI was the hydrolysis of pNP-Arap and pNP-Araf (Table 2). TLC and HPLC product analysis showed that arabinose was the only monosaccharide produced from both arabinosides. At high pNP-Ara*p* concentrations the liberation of arabinose was accompanied by the formation of an aryl glycoside having the mobility of an pNP-arabinobioside (Fig. 7). No evidence was obtained for the formation of a free disaccharide (Fig. 8). pNP-Ara*f* was found also to be the better substrate than pNPX (Table 2), but served just as a substrate for hydrolytic cleavage. The enzyme hydrolysed MeUmb-Xyl_2 and MeUmb-Xyl_3 at the aglyconic linkage. Kinetic parameters determined for their hydrolysis are summarised in Table 2. The affinity of XYLI towards MeUmb-Xyl_3 was surprisingly 2 times lower than that towards MeUmb-Xyl_2. In contrast, the comparison of turnover numbers clearly shows that the enzyme prefers MeUmb-Xyl_2 to MeUmb-Xyl_3. Also, the comparison of k_{cat}/K_m ratio revealed that the enzyme hydrolysed MeUmb-Xyl_2 1.6 times more efficiently than MeUmb-Xyl_3 (Table 2). Hence, it is believed that the observed difference in the affinity and turnover numbers could be due to the nature of interaction and orientation of a particular substrate in

Table 2
Kinetic constants of XYLI from *T. aurantiacus* for model substrates

Substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m
PNPX	297	769	2.6
PNP-Arap	59.4	133.3	2.2
PNP-Araf	65.6	85.5	1.3
PNPC	82	5000	61
$MeUmbX_2$	0.167	3333	19,958
$MeUmbX_3$	0.067	833	12,433



Scheme 1. Mode of attack of 4-*O*-methyl-D-glucuronoxylan by XYLI.

the active site of the enzyme. With 100 times higher enzyme concentration and 50 times longer incubation as those used for MeUmb-Xyl_2 and MeUmb-Xyl_3, an evidence for hydrolysis of MeUmb-Glc_2, MeUmb-Glc_3, MeUmb-Glc_4 and MeUmb-Glc_5 at the agluconic linkage has been obtained.

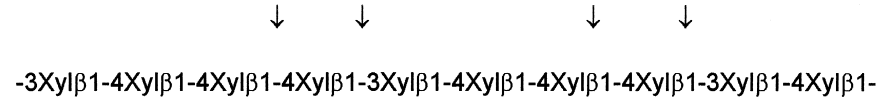
4. Discussion

The molecular weight (33 kDa) of the major endoxylanase purified from *T. aurantiacus* IMI 216529 was in accordance with those reported for purified fungal xylanases belonging to family F/10, e.g. from *Penicillium chrysogenum* [15], *Aspergillus awamori* [16] *Filobasidium floriformei* [17] and *Fusarium oxysporum* [18].

Until the present study, the catalytic properties of XYLI from *T. aurantiacus* have not been investigated in detail. The results of preliminary investigations by Bhat et al. [19], suggesting that the enzyme belongs to the family 10 of glycosyl hydrolases, were confirmed in the present study. The nature of the fragments liberated from 4-*O*-methylglucuronoxylan, rhodymenan, arabinoxylan and acetyl-4-*O*-methylglucuronoxylan is fully compatible with the fact that XYLI of *T. aurantiacus* IMI 216529 belongs to family 10. The enzyme liberates from polysaccharides shorter fragments than EXs of family 10. This is in agreement with its relatively high activity towards short xylooligosaccharides such as Xyl_3. At 0.25 mM substrate concentration, Xyl_3 is hydrolyzed by XYLI 4250 times faster than by the same number of units of EX from *T. lanuginosus* ATCC 46882, a member of family 11 [20].

The identification of the shortest acidic fragment released from 4-*O*-methylglucuronoxylan allows to propose that the enzyme, in contrast to EXs of family 11, is capable of attacking the xylosidic linkage next to the branch and towards the non-reducing end. Similarly, as other xylanases of family 10, the *T. aurantiacus* XYLI requires two unsubstituted consecutive xylopyranosyl residues (Scheme 1).

Endoxylanases of family 11 require in glucuronoxylan three unsubstituted consecutive xylopyranosyl residues [6]. Since the isomeric xylotriose, Xylβ1-3Xylβ1-4Xyl, occurs among the products of rhodymenan hydrolysis by the xylanase of *T. aurantiacus*, it can be proposed that the enzyme similarly as other EXs of family 10 [21], does not attack in the polysaccharide β-1,4-linkages which follow a β-1,3-linkage towards the reducing end (Scheme 2).



Scheme 2. Mode of attack of rhodymenan by XYLI.

$$PNPX \rightarrow Xyl + PNP$$

$$PNPX + PNPX \rightarrow PNPX_2 + PNP$$

$$pNPX_2 + pNPX \rightarrow pNPX_3 + pNP$$

$$pNPX_2 \rightarrow Xyl_2 + pNP$$

$$pNPX_3 \rightarrow Xyl_3 + pNP$$

$$pNPX + Xyl_3 \rightarrow Xyl_2 + pNPX_2$$

Scheme 3. The degradation of pNPX by XYLI.

Action pattern on acetylxylan may become an additional criterium for a finer classification of EXs. Mono-acetylated Xyl_2 liberated from acetylxylan by XYLI of *T. aurantiacus* is a characteristic fragment of hydrolysis of acetylxylan by EXs of family 10 [6]. Little or no acetylated Xyl_2 was found in the hydrolysates by XlnB and XlnC of *S. lividans* [13], EXII of *T. reesei* [8] and EXI of *Schizophyllum commune* [22], all members of family 11.

The degradation of pNPX by EXs of family 10 proceeds by a complex reaction pathway that involves a series of hydrolytic and glycosyl transfer reactions as shown below in Scheme 3 [6]. Similar complex reaction pathway of pNPX degradation was observed with *Cryptococus albidus* EX and *S. lividans* XlnA, family 10 EXs [6].

XYLI exhibited 6590 times higher catalytic efficiency for MeUmb-Xyl_2 than for MeUmb-Glc_2. A family 10 EX of *Cellulomonas fimi* was also reported to be 50 times more active on $pNPX_2$ than pNPC [23,24]. Although we do not have the comparison of the hydrolysis of pNP-glycosides of cellobiose and xylobiose, hydrolysis of aryl β-D-cellobiosides at the agluconic bond by XYLI points to the fact that the enzyme contains in its substrate binding site at least two subsites, -II and -I, that can accommodate a cellobiosyl moiety. The smaller K_m value for pNPC than for pNPG suggests that the decisive affinity in productive binding of pNPC is exhibited by subsite-II, although this affinity is much lower for β-D-glucopyranosyl residue than for β-D-xylopyranosyl residue. Clear evidence has been obtained for the capability of the enzyme of catalyzing transfer of cellobiosyl moiety to pNPC or cellobiose. The type of the newly formed linkage in such products have not been established yet. The 6′-*O*-glycosylation of cellobiose by *C. albidus* EX reported earlier [25], leads us to assume that the cellobiosyl transfer may also result in the formation of other than 4′-O-*β*-glycosidic linkage.

As it has been demonstrated [26], some high molecular-weight EXs hydrolyse agluconic bond in substrates containing more than two consecutive glucopyranosyl residues, namely in aryl β-D-glycosides of larger cellooligosaccharides. Similarly, the EX from *T. aurantiacus* under extreme conditions can attack the agluconic bond in MeUmb-glycosides of cellotriose, cellotetraose and cellopentaose, which indicates that it has the ability to tolerate the replacement of more than two D-xylopyranosyl residues by D-glucopyranosyl residues.

Finally, based on the molecular weight and the above catalytic properties, we suggest that the major endoxylanase purified from *T. aurantiacus* IMI 216529 (XYLI) belongs to glycosyl hydrolases of family 10. The tolerance of the enzyme to replacement of β-D-xylopyranosyl residues in the substrates by β-D-glucopyranosyl residues is in agreement with the suggestion that EXs of family 10 evolved from a common ancestor with endo-β-1,4-glucanases of family 5 (formerly A) [27,28].

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