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Xylanase XYN IV from *Trichoderma reesei* showing exo- and endo-xylanase activity

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A minor xylanase, named XYN IV, was purified from the cellulolytic system of the fungus *Trichoderma reesei* Rut C30. The enzyme was discovered on the basis of its ability to attack aldotetraohexenuronic acid (HexA-2Xyl-4Xyl-4Xyl, HexA^{3}Xyl$_3$), releasing the reducing-end xylose residue. XYN IV exhibited catalytic properties incompatible with previously described endo-β-1,4-xylanases of this fungus, XYN I, XYN II and XYN III, and the xylan-hydrolyzing endo-β-1,4-glucanase EG I. XYN IV was able to degrade several different β-1,4-xylans, but was inactive on β-1,4-mannans and β-1,4-glucans. It showed both exo-and endo-xylanase activity. Rhodymenan, a linear soluble β-1,3-β-1,4-xylan, was as the best substrate. Linear xylooligosaccharides were attacked exclusively at the first glycosidic linkage from the reducing end. The gene *xyn4*, encoding XYN IV, was also isolated. It showed clear homology with xylanases classified in glycoside hydrolase family 30, which also includes glucanases and mannanases. The *xyn4* gene was expressed slightly when grown on xylose and xylitol, clearly on arabinose, arabitol, sophorose, xylobiose, xylan and cellulose, but not on glucose or sorbitol, resembling induction of other xylanolytic enzymes from *T. reesei*. A recombinant enzyme prepared in a *Pichia pastoris* expression system exhibited identical catalytic properties to the enzyme isolated from the *T. reesei* culture medium. The physiological role of this unique enzyme remains unknown, but it may involve liberation of xylose from the reducing end of branched oligosaccharides that are resistant toward β-xylosidase and other types of endoxylanases. In terms of its catalytic properties, XYN IV differs from bacterial GH family 30 glucuronoxylanases that recognize 4-*O*-methyl-D-glucuronic acid (MeGlcA) substituents as substrate specificity determinants.

Introduction

Xylans are the main hemicelluloses in plants, and exist in plant cell walls both in trees and in annual plants. Some algae also produce xylans, but there are no reports regarding microbial xylans. The plant xylans have a backbone consisting of β-1,4-linked D-xylopyranosyl units, which are further substituted with L-arabinofuranose

Abbreviations
Ara*f*, arabinofuranose; EG I, endo-β-1,4-glucanase I from *Trichoderma reesei*; GH, glycoside hydrolase; HexA, hexenuronic acid; HexA-2Xyl-4Xyl-4Xyl (HexA^{3}Xyl$_3$), aldotetraohexenuronic acid; MeGlcA, 4-*O*-methyl-D-glucuronic acid; MeGlcA-2Xyl-4Xyl-4Xyl (MeGlcA^{3}Xyl$_3$), aldotetraouronic acid; Xyl, D-xylose; Xyl$_2$–Xyl$_5$, β-1,4-xylobiose–β-1,4-xylopentaose; Xyl-4Xyl-4Xyl-Me, methyl β-xylotrioside; Xyl-Me, methyl β-D-xylopyranoside; XYN, endo-β-1,4-xylanase.

(L-Ara*f*), 4-*O*-methyl-D-glucuronic acid (MeGlcA) or esterified with acetic acid [1]. MeGlcA is normally linked to the O2 position, but L-Ara*f* and acetic acid may be linked to O2, O3 or both on the same xylosyl residue. The structure of xylan varies between plants, and may even vary in different parts of the same plant. Algae, such as red seaweed, produce mainly an unsubstituted linear xylan with β-1,3- and β-1,4-linkages in the ratio 1 : 2 [2].

The main enzymes degrading xylans are endo-β-1,4-D-xylanases (EC 3.2.1.8), which make random cuts in the xylan backbone. The best known microbial xylanases hydrolyzing all types of xylan belong to the glycoside hydrolase (GH) families 10 or 11 (http://www.cazy.org) [3]. Endoxylanases also occur in GH family 30 [4–10] (originally classified in GH family 5 [11]), GH family 8 [12–14] and GH family 5 [15]. Bacterial GH family 30 xylanases are specialized for hydrolysis of glucuronoxylan [6–9], while the fungal enzyme from *Bispora* sp. was shown to be more versatile [10]. The GH family 5 enzymes appear to be specialized for hydrolysis of arabinoxylan [15]. The side groups in xylans are cleaved by α-L-arabinofuranosidases (EC 3.2.1.55), α-glucuronidases (EC 3.2.1.139) and acetyl xylan esterases (EC 3.1.1.72). The formed oligosaccharides are degraded to xylose by β-xylosidases (EC 3.2.1.37), which remove xylose units from the non-reducing end of the xylooligosaccharides [16]. The side groups restrict the action of β-xylosidases, and thus only non-substituted oligosaccharides are degraded totally. Some β-xylosidases are reported to act also on polymeric xylan [17–19].

There are only a few reports on specific exo-β-1,4-xylanases, and information on their catalytic properties is limited. These enzymes differ from β-xylosidases as they are inactive on xylobiose. Xylanase II from *Chaetomium thermophile* var. *coprophile* is reported to produce mainly xylobiose as the final product [20]. Several xylanases from *Aeromonas caviae* ME-1 are also exo-acting, but it remains unknown which end of the oligosaccharides is their target [21–24]. The xylanases Xyn IV, Xyn V and Xyn X formed exclusively xylobiose, xylotetraose or both, respectively [21–24]. Another type of intracellular GH family 8 enzyme was recently discovered in *Bacillus halodurans* C-125 [13]. It was able to hydrolyze xylooligosaccharides with DP $\geq$ 3 by exo-action, releasing xylose units from the reducing end. However, it did not show activity on polymeric xylan and was thus named exo-oligoxylanase [14].

The filamentous fungus *Trichoderma reesei* is one of the best producers of cellulolytic and hemicellulolytic enzymes, and produces all necessary enzymes for xylan degradation [25]. Several commercial enzyme preparations are presently manufactured using strains of *T. reesei*. The fungus is reported to produce at least three specific endo-β-1,4-xylanases [26–28]. The two main xylanases, XYN I and XYN II, with molecular masses of 19 and 20 kDa and isoelectric points of 5.5 and 9.0, respectively, belong to GH family 11. The third xylanase, XYN III, is larger, with a molecular mass of 32 kDa. Its isoelectric point is 9.1, and it has been classified in GH family 10. *T. reesei* also possesses a fourth enzyme that is able to act on the internal β-1,4-linkages in xylans. This endo-β-1,4-glucanase I (EG I) of GH family 7, which cleaves internal β-1,4-glucosidic linkages in cellulose, is also highly active on xylans [29,30].

An interesting xylanolytic activity was previously detected as a contaminant in a *T. reesei* α-glucuronidase preparation [31]. This enzyme was able to liberate xylose from a xylooligosaccharide, that was not degraded by XYN I, XYN II or β-xylosidase from *T. reesei*. Here we report purification of this enzyme, named XYN IV, which is an additional specific xylan-degrading enzyme from *T. reesei*. The corresponding gene, *xyn4*, was also isolated, and, based on amino acid homology, XYN IV was found not to belong to the two major xylanase families, but to GH family 30, which is a very diverse family, including glucanases, mannanases and xylanases. As the catalytic properties of the XYN IV purified from a culture fluid were a combination of clear exo-action and weak endo-action, they were further confirmed for a recombinant enzyme produced in *Pichia pastoris*.

Results

Discovery of the new xylanase

In our previous study of an α-glucuronidase preparation from *T. reesei* [31], it was found that the enzyme did not cleave the glycosidic linkage between hexenuronic acid (HexA) and xylose in the tetrasaccharide $HexA^3Xyl_3$ [32]. Instead, $HexA^3Xyl_3$ was converted into $HexA^2Xyl_2$ by liberation of one xylose unit from the reducing end. This action on $HexA^3Xyl_3$ was a new catalytic activity not exhibited by any of the known xylan-depolymerizing enzymes from *T. reesei*, i.e. the specific endo-β-1,4-xylanases XYN I, XYN II and XYN III, the xylan-degrading EG I and one β-xylosidase. Consequently, this new enzyme was named XYN IV, and $HexA^3Xyl_3$ was used as a substrate throughout for its purification.

Production and purification of XYN IV

T. reesei Rut C30 was cultivated in a similar way as described previously for production of α-glucuronidase. The two-first chromatographic purification steps were performed according to the procedure for α-glucuronidase, with minor modifications [33]. The major xylanases of *T. reesei* were separated from XYN IV in the two-first steps, resulting in more than 10 000-fold reduction in the amount of xylanase activity in the sample. XYN IV was separated from α-glucuronidase and residual β-xylosidase in the last two steps using DEAE-Sepharose and gel filtration on Sephacryl S-100. As XYN IV is a very minor xylanase of *T. reesei* in terms of common xylanase assays, its purification degree and yield were not calculated.

Protein properties

The molecular weight of XYN IV is 43.0 kDa according to SDS/PAGE (Fig. 1). XYN IV had several isoforms with pI values of ~ 7 (results not shown). The N-terminal amino acid sequence was ?-S-Y-A-T-?-S-Q-Y-?-A-N-I-?-I (? = unclear or very weak signal). The amino acid sequences of two internal peptides were G-A-T-S-D-D-E-N-N and T-N-P-N-E-Y-V-Y-A-D-V-?-S-A-P. These partial sequences were sufficient to identify the corresponding *xyn4* gene in the *T. reesei* genome, and the correct amino acid sequence was obtained based on the gene sequence (see below).

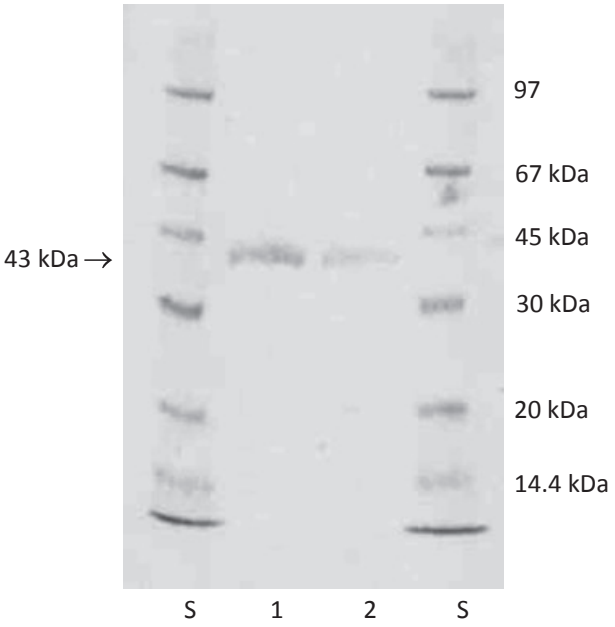

Fig. 1. SDS/PAGE of purified XYN IV. Samples: S, standards; lane 1, 5 μg XYN IV; lane 2, 2.4 μg XYN IV.

The specific β-1,4-xylanase activity of purified XYN IV on birch glucuronoxylan was only 16 nkat·mg^{-1}, which is < 1% of the specific activity of other *T. reesei* xylanases. The optimum pH for XYN IV was between pH 3.5 and 4.0 (5 min xylanase activity assay at 40 °C). The optimal temperature in a 5 min assay at pH 4.0 was between 40 and 50 °C. At room temperature and at pH values between 3.0 and 5.0 the activity of XYN IV remained unchanged in 24 h incubation. At temperatures higher than 50 °C, XYN IV lost activity after a few hours (results not shown). At 40 °C, it was most stable at pH 4.5, and at 50 °C it was most stable at pH 4.0.

Hydrolysis of xylans by XYN IV

The purified XYN IV was a specific xylanase. It did not show any action on glucomannan, galactomannan, β-1,3-β-1,4-glucan, laminarin or carboxymethyl cellulose. Nor did it show any α-arabinofuranosidase, α-galactosidase, β-mannosidase or β-xylosidase activity on the corresponding 4-nitrophenyl glycosides as substrates.

XYN IV was able to act on all three tested xylans (hardwood glucuronoxylan, wheat arabinoxylan and rhodymenan, the latter being the most rapidly hydrolyzed substrate) (Fig. 2). The main products of glucuronoxylan hydrolysis as shown by TLC were xylose, xylobiose and an aldotetraouronic acid (Fig. 3). Longer acidic xylooligosaccharides were also detected in small amounts. The enzyme also hydrolyzed

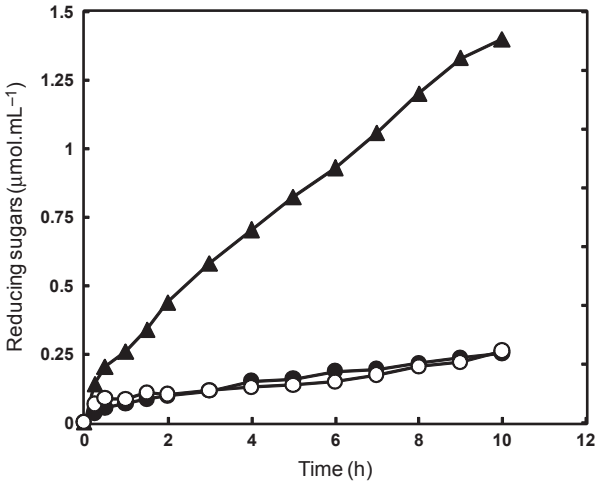

Fig. 2. Rate of hydrolysis of various xylans by XYN IV from *T. reesei* followed as liberation of reducing sugars expressed as xylose equivalents. Substrate concentration 10 mg·mL^{-1}; enzyme concentration 11.4 nkat·mL^{-1}. Black circle, glucuronoxylan; white circle, wheat arabinoxylan; black triangle, rhodymenan.

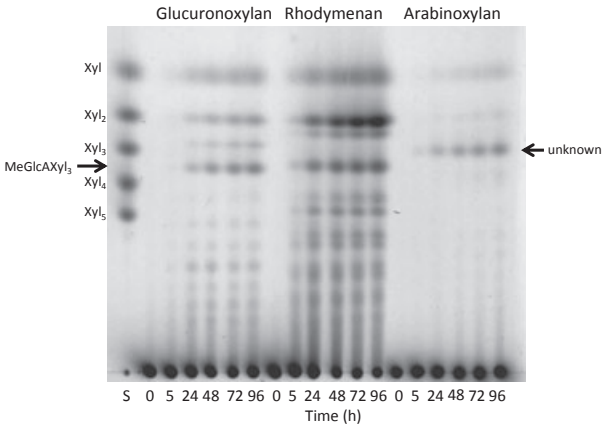

Fig. 3. Hydrolysis by XYN IV of glucuronoxylan, rhodymenan and wheat arabinoxylan, followed by TLC on silica gels developed twice in ethyl acetate/acetic acid/2-propanol/formic acid/water (25 : 10 : 5 : 1 : 15 v/v). Substrate concentration 10 mg·mL^{-1}; enzyme concentration 1.4 nkat·mL^{-1}. S, standards. Total sugars were detected using *N*-(1-naphthyl)ethylenediamine reagent.

arabinoxylan but to a limited extent, liberating xylose, Xyl_2 and an oligosaccharide. The latter product, presumably an $AraXyl_3$, showed similar migration as Xyl_3 on TLC. The much higher degree of endo-type hydrolysis observed in the case of rhodymenan requires a separate investigation. The endo-action of XYN IV was also demonstrated using xylan dyed with Remazol Brilliant Blue (RBB-xylan), a specific substrate for endoxylanases [34].

The structure of aldotetraouronic acid liberated from glucuronoxylan was established by enzyme-assisted analysis (Fig. 4). The compound did not serve as a substrate of the GH family 67 α-glucuronidase of *Aspergillus tubingensis* [35], which requires MeGlcA to be linked to the non-reducing xylopyranosyl residue of the xylooligosacccharide [35,36]. The aldotetraouronic acid was hydrolyzed by β-xylosidase to yield a product that is one xylose residue shorter, $MeGlcA^2Xyl_2$, that was readily hydrolyzed by GH family 67 α-glucuronidase to MeGlcA and xylobiose. The aldotetraouronic acid was also found to be attacked by *Pichia stipitis* α-glucuronidase of GH family 115 [37], to give free uronic acid and Xyl_3. The major acidic oligosaccharides liberated from glucuronoxylan therefore appear to have the structure Xyl-4(MeGlcA-2)Xyl-4Xyl ($MeGlcA^2Xyl_3$), which differs from the aldouronic acid liberated by GH family 10 endoxylanases ($MeGlcA^3Xyl_3$) [38]. Treatment of the whole glucuronoxylan hydrolysate with β-xylosidase resulted in the disappearance of xylobiose, an increase in the Xyl concentration, and conversion of the aldotetraouronic acid $MeGlcA^2Xyl_3$ into aldotriouronic acid $MeGlcA^2Xyl_2$. However, the minor products migrating as acidic oligosaccharides did not change significantly (data not shown). A parallel experiment with glucuronoxylan hydrolysate using *Erwinia chrysanthemi* GH family 30 xylanase showed clearly that all acidic oligosaccharides were converted to aldotriouronic acid $MeGlcA^2Xyl_2$, as described previously [8]. This suggests that XYN IV does not liberate aldouronic acids of the structure $MeGlcA^2Xyl_n$, as bacterial GH family 30 xylanases do. The formation of $MeGlcA^2Xyl_3$ during hydrolysis of glucuronoxylan is a unique property of the XYN IV enzyme.

The structure of the major xylooligosaccharide generated from wheat arabinoxylan was solved as Xyl-4(Ara-3)Xyl-4Xyl (Ara^2Xyl_3) by applying pure α-arabinofuranosidase and β-xylosidase on arabinoxylan hydrolysate in which XYN IV was denaturated by heating. The oligosaccharide was resistant to β-xylosidase and was converted to arabinose and xylotriose by α-arabinofuranosidase. Xylotriose was hydrolyzed to xylose in the second step by β-xylosidase. The oligosaccharide also showed identical chromatographic mobility as the authentic compound. As shown below, XYN IV generates the same tetrasaccharide from Ara^3Xyl_4, which excludes the structure Ara^3Xyl_3.

Action on linear xylooligosaccharides

XYN IV showed a typical exo-action on linear β-1,4-xylooligosaccharides. The liberation of xylose was accom-

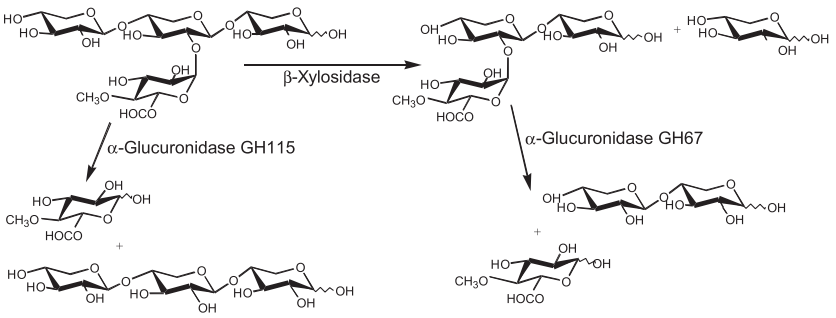

Fig. 4. Enzyme-assisted elucidation of the structure of the main aldouronic acid liberated from glucuronoxylan by *T. reesei* XYN IV.

panied by formation of shorter oligosaccharides, which were further digested to xylose and Xyl_2 (Table 1). An example of hydrolysis of 20 mM Xyl_5 followed by TLC is shown in Fig. 5A. Longer incubations provided clear evidence that Xyl_2 is subject to slow hydrolysis. No products larger than the substrate were observed in reaction mixtures of 20 mM oligosaccharides, indicating no significant transglycosylation activity of XYN IV.

The question of whether XYN IV attack of linear xylooligosaccharides occurs at the reducing or non-reducing end was answered using [1-^{3}H]-reducing end-labeled β-1,4-xylooligosaccharides. The radioactive label enabled determination of the initial bond cleavage frequencies of the substrates. [1-^{3}H]-xylotriose, -xylotetraose and -xylopentaose were attacked by the enzyme exclusively at the first glycosidic linkage from the reducing end, as [1-^{3}H]-xylose was found to be the only radioactive product generated from all labeled substrates (Fig. 6A). The bond cleavage frequencies of the first linkage from the reducing end were 1.0 in all three cases, which means that the oligosaccharides were strictly degraded from the reducing end by releasing the monomer. The pattern of attack of oligosaccharides was found to be the same at 0.025, 0.25, 5 and 20 mM substrate concentration, indicating that the enzyme forms only one type of productive complex with linear substrates, suggesting a small number of subsites of the substrate-binding site. This is in contrast to xylanases of GH families 10 and 11, which form productive complexes with two substrate molecules, resulting in shifted substrate binding and catalysis of glycosyl transfer reactions at a 20 mM concentration of linear xylooligosaccharides [39,40].

Determination of the kinetic parameter k_o/K_m at a 0.025 mM concentration of [1-^{3}H]-labeled substrates (Table 2) provided unambiguous evidence that xylotriose and xylotetraose are the best substrates for the enzyme, and that xylobiose serves as a poor substrate. Based on the kinetic parameters and bond cleavage frequencies, it may be deduced that the enzyme substrate-binding site consists of one subsite on the right of the catalytic groups (+I) and at least two subsites to the left of the catalytic groups (−I and −II). Calculation of subsite affinities [41] based on the k_o/K_m parameters (with a bond cleavage frequency of 1.0 in all cases) gave the following values: subsite −II, 4.1 kcal·mol^{-1}; subsite −III, −0.05 kcal·mol^{-1}; subsite −IV, −1.5 kcal·mol^{-1}. Thus subsite −II appears to be decisive for binding of linear xylooligosaccharides. The affinities of subsites +I and −I, or the sum of affinities of these two subsites, could not be calculated on the basis of obtained data. However, the fact that xylobiose is a very poor substrate suggests that the value will be very low.

Table 1. Products of hydrolysis of various substrates at 20 and 2 mM concentrations as indicated, with XYN IV at a concentration 1.4 nkat·mL^{-1}. Aliquots were taken at intervals for TLC for product analysis and semi-quantitative evaluation of the hydrolysis rate.

Compound	Cleavage products	Disappearance of the substrate
Xyl-Me	No hydrolysis	–
Xyl-4NPh	No hydrolysis	–
Xyl_2	Xyl	(+)
Xyl_3	Xyl + Xyl_2	+++
Xyl_4	(Xyl_3 + Xyl) → Xyl_2 + Xyl	+++
Xyl_5	(Xyl_4 + Xyl) → (Xyl_3 + Xyl) → etc.	+
Xyl-4Xyl-Me	Xyl_2 + MeOH	(+)
Xyl-4Xyl-4Xyl-Me	Xyl_2 + Me-Xyl	+++
Xylotetraitol (Xyl_4-ol)	(Xylitol + Xyl_3)[a] → Xyl_2 + Xyl + Xylitol	(+)
Xyl-4Xyl-4NPh	Xyl_2 + 4NPh-OH	++
Xyl-4Xyl-MeUmb[b]	Xyl_2 + MeUmb-OH	+
Xyl-4Xyl-4Xyl-MeUmb[b]	Xyl_3 + MeUmb-OH	+
$HexA^3Xyl_3$[b]	Xyl + $HexA^2Xyl_2$	++
$MeGlcA^3Xyl_3$[b]	Xyl + $MeGlcA^2Xyl_2$	++
$MeGlcA^3Xyl_4$[b]	Xyl + $MeGlcA^2Xyl_3$	++
Ara^2Xyl_4[b]	Ara^2Xyl_3 + Xyl	+
Ara^2Xyl_3[b]	No hydrolysis	–

[a] Xyl_3 was not observed as an intermediate due to fast hydrolysis. [b] Substrate concentration 2 mM. (+), poor substrate; +++, excellent substrate.

Hydrolysis of modified and non-linear xylooligosaccharides

Table 1 also shows the action of XYN IV on a series of glycosides and acidic oligosaccharides. The product analysis and semi-quantitative evaluation of the rate of hydrolysis was performed by TLC. XYN IV did not attack methyl and 4-nitrophenyl β-D-xylopyranoside. These substrates are perhaps too small to be bound productively in the substrate-binding site, or may contain an unfavorable hydrophobic aglycon. Xyl-4Xyl-Me hydrolysis was as slow as that of Xyl_2, but produced mainly methanol and Xyl_2. As may be expected, the substrate that is one xylopyranosyl residue longer, Xyl-4Xyl-4Xyl-Me, was hydrolyzed much faster to give Xyl_2 and methyl β-D-xylopyranoside. A cleavage mode that allowed liberation of a fragment larger than Me-Xyl from the reducing end was not observed. This result indicates that the subsite of the enzyme substrate-binding site to the right of the catalytic groups (subsite +I) recognizes the methyl glycoside part of the substrate similarly to the reducing-end xylopyranose. Additional information regarding the

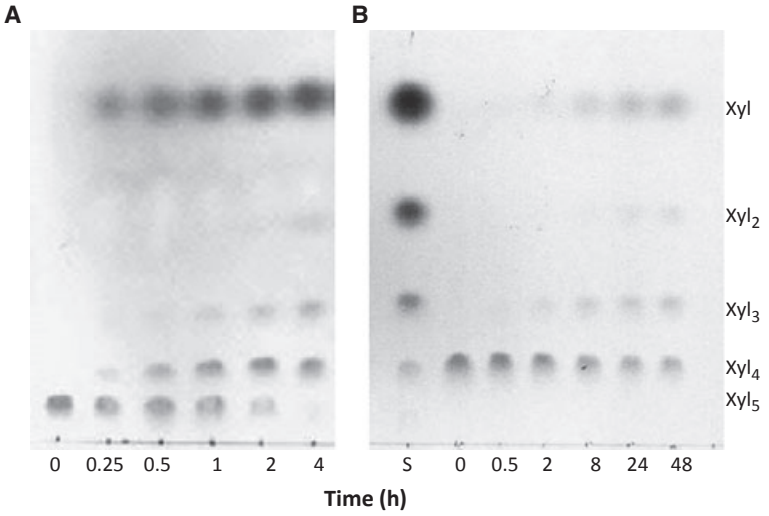

Fig. 5. TLC analysis (on microcrystalline cellulose) of the products of hydrolysis of 20 mM Xyl_5 by XYN IV isolated from the growth medium (A) and of 20 mM Xyl_4 by recombinant XYN IV (B) in 0.05 M sodium acetate buffer (pH 4.0). Enzyme concentration 1.4 nkat·mL^{-1}. S, standards.

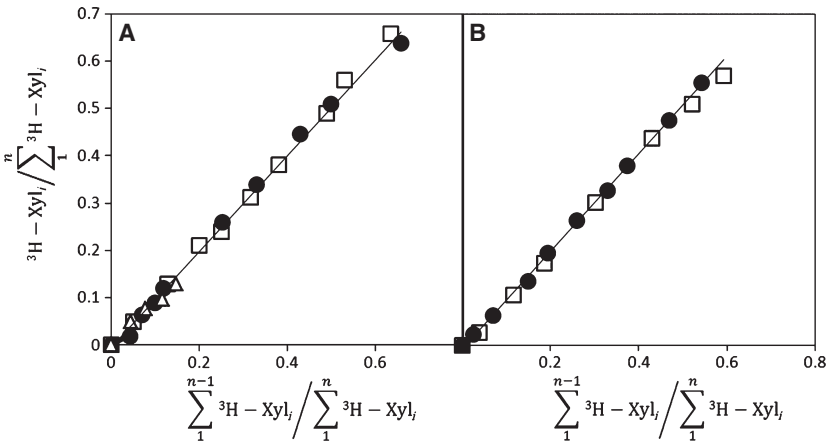

Fig. 6. (A) Liberation of [1-^{3}H]-xylose as the only radioactive product of hydrolysis of 0.025 mM [1-^{3}H]-labeled Xyl_3 (black circle), Xyl_4 (white square) and Xyl_5 (black triangle) by XYN IV isolated from the growth medium. (B) Liberation of [1-^{3}H]-xylose as the only radioactive product of hydrolysis of 0.025 mM [1-^{3}H]-labeled Xyl_4 (white square) by recombinant XYN IV. The concentration of the enzyme was in the range 0.35–7.0 nkat·mL^{-1}. The slope of the function corresponds to the initial bond cleavage frequency of oligosaccharides, which, in this case, is equal to 1.0.

Table 2. Kinetic parameters k_o/K_m of XYN IV for β-1,4-xylooligosaccharides obtained at 0.025 mM substrate concentration. The enzyme concentration varied from 0.35 to 7 nkat·mL^{-1}.

Xylooligosaccharide	k_o/K_m (M^{-1} s^{-1})
Xylobiose	5.1×10^1
Xylotriose	4.7×10^4
Xylotetraose	4.3×10^4
Xylopentaose	3.7×10^3

'plus' subsites of the substrate-binding site was obtained using xylotetraitol as a substrate. The reduced tetrasaccharide was hydrolyzed much more slowly than Xyl_4. The first step was formation of xylitol and Xyl_3, which, because it is a much better substrate than xylotetraitol, was cleaved immediately at the reducing end to xylose and Xyl_2.

Aryl glycosides of xylobiose and xylotriose, Xyl-4Xyl-NPh, Xyl-4Xyl-MeUmb and Xyl-4Xyl-4Xyl-MeUmb, were cleaved by XYN IV exclusively at the aglyconic linkage.

$HexA^3Xyl_3$, $MeGlcA^3Xyl_3$ and $MeGlcA^3Xyl_4$ were shortened to $HexA^2Xyl_2$, $MeGlcA^2Xyl_2$ and $MeGlcA^2Xyl_3$, respectively, by liberation of one xylose residue from the reducing end (Table 1). Similar behavior of XYN IV on the side-chain sugar was observed with 3-*O*-L-arabinofuranose-substituted xylooligosaccharides. Of Ara^2Xyl_4 and Ara^3Xyl_4, only the former oligosaccharide was attacked to give Ara^2Xyl_3 and xylose (Table 1).

Stereochemical course of hydrolysis of glycosidic linkages

Xyl-4Xyl-4Xyl-Me was found to be a suitable substrate for the ^{1}H-NMR study of the stereochemical course of hydrolysis of glycosidic linkages by XYN IV. The glycoside does not have reducing end, and

consequently does not produce NMR signals in the anomeric region of the spectrum that interfere with monitoring of the newly formed reducing end. The compound was rapidly hydrolyzed to the β-anomer Xyl_2 and Xyl-Me. The β-anomer was slowly mutarotated to the α-anomer. This result confirms that XYN IV, similar to other GH family 30 enzymes, is a retaining glycoside hydrolase that uses a double displacement reaction mechanism for hydrolysis of glycosidic linkages.

Isolation and sequence analysis of the *xyn4* cDNA

In parallel with purification and characterization of XYN IV, we studied the regulation of protein expression in *T. reesei*. The *xyn4* cDNA was incidentally isolated in a screen of *Saccharomyces cerevisiae* in which cloning of transcription factors activating the *T. reesei pdi1* promoter was attempted. Among the cDNA clones obtained in the screening, four were found to be derived from the same gene, and one of them was sequenced. A sequence database search revealed that the cDNA has homology with GH family 30 xylanases from *Aeromonas caviae* and *Erwinia chrysanthemi*. Comparison of the putative amino acid sequence encoded by the cDNA with peptide sequencing data from the isolated XYN IV enzyme revealed that the cDNA encodes this xylanase. Thus the gene was named *xyn4*.

The *xyn4* cDNA encodes a protein of 465 amino acids. The N-terminal sequence obtained from XYN IV matches the *xyn4* gene from amino acid position 18 onwards, and thus the signal peptide is 17 amino acids in length. The predicted molecular weight of the mature XYN IV is 50.3 kDa. *T. reesei* XYN IV has clear homology with the GH family 30 xylanases XynA from *Erwinia chrysanthemi* (25% identity, 41% similarity [4]), XynD and Xyn3 from *Aeromonas caviae* (24% identity, 40% similarity [5]), GH family 30 xylanase from *Bacillus subtilis* (25% identity, 41% similarity [7]) and *Paenibacillus barcinonensis* Xyn30D (23% identity, 37% similarity [9]) (Fig. 7). The closest homology was observed with XYLD from *Bispora* sp. MEY-1 (37% identity, 54% similarity) [10] and with the N-terminal part of a putative xylanase from the fungus *Leptosphaeria maculans* (37% identity, 54% similarity; Genbank accession number AAO49459.1) that has not been studied at the enzyme level. The *Leptosphaeria maculans* GH family 30 xylanase is much longer than XYN IV. Alignment of the *T. reesei* XYN IV with bacterial GH family 30 xylanases suggests that the proton donor and the nucleophile are Glu201 and Glu293, respectively (Fig. 7). The amino acids around the proton donor (Asn-Glu-Pro-Asp, NEPD) are conserved, but this appears not to be the case for the nucleophile amino acid. Of six aromatic amino acids contributing to substrate binding in bacterial enzymes (several tryptophans and tyrosines) that are conserved in bacterial enzymes, only one is conserved in fungal enzymes, and two are replaced with other aromatic amino acids in XYN IV (data not shown). The most interesting finding relevant to the mode of action of fungal GH family 30 xylanases was that Arg293 in *Erwinia chrysanthemi* and Arg303 in *Bacillus subtilis*, which are responsible for recognition of the MeGlcA residue in the substrate via ionic interaction with the MeGlcA carboxyl group [42,43] and are conserved in all bacterial GH family 30 xylanases, do not occur in fungal enzymes (Fig. 7).

A BLAST search of the *T. reesei* genome database (http://genome.jgi-psf.org/Trire2/Trire2.home.html) retrieved two gene models with significant homology to XYN IV. One of them (e_gw1.30.36.1) shows high homology with fungal β-1,6-glucanases, and the function of the second one (e_gw1.28.211.1) cannot be predicted based on homology.

The *xyn4* expression pattern

The expression level of *xyn4* as a function of culture conditions was investigated in *T. reesei* strain QM9414 by Northern hybridization. No *xyn4* expression was detected on glucose, suggesting that the gene is under catabolite repression. *xyn4* mRNA was not detectable in mycelia grown on sorbitol, or in a glucose depletion sample, which is unusual for *T. reesei* cellulases and hemicellulases [44]. A low *xyn4* mRNA level was detected in mycelia grown on xylose or xylitol, and a clearly higher level was detected in mycelia grown on L-arabinose and L-arabitol. The highest expression levels were detected on cellulose and the three xylans that were tested. When mycelia were grown with added sorbitol and either sophorose or xylobiose in the culture, the *xyn4* gene was clearly induced, more strongly with xylobiose than with sophorose.

In many respects, the expression pattern of *xyn4* resembles that of the other xylanase genes *xyn1* and *xyn2*, the β-xylosidase gene *bxl1* and the α-glucuronidase gene *glr1* (Table 3) [44], but is closest to that of *bxl1*. Both *xyn4* and *bxl1* are expressed on L-arabinose and are strongly induced by xylobiose, whereas *xyn1* and *xyn2* are not. The low expression level detected on xylose and xylitol is a particular feature of the *xyn4* gene (Table 3). None of the 12 cellulase and hemicellulase genes studied previously [44] was expressed in a xylose culture. Surprisingly, no *xyn3*

Fig. 7. Amino acid sequence alignment of the N-terminal parts (~320 amino acids) of the following GH family 30 xylanases: *Leptosphaeria maculans* Xyl38 (accession number Q873Z0), *Bispora* sp. MEY-1 XylD (accession number D6MYS9), *Trichoderma reesei* XYN IV (accession number AAP64786.1), *Aeromonas caviae* W-61 Xyn3 (accession number P70733), *Paenibacillus barcinonensis* Xyn30D (accession number AEY82463.1), *Bacillus subtilis* XynC (accession number Q45070) and *Erwinia chrysanthemi* XynA (accession number Q46961). The amino acid numbering shown on the right side is according to the native protein sequences. Catalytic glutamic acids acting as catalytic acid/base and nucleophile, are shown in bold and underlined. The amino acids corresponding to arginine conserved in bacterial sequences (e.g. Arg293 of *Erwinia chrysanthemi* XylA) and responsible for recognition of the MeGlcA residue in the substrate [42,43] are shown in bold, underlined and boxed. Aromatic amino acids lining the catalytic cleft and conserved in bacterial proteins are shaded in gray. Identical amino acids are indicated by asterisks, similar amino acids are indicated by colons, and conserved polar or non-polar amino acids are indicated by dots.

expression on any of the studied carbon sources was detected in the strain studied here. The expression pattern of the *xyn3* gene is known only for strain PC-3-7 [45], being expressed on cellulose, sorbose and sophorose but not on xylose, xylan or xylooligosaccharides.

Recombinant XYN IV

The unique catalytic properties of XYN IV, namely the combination of weak endo-action with clear exohydrolytic activity, prompted us to verify the catalytic properties of XYN IV isolated from the *T. reesei* growth medium using a recombinant enzyme. The XYN IV gene was expressed in *Pichia pastoris*, and the enzyme product was purified. The recombinant enzyme showed identical catalytic properties to the enzyme isolated from the *T. reesei* growth medium. It hydrolyzed xylans and linear and acidic xylooligosaccharides to the same products as XYN IV purified from *T. reesei*. The exo-action of the recombinant enzyme on Xyl_4 is shown in Fig. 5B. The experiment with [1-^{3}H]-reducing end-labeled xylooligosaccharides confirmed strict exo-action from the reducing end on linear xylooligosaccharides (Fig. 6B). Similar to the enzyme isolated from the culture medium, the recombinant enzyme showed a higher specific activity on rhodymenan than on glucuronoxylan or arabinoxylan. The aldouronic acids MeGlcA^{3}Xyl$_3$ and MeGlcA^{3}Xyl$_4$ were shortened by the recombinant enzyme, with the removal of one xylose residue from the reducing end.

Table 3. Expression patterns of five *T. reesei* genes involved in xylan degradation.The data on *xyn1*, *xyn2*, *glr1* and *bxl1* are from Margolles-Clark *et al.* [44]. The signal intensities based on visual judgment varied from non-detectable (–) to very strong (+++).

Carbon source	*xyn1*	*xyn2*	*xyn4*	*bxl1*	*glr1*
Glucose	–	–	–	–	–
Glucose depletion	–	++	–	++	+
Cellulose	++	++	+++	++	+
L-arabinose	–	–	++	+	–
L-arabitol	++	++	++	+++	–
Xylose	–	–	+	–	–
Xylitol	–	–	+	–	–
Lenzing xylan	++	–	+++	++	+
Glucuronoxylan	+	++	++	+(+)	(+)
Oat spelt xylan	+++	+	++	++	+
Sorbitol	–	(+)	–	–	–
Sorbitol+sophorose	+	+(+)	+	++	–
Sorbitol+xylobiose	–	(+)	++	++	–

The endo-action of the recombinant XYN IV was also confirmed on RBB-xylan. These results firmly excluded the possibility that any of the catalytic properties of XYN IV isolated from the growth medium of *T. reesei*, such as the weak endo-action, are due to contamination by another type of xylanase.

Discussion

$HexA^3Xyl_3$ is a substrate for xylanases that catalyze cleavage from the reducing end

Here we show that the non-natural hexenuronic acid-containing xylooligosaccharide, tetrasaccharide $HexA^3Xyl_3$, may serve as a specific substrate for screening of exoxylanases operating on the reducing end because this oligosaccharide is not attacked by α-glucuronidases. In contrast, the natural oligosaccharide $MeGlcA^3Xyl_3$ is attacked by α-glucuronidases [33, 35–37] and thus is not suitable for screening of exoxylanases in the presence of α-glucuronidases. In the present work, use of $HexA^3Xyl_3$ enabled discovery of a new type of xylanase from *T. reesei* RUT-C30. The enzyme catalyzed release of Xyl from the reducing end of $HexA^3Xyl_3$.

T. reesei XYN IV: exo- versus endo- mode of action

XYN IV exhibited completely different catalytic properties to previously described endo-β-1,4-xylanases of this fungus, or any other currently classified xylanase. An exception is a non-classified XynC from *Schizophyllum commune*. This enzyme has the ability to hydrolyze $MeGlcA^3Xyl_3$ to $MeGlcA^2Xyl_2$ and xylose; however, in contrast to XYN IV, the enzyme behaves as a typical endo-acting xylanase [46]. The exoxylanase from *Bacillus halodurans* (GH family 8) was shown to attack linear xylooligosaccharides in a similar manner but is an inverting hydrolase [13,14].

One of the major products formed by XYN IV from various xylans and xylooligosaccharides was xylose. All linear xylooligosaccharides studied were exclusively cleaved at the first glycosidic linkage from the reducing end. These data suggest that the XYN IV may be a unique exoxylanase. However, this mode of action may also be an activity of the enzyme that is limited to the tested substrates, which differ significantly from those that the enzyme encounters in nature, as xylans and derived xylooligosaccharides are often considerably acetylated.

The possibility that the low endo-action of XYN IV may be the result of minor contamination of the exoxylanase by an endoxylanase was the main reason why XYN IV isolated from the culture fluid was compared with a recombinant *T. reesei* XYN IV expressed in *Pichia pastoris*. The catalytic properties of both enzyme preparations were found to be identical, confirming that both the endo- and exo-action are inherent properties of this *T. reesei* GH family 30 xylanase. These properties are different from the properties of bacterial GH family 30 appendage-dependent endoxylanases that recognize MeGlcA substituents as substrate specificity determinants [6–9].

The specific β-1,4-xylanase activity of XYN IV on glucuronoxylan was extremely low, just a fraction of the values exhibited by the *T. reesei* XYN I and XYN II on the same polysaccharide substrate. In addition, the action pattern of XYN IV appears to be different from GH family 10 xylanases, which always produce $MeGlcA^3Xyl_3$ from glucuronoxylan, whereas GH family 11 xylanases produce $MeGlcA^3Xyl_4$ [38]. The main acidic oligosaccharide produced by XYN IV or by XynC from *S. commune* is $MeGlcA^2Xyl_3$ [46]. XYN IV also acted on arabinoxylan, releasing mainly xylose, xylobiose and one major Ara-substituted xylooligosaccharide, the structure of which was established as Ara^2Xyl_3. As in the case of the main aldotetraouronic acid generated from glucuronoxylan, the Ara-containing tetrasaccharide also contains Ara*f* linked to the middle xylopyranosyl residue of Xyl_3.

Information on the substrate-binding site of XYN IV was obtained by determining the bond cleavage frequencies and kinetic parameters k_o/K_m of the labeled xylooligosaccharides. The enzyme appears to have a substrate-binding site composed of three subsites: −II, −I and +I (Fig. 8). Subsite −II showed the

highest affinity for xylopyranosyl residues. This interaction appears to be decisive for hydrolysis of low-molecular-mass substrates. For unsubstituted β-1,4-linked xylooligosaccharide, there appears to be either a serious steric barrier or a xylose repulsive force at the position of the hypothetical subsite +II. In the case of polymeric substrates, this repulsive force must be overcome by additional enzyme–substrate interactions. This idea is supported by the facts that the endo-action of the enzyme is undisputable, and that the structure of the main aldouronic acid produced from glucuronoxylan is $MeGlcA^2Xyl_3$, suggesting accommodation of the MeGlcA-substituted xylopyranosyl residues at hypothetical subsite −II. Based on the ability of XYN IV to shorten the tetrasaccharide Ara^3Xyl_4 to Ara^2Xyl_3, it may be assumed that subsite −II can also accommodate 3-*O*-L-arabinosylated xylopyranosyl residues.

Additional considerations concern the subsites of the substrate-binding site around the catalytic groups of XYN IV. Xylobiose, which is an excellent substrate for β-xylosidases, was hydrolyzed three orders of magnitude more slowly than longer oligosaccharides. This result suggests that the sum of the affinities of subsites −I and +I must be negative, resulting in repulsion of xylobiose. The fact that conversion of xylotetraose to xylotetraitol led to a considerable reduction in the rate of hydrolysis of the same glycosidic linkage suggests that the reducing-end xylopyranose in linear oligosaccharides plays an important role in binding of the substrate at subsite +I, and that the binding affinity of this subsite is positive. The fact that Xyl_4 was almost as good a substrate as Xyl_3 (Table 1) is consistent with

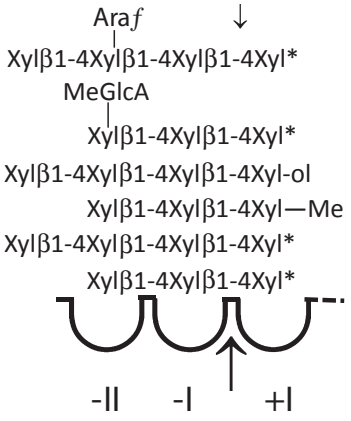

Fig. 8. Hypothetical subsites in the substrate-binding site of XYN IV, and productive complexes with the following substrates from bottom to top: Xyl_3, Xyl_4, methyl β-xylotrioside, xylotetraitol, aldotetraouronic acid $MeGlcA^3Xyl_3$ and arabinoxylotetraose Ara^2Xyl_4. The larger arrow indicates the enzyme catalytic groups, and the asterisks indicate the reducing end of oligosaccharides. The smaller arrow marks the points of cleavage.

the lack of substrate binding at hypothetical subsite −III. Cleavage of 4-nitrophenyl-β-D-xylobioside and 4-methylumbelliferyl-β-D-xylobioside at the aglyconic bond, in contrast to the resistance of 4-nitrophenyl-β-D-xyloside and 4-methylumbelliferyl-β-D-xyloside (Table 1), may be explained by a strong affinity of subsite −II for the xylopyranosyl residue.

XYN IV as a non-transglycosylating xylanase

The retaining character of XYN IV as an enzyme belonging to GH family 30 was confirmed by determination of the anomeric configuration of the newly formed xylobiose from methyl β-xylotrioside by ^{1}H-NMR spectroscopy. In this regard, it is interesting to note that no glycosyl transfer reactions were observed even at high substrate concentration. Given the existence of three subsites of the enzyme substrate-binding site, it is difficult to imagine how the transfer reaction may take place when the acceptor site is available to just the monomer, which is released after cleavage of the glycosidic linkage to the reducing-end xylose of the substrate. If the transfer reaction is catalyzed at high substrate concentrations, such transfer will always lead to resynthesis of the substrate molecule. Such a reaction may be demonstrated by incubation of the enzyme with radioactively labeled xylose and unlabeled xyloligosaccharide. The radioactive xylose will be incorporated into the resynthesized substrate molecules. However, such experiments have not yet been performed. It is well known that glycosyl transfer reactions are catalyzed by both GH family 10 and 11 endoxylanases at higher substrate concentrations. Under such conditions, the enzymes form a productive complex with two substrate molecules, a so-called termolecular (ternary) shifted complex [39–41,47,48]. Such enzymes have a larger number of subsites around the catalytic groups than XYN IV.

Position of XYN IV among xylanases and its physiological function

Currently all known bacterial GH family 30 xylanases are 'appendage-dependent' endoxylanases [49]. Their activity is strictly dependent on the presence of MeGlcA or GlcA side residues [6–9,49,50]. The decisive enzyme –substrate interaction for this substrate specificity was shown to be ionic interaction of one conserved arginine with the carboxyl group of the MeGlcA side residue [42,43]. The fungal GH family 30 xylanase from *Bispora* sp. also hydrolyzes arabinoxylan, with xylose and xylobiose as the main products of hydrolysis [10]. Similar to the *Bispora* sp. enzyme, *T. reesei* XYN IV

also does not recognize MeGlcA side chains as substrate specificity determinants. This maybe due to lack of the positively charged Arg close to the active site that is conserved in bacterial enzymes (e.g. Arg293 of *Erwinia chrysanthemi*) but is replaced by Gln or Glu in fungal enzymes (Fig. 7). The glutamic acid acting as the catalytic acid/base and the surrounding residues are quite conserved between bacterial and fungal enzymes. In contrast, a region around the catalytic nucleophile (Glu293 in XYN IV) does not show any significant homology with bacterial enzymes. The low degree of conservation of aromatic amino acids lining the substrate-binding site in bacterial enzymes also supports the view that the fine architecture of the substrate-binding site of XYN IV differs from that of bacterial enzymes. This is mainly reflected in a strict exo-action action of XYN IV on short linear xylooligosaccharides, in contrast to negligible activity of bacterial enzymes on substrates that do not contain uronic acid side chains. The abundance of xylose in xylan hydrolysates produced by the *Bispora* sp. GH family 30 xylanase [10] suggests that the *Bispora* sp. enzyme has a similar exo-acting mode of action on xylooligosaccharides to *T. reesei* XYN IV. The fungal GH family 30 xylanases clearly form a distinct sub-family as indicated previously [11]. However, the overall structure of fungal GH family 30 xylanases does not differ very much from bacterial enzymes. Of the GH family 30 xylanases, only the enzymes from *Erwinia chrysanthemi* and *Bacillus subtilis* have been crystallized and have been shown to consist of two domains: a catalytic domain and a putative xylan-binding domain composed of nine β-strands [42,43,51]. The auxiliary domain was suggested to be present in all GH family 30 enzymes, regardless their catalytic properties [11]. The modeled structure of the *Bispora* sp. enzyme confirms this suggestion [10]. In the case of the *Paenibacillus barcinonesis* enzyme, this domain was shown to be indispensable for the enzyme activity [9]. Its deletion abolished the enzyme activity [9]. All these facts suggest that the putative xylan-binding domain is also present in XYN IV and is crucial for its activity.

The exo-oligoxylanase of *Bacillus halodurans*, which also acts from the reducing end of the xylooligosaccharides, is an intracellular GH family 8 enzyme, but is inverting and inactive towards polymeric xylan [13,14]. Homologous GH family 30 Xyn3 from *Aeromonas caviae* strain W-61 has been reported to be a specific endo-1,4-β-xylanase that produces mainly long oligosaccharides with six or more xylose units [52,53].

Several questions concerning XYN IV remain open. What is the specific role of the enzyme in plant cell-wall degradation? Does the enzyme have a substrate recognition group? The recognition group does not seem to be MeGlcA, at least not to such an extent as in the case of bacterial GH family 30 xylanases. The present study provides evidence for the fact that, on xylooligosaccharides, the enzyme behaves mainly as a xylose monomer-producing catalyst. There is no apparent structural explanation for these catalytic activities of XYN IV.

The major role of the enzyme may therefore be release of xylose from the reducing end of xylooligosaccharides carrying a substituent close to the non-reducing end. This catalytic property is not exhibited by any known xylanolytic enzyme except the *Bacillus halodurans* intracellular xylanase, which does not recognize polymeric xylan as a substrate [13]. In this regard, the enzyme may be an important enzyme for saccharification of the major plant hemicellulose. However, it is difficult to explain the more pronounced endo-action of XYN IV on rhodymenan. Rhodymenan does not occur in the habitats of the fungus and is therefore not considered a natural substrate. It is obvious that further study is required to clarify the role of XYN IV in the life cycle of *T. reesei*. Of special interest remains elucidation of the mode of action of XYN IV on native partially acetylated plant xylans. The fact that all endoxylanases were designed by nature to attack partially but significantly acetylated polysaccharides is often neglected. Acetylation of xylan is not restricted to hardwood xylan; considerable acetylation has also been reported for xylans from cereals and annual plants, including *Arabidopsis thaliana* [54–56].

Experimental procedures

Substrates

Hardwood xylan with a reduced content of MeGlcA (Xyl:MeGlcA ratio of $\sim$ 27 : 1) was obtained from Lenzing AG (Lenzing, Austria). 4-*O*-Methylglucuronoxylan from birchwood (Xyl:MeGlcA ratio of $\sim$ 9 : 1, referred to throughout as glucuronoxylan) was purchased from Roth (Karlsruhe, Germany). Rhodymenan, an algal unsubstituted β-1,3-β-1,4-xylan, was generously supplied by A.I. Usov (Zelinski Institute of Organic Chemistry, Russian Academy of Sciences, Moscow, Russia). RBB-xylan was prepared as described previously [34]. Wheat arabinoxylan with a Ara:Xyl ratio of 41 : 59, glucomannan from konjac, galactomannan from locust beans and barley β-glucan were purchased from Megazyme (Wicklow, Ireland). Oat spelt arabinoxylan and laminarin were obtained from Sigma (St. Louis, MO) and carboxymethyl cellulose was obtained from Fluka (Lausanne, Switzerland).

β-1,4-xylooligosaccharides, from xylobiose (Xyl_2) to xylopentaose (Xyl_5), were obtained from Megazyme.

Xylotetraitol was prepared by reduction of the corresponding tetramer using $NaBH_4$. [1-^{3}H]-reducing end-labeled β-1,4-xylooligosaccharides (specific radioactivity ~ 50 MBq·μmol^{-1}) were prepared by custom catalytic tritiation performed at Nycom Ltd (Prague, Czech Republic), followed by chromatographic purification.

A xylotriose with an α-1,2-linked hexenuronic acid at the non-reducing end xylose unit ($HexA^3Xyl_3$) was isolated by anion exchange chromatography and gel filtration after enzymatic hydrolysis of alkali-cooked birch pulp [32]. MeGlcA-substituted oligosaccharides ($MeGlcA^3Xyl_3$, $MeGlcA^3Xyl_4$) and arabinose-substituted xylooligosaccharides (Ara^2Xyl_3, Ara^2Xyl_4 and Ara^3Xyl_4) were obtained as described previously [17,40].

The oligosaccharide methyl glycosides β-D-Xylp-(1,4)-β-D-Xylp-*O*-Me (Xyl-4Xyl-Me) (methyl β-xylobioside) and β-D-Xylp-(1,4)-β-D-Xylp-(1,4)-β-D-Xylp-*O*-Me (Xyl-4Xyl-4Xyl-Me) (methyl β-xylotrioside) [57,58] were generous gifts from P. Kovac and J. Hirsch (Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia). 4-nitrophenyl and methyl β-D-xylopyranosides (Me-β-Xyl) were obtained from Sigma. 4-methylumbelliferyl β-xylobioside and β-xylotrioside were prepared as described previously [59].

Production and purification of XYN IV

T. reesei strain Rut C30 was used as an enzyme producer. It was cultivated in a bioreactor with a working volume of 28 L. The fermentation conditions were: temperature 29 °C, pH controlled between 6.0 and 6.5 by automatic addition of NH_4OH or H_3PO_4, $pO_2 \geq 20\%$ controlled by agitation, aeration 5 L·min^{-1}, cultivation time 75 h. The culture medium used contained 30 g·L^{-1} xylan from Lenzing AG, 15 g·L^{-1} distiller's spent grain, 5 g·L^{-1} KH_2PO_4 and 5 g·L^{-1} $(NH_4)_2SO_4$. After cultivation, the mycelium was separated by centrifugation for 20 min, 4000 ***g*** in Cryofuge 8000 centrifuge (Heraeus, Sepatech, Osterode, Germany), and the culture filtrate was concentrated to 1.8 L by ultrafiltration (20 kDa cut-off, type ES 625, PCI Membrane Systems Ltd, Basingstoke, Hampshire, UK).

The concentrated culture filtrate (1800 mL) was adjusted to pH 4.5 and conductivity 17 mS·cm^{-1}, after which it was loaded onto a CM-Sepharose FF column (2 L, height 20 cm) equilibrated with 50 mM sodium acetate buffer pH 4.5 containing 0.14 M NaCl. The chromatografic columns and media for protein were acquired from Pharmacia, presently GE Healthcare (Little Chalfont, UK). After washing unbound materials from the column, bound proteins were eluted using a linear gradient of 0.14–0.40 M NaCl in 50 mM sodium acetate buffer pH 4.5 at a flow rate of 150 mL·min^{-1}. Fractions in 450 mL volumes were collected and measured for activity against $HexA^3Xyl_3$. The total volume of combined active fractions was 3150 mL, containing 0.6 mg·mL^{-1} protein.

Before the next purification step, the pH of the pool was adjusted to pH 5.5 and the conductivity was increased to 133 mS·cm^{-1} by addition of $(NH_4)_2SO_4$. The adjusted sample was loaded on a phenyl Sepharose FF column (1100 mL, height 11 cm; GE Healthcare, Little Chalfont, UK) equilibrated by 25 mM sodium acetate buffer pH 5.5, containing 0.85 M $(NH_4)_2SO_4$. Elution of XYN IV was performed by reducing the salt concentration linearly from 0.85 to 0.6 M. The flow rate was 90 mL·min^{-1}. Fractions of 450 mL were collected and analyzed for the presence of XYN IV. Active fractions were pooled (8550 mL) and concentrated by ultrafiltration to 1200 mL.

The concentrate was further buffer-exchanged into 20 mM sodium phosphate buffer pH 6.0 by gel filtration on a Sephadex G-25 column (4 L, height 40 cm). Then the sample was applied to a CM-Sepharose FF column (350 mL, height 18 cm high; GE Healthcare, Little Chalfont, UK). After washing the unbound impurities from the column, XYN IV was eluted by an increasing NaCl gradient from 0 to 0.4 M. The flow rate used was 45 mL·min^{-1}, and the fraction size was 90 mL. XYN IV activity was shown in two peaks, which were first pooled separately but later combined after final purification step (gel filtration) as they both contained XYN IV (different isoforms). Both pooled fractions (1350 and 630 mL) were concentrated by ultrafiltration to 100 mL.

Final purification of XYN IV was performed by gel filtration on a Sephacryl S-100 HR column (width 5.7 cm, height 100 cm; GE Healthcare, Little Chalfont, UK). The eluent used was 50 mM sodium acetate buffer pH 4.5 containing 0.1 M NaCl, and the flow rate was 33 mL·min^{-1}. The combined active fractions were concentrated and used for XYN IV characterization.

Auxiliary enzymes

Purified GH family 67 α-glucuronidase from *Aspergillus tubingensis* [35] was generously supplied by J. Visser and R.P. de Vries (Wageningen Agricultural University, The Netherlands). Purification of GH family 115 α-glucuronidase from *Pichia stipitis* was performed as described by Ryabova *et al.* [37]. The β-xylosidase was *Aspergillus niger* enzyme produced in recombinant *Saccharomyces cerevisiae* [36]. The GH family 51 α-L-arabinofuranosidase from *A. niger* was obtained from Megazyme. The GH family 30 glucuronoxylanase from *Erwinia chrysanthemi* was supplied by J.F. Preston (University of Florida, Department of Microbiology and Cell Science, Gainesville, FL, USA).

Activity assays

The activity of XYN IV during its purification was followed qualitatively using $HexA^3Xyl_3$ as substrate. $HexA^3Xyl_3$ (0.5 mg·mL^{-1}) in 0.1 M sodium citrate buffer, pH 4, was incubated with the enzyme overnight at 40 °C, after which the reaction was terminated by boiling. The samples were analyzed for liberated xylose and $HexA^2Xyl_2$ by TLC on

silica gel-coated sheets (Merck, Darmstadt, Germany) developed in ethyl acetate/acetic acid/water (6 : 4 : 1 v/v). Sugars were visualized using orcinol reagent (Sigma, St. Louis, MO, USA). β-1,4-xylanase activity was determined as described by Bailey *et al*. [60] using 1% 4-*O*-methylglucuronoxylan as the substrate. After determination of the pH optimum of purified XYN IV, the xylanase assay was performed at pH 4.0. The activity is expressed in katals (kat).

The activity of XYN IV on konjac glucomannan, locust bean galactomannan, barley β-glucan, carboxymethyl cellulose and laminarin was tested by incubating 0.5% polysaccharide solution in 0.1 M sodium citrate buffer, pH 4.0, with XYN IV (35 $mg \cdot g^{-1}$) for 24 h at 40 °C.

The α-L-arabinofuranosidase, α-galactosidase, β-xylosidase and β-mannosidase activities of XYN IV were examined using the corresponding 4-nitrophenyl glycosides as substrates [61].

Investigation of protein properties

SDS/PAGE was performed on a 10% gel slab as described previously [62]. Isoelectric focusing was performed on a 5% polyacrylamide gel using a Multiphor II system (Pharmacia, Uppsala, Sweden) with pH gradient 3.5–9.5. Proteins were stained by silver staining (silver stain kit; Bio-Rad, Hercules, CA, USA).

The N-terminal amino acid sequence of the intact XYN IV and the amino acid sequence of two internal peptides (produced by trypsin degradation and purified by HPLC using C18 reversed-phase column) were analyzed by automated Edman degradation at the University of Kuopio (Finland). Protein content was analyzed as described previously [63] using bovine serum albumin as standard.

The pH optimum of XYN IV was determined at 40 °C in McIlvaine buffers with pH values between 3.0 and 8.0. The effect of different temperature (30, 40, 50 and 60 °C) on enzyme activity was followed at pH 4.0. The stability of XYN IV was studied at pH 3.0 and 5.0 at room temperature, 40 and 50 °C, by incubating purified XYN IV for 24 h.

Hydrolysis experiments with xylans and xylooligosaccharides

Various xylans (5 or 10 $g \cdot L^{-1}$) dissolved in 0.1 M sodium citrate buffer or 0.05 M sodium acetate buffer, pH 4.0, were treated with 500 $nkat \cdot g^{-1}$ (31 $mg \cdot g^{-1}$) of XYN IV at 40 °C. Liberation of reducing sugars was determined according to the Somogyi–Nelson procedure [64]. The products of hydrolysis of various xylans, linear xylooligosaccharides, their glycosides, hexenuronic acids and aldouronic acids (at 2 or 20 mM substrate concentrations as indicated) with XYN IV (10–500 $nkat \cdot g^{-1}$ substrate) in 0.1 M sodium citrate buffer or 0.05 M sodium acetate buffer (both pH 4.0) were analyzed by TLC on silica gel-coated sheets (Merck) developed in 1-butanol/ethanol/water (10 : 8 : 5 v/v) or in ethyl acetate/acetic acid/2-propanol/formic acid/water (25 : 10 : 5 : 1 : 15 v/v), or on microcrystalline cellulose (Merck) using ethyl acetate/acetic acid/water (18 : 7 : 10 or 3 : 2 : 2 v/v). On silica gel-coated sheets, sugars were visualized using orcinol or *N*-(1-naphthyl)ethylenediamine reagent. On cellulose, reducing sugars were detected using aniline-hydrogenphthalate reagent. The main aldouronic acid liberated by XYN IV from glucuronoxylan was purified from the hydrolysate by preparative TLC. On chromatograms, the compound was localized by detection of sugars on guide strips and eluted from the silica gel by methanol.

Bond cleavage frequencies and kinetic parameters

Bond cleavage frequencies [39–41,48,49] of [1-^{3}H]-reducing end-labeled β-1,4-xylooligosaccharides were determined in 0.05 M sodium acetate buffer, pH 4.0, at 30 °C at four substrate concentrations (0.25, 2, 5 and 20 mM) using varying enzyme concentrations. Aliquots of the mixture were taken after various time intervals and analyzed by TLC on cellulose-coated sheets. After detection of guide strips with xylose and xylooligosaccharides as standards, the corresponding areas of the chromatograms were cut out and the radioactivity of the substrate and its hydrolysis products was counted in toluene scintillation fluid using a 1214 RACKBETA liquid scintillation counter (LKB Wallac, Turku, Finland). The fraction of total radioactivity for all compounds was calculated using

$$^3H - Xyl_i / \sum_1^n {}^3H - Xyl_i$$

where ^{3}H-Xyl$_i$ is the radioactivity of the product or substrate with degree of polymerization i, and n is the degree of substrate polymerization. This ratio was plotted against the extent of the reaction obtained by dividing the radioactivity in all products by the total radioactivity in the sample

$$\sum_1^{n-1} {}^3H - Xyl_i / \sum_1^n {}^3H - Xyl_i$$

The slopes of the lines

$$^3H - Xyl_i / \sum_1^{n-1} {}^3H - Xyl_i$$

give the initial product ratios, which are equal to the initial bond cleavage frequencies. A relationship between the natural logarithm of the ratio of total radioactivity to the radioactivity of the substrate

$$\ln \sum_1^n {}^3H - Xyl_i / {}^3H - Xyl_n$$

and time was used to determine the k_o/K_m parameters [41]. The k_o/K_m parameters and bond cleavage frequencies were

then used to calculate the affinity of hypothetical subsites of the substrate-binding site for xylopyranosyl residues [41].

Stereochemistry of hydrolysis

The stereochemical course of cleavage of glycosidic bonds was followed by ^{1}H-NMR spectroscopy using methyl β-D-xylotrioside as a substrate. A 0.5 mL aliquot of a 5 mM solution of the xylotrioside in 0.05 M sodium acetate buffer in D_2O, pD 4.5, prepared from deuterized acetate and acetic acid (Aldrich Chemicals, St. Louis, MO, USA), was incubated with XYN IV lyophilized twice from D_2O (85 nkat·mL^{-1}) at 35 °C in a NMR test tube. ^{1}H-NMR spectra were recorded after various times on a Bruker AM 300 spectrometer (Bruker, Tübingen, Germany). The concentration of the enzyme required for rapid hydrolysis of the substrate was established in a preliminary experiment followed by TLC.

Isolation of the *xyn4* gene

The screening of a cDNA expression library [65] constructed from *T. reesei* RUT-C30 was performed essentially as described previously [66]. The screening construct contained a 1.2 kb fragment from the promoter of the *T. reesei pdi1* gene cloned upstream of the *Saccharomyces cerevisiae HIS3* gene. The *T. reesei* cDNA expression library was transformed into yeast strain DBY746 (α, *his3Δ1*, *leu2-3*, *ura3-52*, *trp1-289*, *Cyh*^{r}) containing the screening construct as described by Gietz *et al.* [67]. Transformants were plated on SC medium lacking Leu, Ura and His [68], and plasmids were isolated from clones that were able to grow on this medium and tranformed into *Escherichia coli* for restriction analysis and sequencing. The *xyn4* cDNA in the plasmid of the selected transformant was sequenced from both strands using internal oligonucleotide primers.

Expression of the *xyn4* gene

For gene expression studies, *T. reesei* strain QM9414 was grown in shake flasks (28 °C, 200 rpm) for 3 days in minimal medium [69] with 6% glucose, 6% arabinose, 6% arabitol, 6% xylose, 6% xylitol, 3% Solka floc cellulose (James River Corp., Richmond, VA, USA), 3% partially debranched xylan from beech (Lenzing AG), 3% 4-*O*-methylglucuronoxylan from birch or 3% arabinoxylan from oat spelts. When induction of the *xyn4* gene was studied, *T. reesei* QM9414 was first grown in 2% sorbitol and *xyn4* expression was induced at 72 h by addition of 1 mM sophorose or at 82 h by 2 mM xylobiose. The mycelium was harvested at 87 h. A culture grown for 87 h with 2% sorbitol served as a control. The glucose-depleted sample originated from a 125 h glucose batch fermentation [70].

Total *T. reesei* QM9414 RNA was isolated as described by Chirgwin *et al.* [71]. Northern hybridization was performed using Hybond N nylon membranes (Amersham, Vienna, Austria) according to the manufacturer's instructions using 0.5 μg of RNA for the cellulose-grown samples and 5 μg RNA for other samples.

Preparation of recombinant XYN IV

The *xyn4* gene (AAP64786) was synthesized by EZBiolab (Carmel, IN) as a 1400 bp ORF ligated in the *Sma*I site of a modified pUC57 plasmid (GenBank: Y14837; https:ncbi.nlm.nih.gov/). The gene was sub-cloned in fusion to the α-mating factor signal peptide in the *Pichia* expression vector pGAPZα (Invitrogen, Carlsbad, CA, USA). The α-mating factor signal peptide was then removed and replaced with the XYN IV native signal peptide (MKSSISVVLALLGHSAA) using a DNA linker. The linker was designed to encode the start Met and native signal peptide sequence with 5′ *Bst*I and 3′ *Hin*dIII overhangs, and was ligated into *Bst*I–*Hin*dIII-digested Xyn IV-pGAPZ. The resulting construct was further PCR-amplified from the unique *Sac*I site of *xyn4* to insert a stop codon at the 3′ end of the gene. Ligation of the PCR fragment into *Sac*I–*Bam*HI-digested Xyn IV-pGAPZ yielded the final gene vector construct with the vector *myc* epitope and the His-tag sequence removed. Transformation of the gene vector into *Pichia pastoris* was achieved by electroporation, and active clones grown on YPD-Zeocin plates were identified by TLC analysis of the hydrolytic activity on Xyl_4.

The active transformant was cultured at 30 °C in medium containing 1% yeast extract, 2% peptone, 2% dextrose, buffered with 100 mM potassium phosphate, pH 6.0 and supplemented with 1.34% yeast nitrogen base and 0.4 μg·mL^{-1} biotin. A 500 mL culture was harvested by centrifugation at 6000 ***g***, and the supernatant was filtered through a 0.45 μm MachV Supor device (Nalgene, Rochester, NY, USA). The filtrate was concentrated tenfold and buffer-exchanged with 10 mM HEPES, 25 mM NaCl, pH 8.0, using a PelliconXL BioMax cassette (10 kDa molecular weight cut-off) on a Labscale TFF system (Millipore, Bedford, MA, USA). The sample was applied to a CM Bio-Gel A column (0.75 cm × 30 cm; Bio-Rad, Hercules, CA, USA) equilibrated with buffer A (10 mM HEPES, 25 mM NaCl, pH 8.0) at a flow rate of 12 mL·h^{-1}. The column was then washed with 80 mL buffer A, followed by elution using a linear gradient of 25–500 mM NaCl. Active fractions were pooled, concentrated and buffer exchanged to 0.1 M Na acetate, pH 4.0, using an Amicon Ultra-15 concentrator (10 kDa molecular weight cut-off; Millipore, Bedford, MA, USA).

The purified enzyme was gel-blotted onto a poly(vinylidene difluoride) membrane for N-terminal sequencing. The result showed correct processing of the signal peptide producing the predicted mature protein.

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