Effect of side groups on the action of β-xylosidase from *Trichoderma reesei* against substituted xylo-oligosaccharides

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Abstract The action of β-xylosidase from *Trichoderma reesei* against different substituted xylo-oligosaccharides was studied. The enzyme cleaved off all unsubstituted xylose units from the non-reducing end of 1,2-linked uronic acid substituted xylo-oligosaccharides. Surprisingly, an L-arabinofuranosyl group linked α-1,3 to the xylopyranosyl ring was found to protect the β-1,4-xylosidic linkage before the substituted xylose unit from being cleaved by the β-xylosidase. Most probably the 1,3-linked substituent sterically hinders the hydrolysis. According to the results of the present work, β-xylosidase of *T. reesei* is not able to remove all unsubstituted xylose units from the non-reducing end of substituted xylo-oligosaccharides, as had been believed previously.

Key words: *Trichoderma reesei* β-xylosidase; Hydrolysis; Substituted xylo-oligosaccharide; High-performance anion-exchange chromatography; NMR

1. Introduction

Xylans are the most abundant hemicelluloses in hardwoods and cereals. The backbone of xylan is formed by β-1,4-linked D-xylopyranose units, to which several side groups are attached. The structure of xylan varies depending on its origin. Hardwood xylan contains α-1,2-linked 4-*O*-methyl-D-glucuronic acid and it is acetylated. Softwood xylan is substituted both by α-1,2-linked 4-*O*-methyl-D-glucuronic acid and by α-1,3-linked L-arabinofuranose [1]. In addition to all the above-mentioned substituents, xylans in annual plants may also contain α-1,2-linked L-arabinofuranose. Some xylans, especially from endosperms of cereals, may even have two arabinofuranose groups linked to a single xylose unit [2,3].

In the degradation of xylans to monomeric sugars several different enzymes are needed [4]. The backbone is randomly hydrolyzed to short xylo-oligosaccharides by endoxylanases. Side groups are cleaved off by α-glucuronidases, α-arabinofuranosidases and acetyl xylan esterases. The xylo-oligosaccharides are further hydrolyzed to xylose by β-xylosidases. β-Xylosidases (EC 3.2.1.37) are enzymes that successively remove the terminal xylose unit from the non-reducing end of xylo-oligosaccharides [5].

Trichoderma reesei is an efficient producer of different xylanolytic enzymes [6]. It produces at least two endoxylanases [ ], one α-arabinofuranosidase [8], one α-glucuronidase [9], one acetyl xylan esterase [10,11] and one β-xylosidase [12]. The β-xylosidase of *T. reesei* is a rather large protein consisting of two identical subunits with molecular masses around 100 kDa [12]. Recently, the gene encoding the subunit has been cloned and sequenced [13]. β-Xylosidases are key enzymes in the production of xylose from different xylo-oligosaccharides. β-Xylosidase of *T. reesei* has also been reported to act slowly on polymeric xylan [13,14]. Substituents, such as an acetyl group attached to the terminal xylose, inhibit the action of this enzyme [15]. In addition to the efficient hydrolysis of linear xylo-oligomers, β-xylosidases are also able to liberate xylose from the non-reducing end of substituted xylo-oligosaccharides. Therefore, β-xylosidases, in combination with side group cleaving enzymes, can be used in the structural determination and controlled production of different substituted xylo-oligomers. In the present work, several substituted xylo-oligosaccharides were treated with β-xylosidase from *T. reesei* in order to investigate its action against different substituted oligosaccharides.

2. Materials and methods

2.1. Enzymes and substrates

β-Xylosidase from *T. reesei* was purified as described by Poutanen and Puls [12]. The arabinose- and uronic acid-containing xylo-oligosaccharides were produced by hydrolysing pine and birch kraft pulps, respectively, with 2000 nkat/g of *Aspergillus oryzae* p*I* 6.9 or *T. reesei* p*I* 5.5 endoxylanase, after which the oligosaccharides were isolated by anion-exchange and gel permeation chromatography [16]. The primary structures of the isolated oligosaccharides were determined by ^{1}H-NMR spectroscopy.

2.2. Hydrolysis experiments

β-Xylosidase (1000 nkat/g) was incubated with oligosaccharides (0.5 g/l) in 50 mM Na-citrate buffer, pH 5.0 for 24 h at 40°C, after which the reaction was terminated by boiling for 10 min. The hydrolysates were analyzed by high-performance anion-exchange chromatography and by ^{1}H-NMR spectroscopy.

2.3. Analytical methods

Analysis with high-performance anion-exchange chromatography on a Dionex 4500i series Chromatograph with pulsed amperometric detection (HPAEC-PAD) was performed as described earlier [17]. For NMR analysis the lyophilised samples were dissolved in D_2O (0.7 ml) and the pH was adjusted to 7 with NaOD or DCl. ^{1}H-NMR spectra were recorded at 27°C using a Varian Unity 600 MHz spectrometer. Typical acquisition parameters were: a 70° pulse of 8 μs, a spectral width of 8000 Hz and a repetition time of 22 s.

3. Results and discussion

Acidic xylo-oligomers used as substrates for β-xylosidase were isolated from a birch kraft pulp hydrolyzate obtained by extensive xylanase treatment [16]. Xylo-oligosaccharides substituted with either 4-*O*-methylglucuronic acid (MeGlcA) or hexenuronic acid (HexA) were obtained. Hexenuronic acid is formed from 4-*O*-methylglucuronic acid by β-elimination of methanol during kraft pulping [18]. Arabinose (Ara)-substituted xylo-oligomers were obtained from a pine kraft pulp

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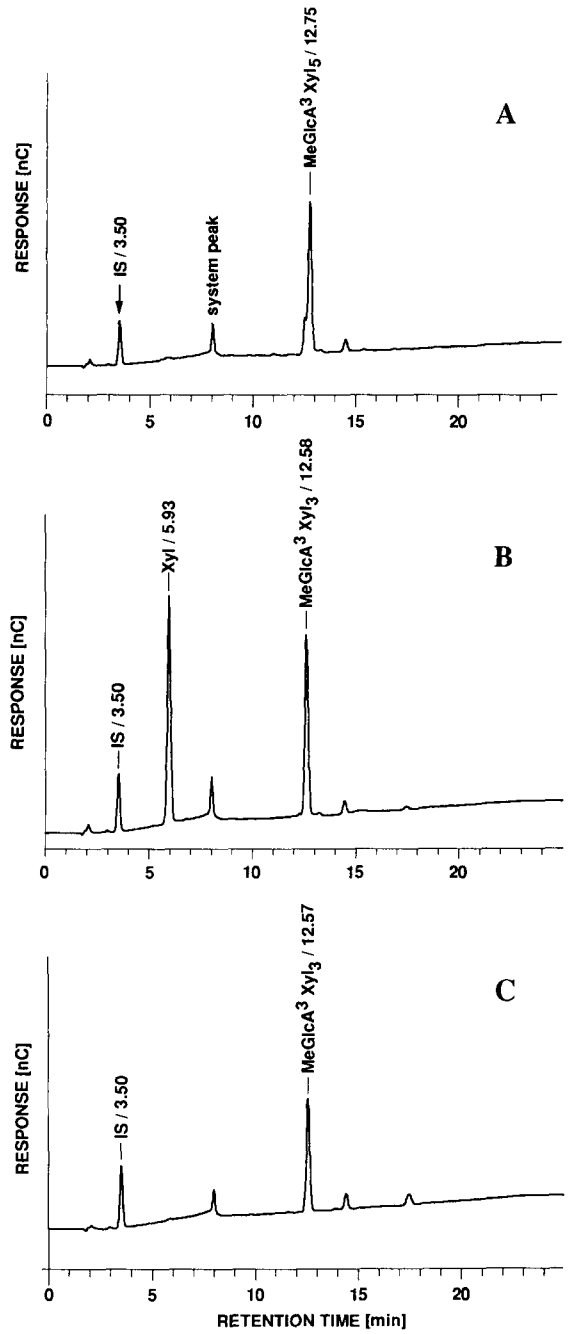

Fig. 1. Degradation of MeGlcA^{3}Xyl$_5$ by *T. reesei* β-xylosidase. HPAEC-PAD chromatogram before (A) and after β-xylosidase hydrolysis (B) and of MeGlcA^{3}Xyl$_3$ (C). IS, internal standard.

hydrolyzate [16]. The primary structures of the isolated oligosaccharides were elucidated by ^{1}H-NMR spectroscopy. Published reference data on chemical shifts and coupling constants for the anomeric protons of arabinoxylo-oligosaccharides [19,20] and 4-*O*-methylglucuronic acid and/or hexenuronic acid xylo-oligosaccharides [18,21,22] were used to distinguish the different structural elements. Integration of the anomeric proton signals revealed the carbohydrate composition and provided an estimation of the purity of the sample. The degree of polymerisation of the oligomers was obtained by division of the integral of all anomeric proton signals with the integral of the reducing end anomeric resonances. Five of the isolated xylo-oligomers (Table 1) were treated with *T. reesei* β-xylosidase.

MeGlcA^{3}Xyl$_3$, in which the substituent is linked to the non-reducing end xylose unit, was not hydrolyzed by β-xylosidase (results not shown) whereas the two other acidic oligomers tested, both carrying the substituent in the internal xylose unit, were readily degraded (Table 1, Figs. 1 and 2). After β-xylosidase treatment the uronic acid substituted xylose was located at the non-reducing end of the xylo-oligosaccharide (Table 1, Fig. 3). Thus, β-xylosidase from *T. reesei* was able to cleave off all unsubstituted xylose units from the non-reducing end of xylo-oligosaccharides carrying a 1,2-linked 4-*O*-methylglucuronic acid or hexenuronic acid side group. The removal of unsubstituted xylose residues prior to the 4-*O*-methylglucuronic acid-substituted xylose unit of MeGlcA^{3}Xyl$_5$ and MeGlcA^{3}Xyl$_4$ by *T. reesei* β-xylosidase has also been suggested previously [9]. β-Glucosidase of *Thermoascus aurantiacus*, which is also able to degrade xylo-oligomers starting from the non-reducing end of the oligosaccharide, has been found not to be able to hydrolyze the linkage between the xylose carrying the MeGlcA group and the adjacent xylose residue [23].

Surprisingly Ara^{2}Xyl$_3$, which has an internal arabinose substituent, was not degraded by the β-xylosidase (results not shown). The AraXyl$_4$ preparation tested contained two different isomers, in which the arabinose was linked to one or other of the internal xylose units. This preparation was partially hydrolyzed by β-xylosidase (Table 1, Fig. 4). The ratio of Ara^{2}Xyl$_4$ to Ara^{3}Xyl$_4$ in the preparation used as substrate was about 2:1 as analyzed by ^{1}H-NMR spectroscopy. As seen from the HPAEC-PAD chromatogram, about 2/3 of the substrate was degraded to Ara^{2}Xyl$_3$ (Fig. 4). The degree of hydrolysis did not increase after longer incubation (results

Table 1
Action of β-xylosidase from *T. reesei* on different substituted xylo-oligosaccharides

Substrate		β-Xylosidase hydrolysis	Products
Abbreviation	Structure[a]		Structure[a]
MeGlcA^{3}Xyl$_4$	MeGlcA | Xyl-Xyl-Xyl	→	
MeGlcA^{3}Xyl$_5$	MeGlcA | Xyl-Xyl-Xyl-Xyl-Xyl	→	MeGlcA | Xyl-Xyl-Xyl + Xyl
HexA^{3}Xyl$_4$	HexA | Xyl-Xyl-Xyl-Xyl	→	HexA | Xyl-Xyl-Xyl + Xyl
Ara^{2}Xyl$_3$	Ara \ Xyl-Xyl-Xyl	→	
Ara^{3}Xyl$_4$	Ara \ Xyl-Xyl-Xyl-Xyl	→	
Ara^{2}Xyl$_4$	Ara \ Xyl-Xyl-Xyl-Xyl	→	Ara \ Xyl-Xyl-Xyl + Xyl

[a]-β-1,4-linkage; | = α-1,2-linkage for MeGlcA and β-1,2-linkage for HexA; \ = α-1,3-linkage. The reducing end is to the right.

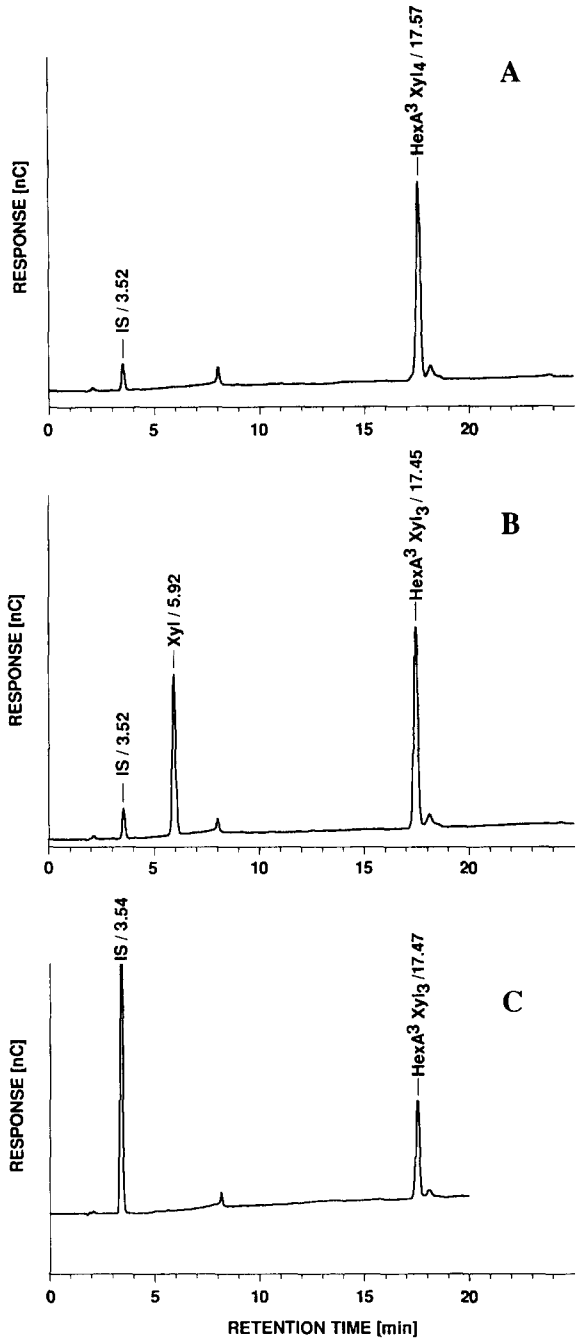

Fig. 2. Degradation of $HexA^3Xyl_4$ by *T. reesei* β-xylosidase. HPAEC-PAD chromatogram before (A) and after β-xylosidase hydrolysis (B) and of $HexA^3Xyl_3$ (C). IS, internal standard.

not shown). Thus, the β-xylosidase was able to hydrolyze Ara^2Xyl_4 to Ara^2Xyl_3 but Ara^3Xyl_4 was not degraded. The β-xylosidase from *T. reesei* has been reported to exhibit the α-arabinofuranosidase activity toward *p*-nitrophenyl-α-L-arabinofuranoside [12,13]. However, no free arabinose was formed in the hydrolysis of Ara^2Xyl_3, Ara^2Xyl_4 or Ara^3Xyl_4 (Fig. 4). Therefore, the enzyme is not able to liberate α-1,3-linked L-arabinofuranose from the xylo-oligosaccharides carrying an internal arabinose side group.

According to the results obtained, the β-xylosidase from *T. reesei* is able to hydrolyze the β-1,4-linkage between an unsubstituted and a 1,2-linked uronic acid substituted xylose unit. The arabinose side group linked at O-3 of the xylopyranose residue appears to protect the β-1,4-xylosidic linkage before the substituted xylose unit from being cleaved by the β-xylosidase. One explanation for the difference between the hydrolysis of arabinose- and uronic acid-containing xylo-oligosaccharides might be the different structures of the substituents. However, this is not a probable reason because oligosaccharides containing either 4-*O*-methylglucuronic acid or hexenuronic acid were both degraded in the same way, although these two acidic groups have clearly different structures [18]. In fact, the results indicate that the position of the substituent affects the specificity of the enzymatic cleavage. Most probably the presence of a monosaccharide unit at O-3 sterically hinders the formation of the enzyme-substrate complex and thus impedes enzymatic hydrolysis. Earlier a β-xylosidase from *Aspergillus awamori* was reported not to remove the non-reducing end xylose adjacent to an arabinose-substituted xylose [24]. Thus, the action of this enzyme, analogously to that of *T. reesei* β-xylosidase, was limited by the α-1,3-linked arabinofuranosyl substituent. On the other hand, Takenishi and Tsujisaka found that *Aspergillus niger* β-xylosidase cleaved off xylose from Ara^1Xyl_2 reduced by sodium borohydride [25]. However, the xylitol formed in the reduction exists in an open-chain form whereas for the substrates used for *T. reesei* and *A. awamori* β-xylosidases the internal xylose with an α-1,3-linked arabinose exists as a pyranose ring. Thus, it seems that the α-1,3-linked arabinose does not block the β-1,4-xylosidic linkage for the action of β-xylosidase when the substrate structure is more flexible as in β-D-Xyl*p*-(1→4)[α-L-Ara*f*-(1→3)]-D-xylitol.

Hardwood xylans contain acetyl substituents esterified both at the O-2 and O-3 positions of the xylose residue [26]. As the acetyl substituent is much smaller than the arabinofuranosyl substituent, it would be interesting to determine whether it is

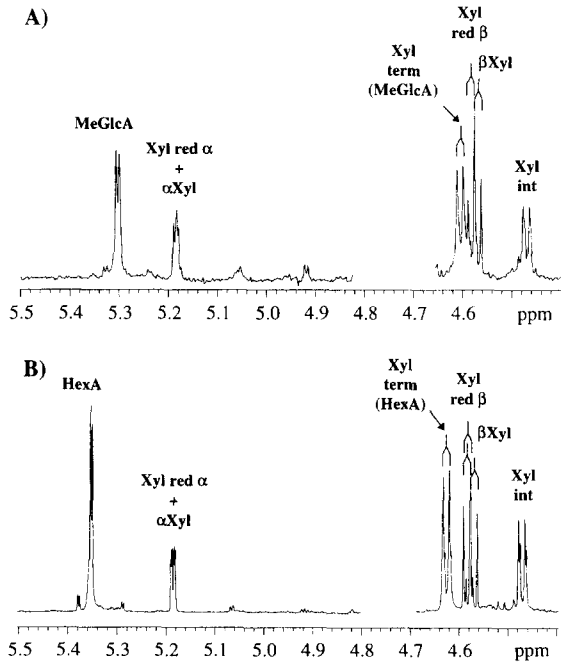

Fig. 3. Anomeric region of ^{1}H-NMR spectra of *T. reesei* β-xylosidase hydrolysate of (A) $MeGlcA^3Xyl_5$ and (B) $HexA^3Xyl_4$. red α, reducing end α; αXyl, α-D-xylose; red β, reducing end β; βXyl, β-D-xylose; int, internal; Xyl term (MeGlcA), terminal xylose with an α-1,2-substituted MeGlcA; Xyl term (HexA), terminal xylose with a β-1,2-substituted HexA. The HDO peak at 4.74 ppm is not shown.

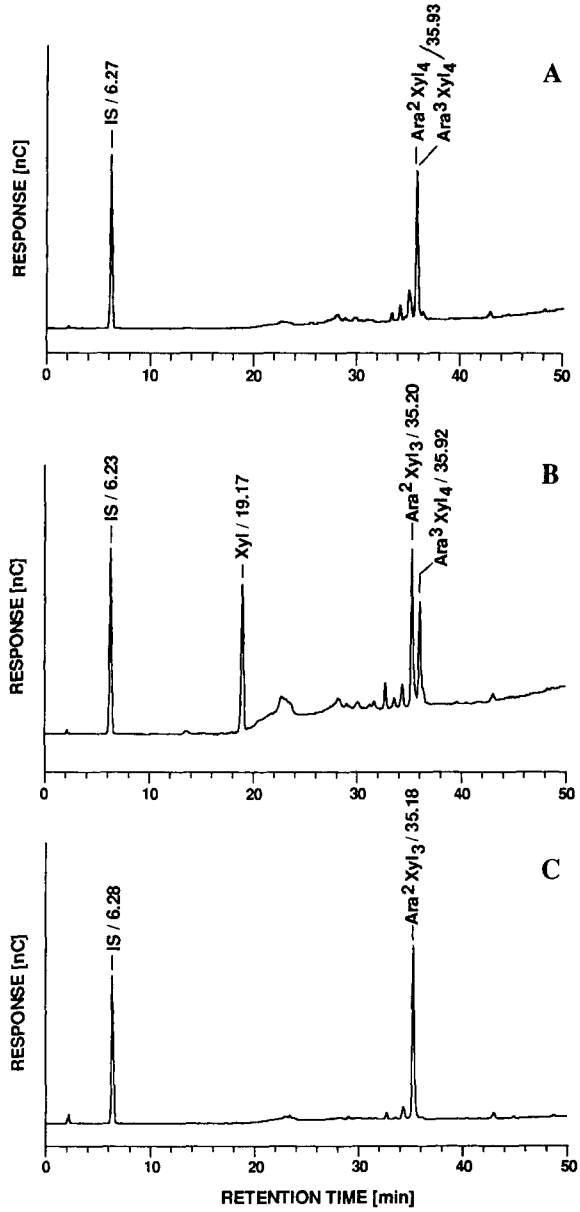

Fig. 4. Degradation of Ara^2Xyl_4 and Ara^3Xyl_4 by *T. reesei* β-xylosidase. HPAEC-PAD chromatogram before (A) and after hydrolysis (B) and of Ara^2Xyl_3 (C). IS, internal standard.

also able to hinder the action of β-xylosidase when linked at O-3.

The differences in the action of β-xylosidase against xylo-oligosaccharides containing 1,3-linked arabinose and 1,2-linked uronic acids were investigated in this study by using one β-xylosidase produced by *T. reesei*. The β-xylosidase from *T. reesei* has been found to belong to a different glycosyl hydrolase family than the other β-xylosidases hitherto classified [13] and it is possible that the β-xylosidases in different families have different substrate specificities, as has been reported for different endoxylanases [27]. The β-xylosidases from *A. awamori* and *A. niger* and the β-glucosidase from *T. aurantiacus* have not been classified. A general picture of the effects of different substituents on the action of β-xylosidases requires the testing of several β-xylosidases belonging to different glycosyl hydrolase families.

The novel information obtained in this work can be used in the structural analysis of different substituted xylo-oligosaccharides as well as in the production of xylo-oligomers with defined structures.

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