The β -D-xylosidase of *Trichoderma reesei* is a multifunctional β -D-xylan xylohydrolase

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An extracellular multifunctional β -D-xylan xylohydrolase, previously described as β -xylosidase, was purified from *Trichoderma reesei* RUT C-30 to physical homogeneity. The active enzyme was a 100 ($\pm$ 5) kDa glycosylated monomer that exhibited a pI of 4.7. Its activity was optimal at pH 4 and it was stable between pH 3 and 6. Its temperature-stability was moderate (70 % of activity remaining after 60 min at 50 °C) and optimal activity was observed at 60 °C. It is capable of hydrolysing β -1,4-xylo-oligosaccharides [degree of polymerization (DP) 2–7], the apparent $V_{\max}$ increasing with increasing chain length. The enzyme also attacked debranched beech-wood (Lenzing) xylan and 4-O-methylglucuronoxylan, forming xylose as the only end product. The K_m for xylan was 0.7 g/l. For this reason we consider the enzyme to be a β -D-xylan xylohydrolase. The enzyme also exhibits

α -L-arabinofuranosidase activity on 4-nitrophenyl α -L-arabinofuranoside, and evidence is presented that this is not caused by an impurity in the enzyme preparation. The β -D-xylan xylohydrolase exhibits glycosyltransferase activity with xylo-oligosaccharides and at high concentrations of 4-nitrophenyl β -D-xylopyranoside (4-Nph- β -Xyl). The enzyme hydrolyses β -1,4-linkages preferentially to β -1,3-linkages, and β -1,2-linked xylo-oligosaccharides are not hydrolysed at all. The enzyme liberates terminal β -1,4-xylopyranose residues linked to a 2-O-substituted xylopyranose residue, but not that linked to a 3-O-substituted xylopyranose residue. The enzyme does not attack methyl, methyl 1-thio-, benzyl or butyl 1-thio- β -D-xylopyranosides and 4-naphthyl, 2-naphthyl and phenyl β -D-xylopyranosides.

INTRODUCTION

Hemicelluloses are the second most abundant polysaccharides in Nature. The major constituents of hemicellulose are the hetero-1,4- β -D-xylans, which are mainly found in grasses, cereals and hardwoods, and the hetero-1,4- β -D-mannans (galactoglucmannans and glucomannans) which are more abundant in softwoods [1]. Interest in the enzymology of hemicellulose degradation has recently reinitiated because of the biotechnological interest in the hydrolysis of hemicelluloses for the pulp and paper or the feedstock industry [2].

Selected strains from the soft-rot fungus *Trichoderma*, particularly *T. reesei*, have been shown to be exceptionally efficient producers of polysaccharide-degrading enzymes, and their hemicellulases have consequently received considerable attention [3]. Significant progress has recently been made on the biochemistry and molecular biology of the xylanases (EC 3.2.1.8) of *T. reesei* [4–10]. However, the β -xylosidase (EC 3.2.1.37) of *Trichoderma*, which is required for complete degradation of xylan to xylose, has so far received only poor attention. β -Xylosidases have been purified from *T. viride* [11] and *T. reesei* [12], but the properties of the enzyme had not been thoroughly investigated. β -Xylosidases have been investigated from several other microorganisms, including fungi [13–18] and bacteria [19–22].

We have recently reported that the formation of β -xylosidase by *T. reesei* can be induced very specifically by xylose at low pH [23]. The availability of a strongly enriched source of β -xylosidase prompted us to purify large amounts of this protein and to investigate its catalytic properties in detail.

MATERIALS AND METHODS

Substrates for enzyme assays

Alkaline pretreated beech-wood xylan (Lenzing AG, Lenzing, Austria), which is unsubstituted and contains terminal succinylation [24], was used as a source of xylan throughout most parts of the present study. In the case of other xylans, they will be specified at the respective experiments.

The following methyl β -D-glycosides were synthesized as described (for abbreviations used in the carbohydrate structures, see the footnote): β -D-Xylp-(14)- β -D-Xylp-OMe (Xyl-4Xyl-Me) [25], β -D-Xylp-(13)- β -D-Xylp-OMe (Xyl-3Xyl-Me) [25], β -D-Xylp-(12)- β -D-Xylp-OMe (Xyl-2Xyl-Me) [25], β -D-Xylp-(14)- β -D-Xylp-(12)- β -D-Xylp-OMe (Xyl-4Xyl-2Xyl-Me) [26], β -D-Xylp-(14)- β -D-Xylp-(13)- β -D-Xylp-OMe (Xyl-4Xyl-3Xyl-Me) [26], β -D-Xylp-(12)- β -D-Xylp-(14)- β -D-Xylp-OMe (Xyl-2Xyl-4Xyl-Me) [27], β -D-Xylp-(14)-[β -D-Xylp-(12)]- β -D-Xylp-OMe (Xyl-4[Xyl-2]Xyl-Me) [27], β -D-Xylp-(13)- β -D-Xylp-(14)- β -D-Xylp-OMe (Xyl-3Xyl-4Xyl-Me) [28], β -D-Xylp-(14)-[β -D-Xylp-(13)]- β -D-Xylp-OMe (Xyl-4[Xyl-3]Xyl-Me) [29], β -D-Xylp-(14)-[β -D-Xylp-(13)]- β -D-Xylp- β -D-(14)-Xylp-OMe (Xyl-4[Xyl-3]Xyl-4Xyl-Me) [30], β -D-Xylp-(14)-[L-Araf-(13)]- β -D-Xylp-OMe (Xyl-4[Ara-3]Xyl-Me) [31]. Methyl β -D-xylopyranoside, 2-nitrophenyl β -D-xylopyranoside and 4-Nph- α -Xyl and 4-Nph- β -Xyl were from Sigma. Methyl 1-thio- β -D-xylopyranoside, 1-butyl 1-thio- β -D-xylopyranoside, benzyl β -D-xylopyranoside and phenyl β -D-xylopyranoside were generously provided by Professor C. K. DeBruyne (State University, Ghent, Belgium).

To prepare unsubstituted xylo-oligosaccharides with chain

Abbreviations used: Xyl, xylose; Xylp, xylopyranose; Araf, arabinofuranose; 4-Nph- β -Xyl, 4-nitrophenyl β -D-xylo(pyrano)side; Glc, glucose; Me, methyl; OMe, methyl ester; GlcUA, glucuronic acid; DP, degree of polymerization.

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lengths between two and seven residues, 5 g of xylan was dissolved in 50 ml of conc. HCl and kept at 25 °C for 18 min. This solution was then added to 150 ml of 50 mM Tris, cooled down to 2 °C, and the pH adjusted to 7 by the slow addition of powdered Ba(OH)₂ under constant stirring. The BaCl₂ precipitate is then removed by centrifugation (10000 g, 20 min), and the supernatant concentrated to 15 ml by flash evaporation. After further centrifugation (see above) the xylo-oligosaccharides were separated on a column (1 cm × 100 cm) of Bio-Gel P2, using distilled water as the eluant (flow rate 2.5 ml/min). Fractions were collected automatically and analysed by the phenol/H₂SO₄ method [32]. The purity of individual oligosaccharides and their chain lengths were assessed by HPLC analysis (see below).

Two acidic oligosaccharides (aldouronic acids) β -D-Xylp-(14)-[4-O-Me-D-GlcUA-(12)]- β -D-Xylp- β -(14)-D-Xylp- β -D-(14)-Xylp (Xyl-4[MeGlcUA-2]Xyl-4Xyl-4Xyl) and -D-Xylp-(14)- β -D-Xylp-(14)-[4-O-Me-D-GlcUA-(12)]- β -D-Xylp- β -(14)-D-Xylp- β -D-(14)-Xylp (Xyl-4Xyl-4[MeGlcUA-2]Xyl-4Xyl-4Xyl) were isolated from hydrolysates of beech-wood 4-O-methyl-D-glucurono-D-xylan by the acidic and alkaline endo-(1,4)- β -xylanases of *Trichoderma reesei* as reported previously [7].

Organism, conditions for cultivation and enzyme purification

T. reesei RUT C-30 (A.T.C.C. 56765) was used throughout the present study. The cultures were grown as described previously [23], and enzyme formation was induced by transfer to 4 mM xylose, pH 2.5, in quarter-strength salt solution [33], lacking urea and phosphate. A 2 litre portion of supernatant from such cultures was filtered through a layer of cotton and then through a G1, and finally a G2, sintered-glass funnel. The clear supernatant was then concentrated to a final volume of 20 ml by ultrafiltration (Filtron Minisette; Amicon P30) and dialysed against 25 mM Bistris buffer, pH 6.3 (Buffer A). This concentrate was loaded on to a Mono P-column (Pharmacia-LKB Ltd., Uppsala, Sweden), and eluted by the application of a 40 ml of pH gradient using Polybuffer 74, diluted 1:10 with distilled water, and adjusted to pH 4.0. The flow rate was 0.5 ml/min. Fractions containing more than 20% of maximal activity were collected, dialysed overnight against 10 mM sodium acetate buffer, pH 6.0 (buffer B), and applied to a 5 mm × 100 mm Mono Q column (Pharmacia-LKB), previously equilibrated in buffer B and connected to an FPLC apparatus. β -Xylosidase was eluted by the application of a linear gradient of 0–0.5 M NaCl in 40 ml of buffer B. The enzyme was stored at –20 °C until further analysis.

Enzyme activity assays

β -Xylosidase activity was assayed by three different methods, depending on the substrate: (a) for routine analysis during enzyme purification and characterization, 4-nitrophenyl β -D-xylopyranoside was used as a substrate, and the activity determined as described in [23]. (b) To follow the hydrolysis of non-chromogenic β -xylo-oligosaccharides, the enzyme was incubated in a total volume of 1.3 ml with different final substrate concentrations up to 3 mM and in the presence of 50 mM citrate buffer, pH 5. After incubation, 20 μ l of the reaction mixture was assayed by HPLC on a Polyspher CHNa (Merck, Darmstadt, Germany) column at 65 °C, using HPLC-grade water (Merck) as an eluant (flow rate 0.5 ml/min). (c) To investigate the activity with various methyl β -D-glycosides of linear and branched oligosaccharides and of two aldouronic acids, they were incubated at a concentration of 1 mM with the enzyme (2.5 munits/ml final concentration) in 0.1 M citrate buffer at 55 °C, and liberated D-xylose was determined chemically [34].

Glycosyltransferase was assayed by incubating the enzyme (final concn. 0.12 unit/ml) at 30 °C in aq. 100 mM (or 200 mM) fresh and warm 4-Nph- β -Xyl. Aliquots of the mixture were analysed by TLC, using aniline/hydrogen phthalate for detection. UV-absorbing compounds were excised from the chromatogram, eluted with water and their absorbance measured at 300 nm to determine the relative abundance of compounds. α -L-Arabinofuranosidase was assayed as described in [35].

All enzyme activities are expressed in units (μ mol of product formed/min of assay under the given conditions). Specific activities are related to mg of enzyme protein quantified by the dye-binding method [36].

Electrophoretic procedures

SDS/PAGE was carried out in 10% polyacrylamide gel slabs at pH 8.3 using 25 mM Tris/glycine buffer containing 0.1% (w/v) SDS as described in [37].

Non-denaturing PAGE was carried out under essentially similar conditions, but omitting SDS from all buffers and solutions, 2-mercaptoethanol from the sample buffer and the heating step prior to sample application.

Isoelectric focusing was carried out in a Multiphor I unit (Pharmacia-LKB), using commercially supplied prepared pH 3–10 isoelectric-focusing gels.

Following electrophoresis, protein bands were revealed either with Coomassie Brilliant Blue or by silver staining [38]. To detect enzyme activities on gels, they were incubated for 30 min at 50 °C in 50 mM sodium citrate buffer, and then overlaid with a liquid film of 1 mM methylumbelliferyl glycoside in 50 mM sodium citrate buffer at 50 °C. The hydrolysis of the glycosides was monitored visually under a UV transilluminator.

For immunological analysis, the gels were blotted on to nitrocellulose [39] and then subjected to immunostaining as described previously [40].

Determination of molecular mass by Superose 12 gel-permeation chromatography

Determination of the molecular mass was carried out with 20 μ g of purified enzyme in a total volume of 100 μ l on a column of Superose 12 (Pharmacia-LKB Ltd., Uppsala, Sweden), previously equilibrated with 50 mM citrate buffer, pH 4.5, containing 0.2 M NaCl.

Peptide generation and sequencing

Generation of tryptic peptides from the purified β -xylosidase and the determination of part of their amino acid sequence was carried out by outdoor custom service (Fa. Toplab, München, Germany).

RESULTS AND DISCUSSION

Enzyme purification

The specific induction of β -xylosidase formation by xylose at low pH [23] produced culture filtrates highly enriched in β -xylosidase, from which the enzyme was purified to apparent physical homogeneity in two steps. The application of 50 μ g of the final preparation yielded a single protein band on SDS/PAGE gels (Figure 1). In comparison with the β -xylosidase protein purified previously [12], the present preparation had an almost-3-fold higher specific activity (1.3 versus 0.47 μ kat/mg of protein). The lack of other protein bands on SDS/PAGE was also confirmed by immunostaining of gels after Western blotting, using anti-

Figure 1 Evidence for β -xylosidase purity

SDS/PAGE of 10 μ g of purified β -xylosidase (lane 2) and of marker proteins [lane 1; molecular masses (M) given on the left-hand side]. Staining was carried out with Coomassie Blue.

bodies against the cellulases CBH I, CBH II and EG I, and the xylanases XYN I and XYN II.

The purified enzyme was also subjected to proteolytic cleavage by trypsin, and the N-terminal amino acid sequences of two major peaks determined. They were:

(1) SNPGQTYWYTGKP

and

(2) VNLSLVWGGYPGQSGGVALFDI

Neither of these two peptides showed significant sequence similarity to any other protein in the Medline protein data banks.

Physical properties of the purified β -xylosidase

The estimation of molecular mass by SDS/PAGE yielded a value of 100 (± 5) kDa. A similar value was calculated according to the relative position of elution of β -xylosidase from Superose 12, suggesting that the enzyme is a monomer. Schiff's staining of 20 μ g of the purified protein band was strongly positive, suggesting that the enzyme contains covalently bound carbohydrate.

The pI was determined by FPLC chromatofocusing as 4.7 (± 0.05).

The highest β -xylosidase activity was observed at pH 4, and more than 90 % of that between pH 3.0 and 4.5. Significantly lower activities were obtained at pH values above 6. The enzyme demonstrated a moderate pH-stability, as more than 90 % of the maximal activity remained following 12 h incubations in buffers within a pH range of 4–6. The enzyme was relatively less stable at lower than at higher pH. The enzyme retained more than 80 % of its original activity during 60 min incubations at temperatures of up to 55 °C.

All these data compare well with those reported previously [12], and their presentation here is therefore omitted. We conclude, therefore, that we are dealing with the same protein as that described in [12].

Substrate specificity

The purified β -xylosidase hydrolysed β -1,4-linked xylo-oligosaccharides with chain lengths from 2–7. The products

Table 1 Products accumulating during reaction of β -D-xylan xylohydrolase with xylo-oligosaccharides (DP 2–7)

Reaction mixtures were incubated at 45 °C and contained 68 μ g of purified enzyme plus 1.0 mg of substrate in 50 mM sodium citrate buffer, pH 5.0 (final volume 1.0 ml). Reactions were stopped after 3 h by boiling, and the products were separated by HPLC as described in the Materials and methods section. The concentrations of the individual products formed in each incubation are given as the molar percentage of the total xylo-oligosaccharides (DP 2–7) present in reaction mixtures after 3 h.

Substrate	DP ...	[Product] (mol % of total xylo-oligosaccharides)						
		1	2	3	4	5	6	7
2		90	10	—	—	—	—	—
3		91	6	3	—	—	—	—
4		95	2	2	1	—	—	—
5		96	—	—	2	2	—	—
6		97	—	—	0.5	1.5	1	—
7		72	—	—	2	10	9	7

accumulating in reaction mixtures were fractionated and quantified by HPLC (Table 1). The experimental conditions were chosen so as to minimize the extent of hydrolysis and so allow the identification of intermediates, if any. Xylose was always the main end product, but the formation of xylo-oligosaccharides with a higher DP was observed. This indicates the occurrence, under the experimental conditions used, of both transfer and hydrolytic reactions. As examples of the time course of substrate conversion, the action of the enzyme on xylotriose, xylotetraose and xylopentaose is shown in Figure 2. At a fixed xylo-oligosaccharide concentration, the activity increased slightly with increasing chain length, which would be atypical for a β -xylosidase and be rather typical for an exoxylanase. This conclusion is supported by the finding that the purified β -xylosidase liberates xylose from various types of xylan, including the partially debranched Lenzing xylan with decreased solubility (Table 2). Consequently we consider the enzyme to be a β -D-xylan xylohydrolase. The K_m value for Lenzing xylan was in the range 0.35–0.71 mg/ml.

The enzyme was capable of hydrolysing 4-nitrophenyl β -D-xyloside, 2-nitrophenyl β -D-xyloside and phenyl β -D-xyloside (Table 3). However, the reactions did not obey Michaelis–Menten kinetics. For instance, two different K_m values could be determined for 4-Nph- β -Xyl. The value 0.42 mM was obtained for the substrate concentration range 1–10 mM, and the value 20.0 mM for concentration range 10–100 mM. The K_m value 8.3 mM obtained for 2-Nph- β -Xyl applies for substrate concentrations larger than 5 mM. For these reasons it is difficult to compare our values with the published K_m value for 4-Nph- β -Xyl (0.08 mM) [12]. When the k_{cat} values for the reactions with high and low concentrations of 4-Nph- β -Xyl are compared, an increase is noted at the high concentrations. Because of the occurrence of transglycosylation (see below), the enzyme's reaction most probably follows a double-displacement mechanism; the differences in K_m and k_{cat} at low and high substrate concentrations could thus be explained by the xylosyl-enzyme intermediate being rate-determining and a second substrate molecule possibly acting as a nucleophile at high substrate concentrations.

The enzyme did not hydrolyse benzyl β -D-xylopyranoside or three alkyl and alkyl 1-thio- β -D-xylopyranosides (Table 3). This observation indicates that the enzyme requires the electron-pulling effect of the aromatic aglycone for hydrolysis of β -D-

Figure 2 Time course of hydrolysis of xylobiose (A), xylotriose (B), xylotetraose (C) and xylopentaose (D) by *T. reesei* β -D-xylan xylohydrolase

Reaction mixtures, incubated at 45 °C, contained 670 ng (A), 67 ng (B), 1200 ng (C) and 50 ng (D) respectively of enzyme plus (A) 1 mg of xylobiose, (B) 1 mg of xylotriose, (C) 0.6 mg of xylotetraose and (D) 0.4 mg of xylopentaose, all in 50 mM sodium citrate buffer, pH 5 (final volume 1 ml). Portions (50 μ l) were taken at intervals, boiled to stop further reaction, and separated by HPLC as described in the Materials and methods section. Key to symbols: $\circ$, xylose; $\bullet$, xylobiose; $\blacksquare$, xylotriose; $\square$, xylotetraose; $\blacktriangle$, xylopentaose.

Table 2 Products released from various types of xylan by β -D-xylan xylohydrolase of *T. reesei*

Reaction mixtures were incubated at 45 °C, and contained 81 μ g of purified enzyme plus 1.0 mg of substrate in 50 mM sodium citrate buffer, pH 5.0 (final volume 1.0 ml). Reactions were stopped after 3 h by boiling, and the products were separated by HPLC as described in the Materials and methods section. The concentrations of the individual products formed in each incubation are given as the molar percentage of the total xylo-oligosaccharides (DP 2–7) present in the reaction mixtures after 3 h. Xylotetraose, xylopentaose and xylohexaose were absent from the mixtures. ND, not detected.

Product	Xylan ...	Amount of product (% by weight of the starting substrate)		
		Lenzing	Wheat straw	Beech-wood
Xylose		59	36	44
Xylobiose		1	ND	0.5
Xylotriose		ND	ND	0.5
Total ...		60	36	45

xylopyranosides. Once this effect is interrupted by introduction of a methylene group, like in benzyl glycoside, the hydrolytic activity of the enzyme is completely blocked. The other reasons for the observed differences of the enzyme's action on various β -D-xylosides may simply be stereochemical ones. The electron-withdrawing effect for the hydrolytic reaction seems to be more important than the configuration of the glycosidic linkage, since the enzyme also hydrolysed 4-Nph- α -Xyl (Table 3) and 4-nitrophenyl α -L-arabinofuranoside. The activity of the purified enzyme towards the latter substrate was reported previously [12]. In order to rule out that this is due to contamination by non-detectable amounts of a highly active α -L-arabinofuranosidase, we subjected the purified enzyme to SDS/PAGE, renatured the enzyme in the gel after electrophoresis, and revealed its activity with the fluorogenic substrates 4-methylumbelliferyl β -D-xylopyranoside and α -L-arabinofuranoside. A band migrating to the position of β -xylosidase was active towards both substrates, and we therefore conclude that both activities are catalysed by the same enzyme. However, although the enzyme exhibits the aryl α -L-arabinofuranosidase activity, it did not liberate L-

Table 3 Summary of the action of the enzyme on alkyl and aryl xylopyranosides

Substrate	K_m (mM)	k_{cat} (s^{-1})	Notes
Me- β -Xyl	—	—	Substrate not hydrolysed at an appreciable rate
Me-1-thio- β -Xyl	—	—	Substrate not hydrolysed at an appreciable rate
1-Butyl-1-thio- β -Xyl	—	—	Slight hydrolysis observed at concentrations greater than 40 mM
4-Nph- β -Xyl	0.42	176	Valid for substrate concentrations in the range 1–10 mM
	20.0	469	Valid for substrate concentrations in the range 10–100 mM
2-Nph- β -Xyl	8.3	90.4	Different kinetics apply below 5 mM substrate concentration
4-Nph- α -Xyl	33.0	14.6	Valid for substrate concentrations larger than 10 mM
Phenyl- β -Xyl	—	—	The reaction does not obey the Michaelis–Menten kinetics over the substrate concentration range 0–100 mM
Benzyl- β -Xyl	—	—	Substrate not hydrolysed at an appreciable rate

Table 4 Time course of the distribution of the aryl aglycone (4-nitrophenol) in products of β -D-xylan xylohydrolase (0.24 unit/ml) degradation of 100 mM 4-Nph- β -D-xylopyranosideAbbreviations: Xyl₂, xylobiose; Xyl₃, xylotriose.

Reaction product or substrate	Relative chromatographic mobility (R_{Xyl})	Proportion (%) in the reaction mixture after:				
		0 h	5 h	27 h	50 h	168 h
4-Nph- β -Xyl	2.12	100	81.8	59.0	34.7	15.1
4-Nitrophenol	2.31		8.5	20.7	35.2	50.0
4-Nph-Xyl ₂						
Minor*	2.00				3.6	5.5
Major	1.80		8.2	16.0	18.0	16.8
4-Nph-Xyl ₃						
Minor*	1.68			1.5	2.5	2.9
Major	1.46		1.2	2.7	5.6	6.7

*Products containing other than β -1,4-linkage, most probably an α -1,3-linkage which is the reason for their higher chromatographic mobility.

arabinofuranose from Xyl-4[Ara-3]-Xyl-OMe. It should be noted that the activity against α -linked substrates cannot be due to impurities of β -linked xylosides present in them, because the reaction approached completeness on prolonged incubation (results not shown).

While this paper was in preparation, the nucleotide sequence of a gene from *T. koningii* encoding a β -xylosidase with α -L-arabinofuranosidase activity was released (GenBank accession no. U38661). It is interesting to note that the amino acid sequence of the two tryptic peptides from the β -D-xylan xylohydrolase were not found in this sequence. On the other hand, the amino acid sequence deduced from the nucleotide sequence of a PCR fragment of the *T. reesei* *xxh1* gene, amplified by the use of primers designed according to these tryptic sequences [40a], showed 65% similarity to a β -D-xylosidase/ α -L-arabinofuranosidase-encoding gene from *Butyrivibrio fibrisolvens* [41]. These findings support our assumptions that the enzyme studied here actually also has an α -L-arabinofuranosidase activity.

Product inhibition

The reaction is inhibited by the product, D-xylose. The inhibition is competitive, with a K_i of 2.4 (± 0.2) mM (4-Nph- β -Xyl as substrate). L-Arabinose, the product of the α -L-arabinosidase activity, has no effect on the activity against 4-Nph- β -Xyl at

concentrations between 5 and 500 mM. Interestingly, the activity against *p*-nitrophenyl α -L-arabinofuranoside is inhibited both by D-xylose and L-arabinose (K_i 1.7 ± 0.3 and 35 ± 6 mM respectively).

Transglycosylation activity

Since the hydrolysis pattern of xylo-oligosaccharides suggested transglycosylation activity of the enzyme, this potential glycosyltransferase activity was studied in more detail: 4-Nph- β -Xyl was chosen as a substrate for this purpose. Incubation of β -D-xylan xylohydrolase at high substrate concentrations yields xylose and 4-nitrophenol, xylobiose, xylotriose and xylotetraose and 4-nitrophenyl xylobioside, as well as 4-nitrophenyl xylotrioside and positional isomers of the compounds as end products (results not shown). The highest yields of 4-nitrophenyl xylobioside and 4-nitrophenyl trioside are of the order of 18 and 6% respectively (Table 4). The yields of positional isomers, showing greater mobility on TLC due to the presence of one β -1,3-linkage, are much lower. As it will be discussed below, the formation of the β -1,2-linkage is not considered, because the enzyme does not seem to be capable of hydrolysing these types of linkages at an appreciable rate.

The β -D-xylan xylohydrolase also catalysed the transfer of xylopyranose residues to acceptors other than water, such as glycerol or other monosaccharides. The reactions were demonstrated by TLC and HPLC; however, the exact structure of the newly formed compounds has not yet been determined. The clear evidence for the glycosyl transfer reactions suggests that *T. reesei* β -D-xylan xylohydrolase is most probably a retaining glycanase, i.e. a glycanase which uses a double-displacement reaction mechanism of the hydrolysis of glycosidic linkage [42]. The synthesis of alkyl β -D-xylosides from primary, secondary, and tertiary alcohols by an unpurified preparation of *T. reesei* ' β -xylosidase' (SP 431; Novo Nordisk Bioindustri S.A., Baksvaerd, Denmark) has recently been reported [43], which principally agrees with our findings. We should also note that the enzyme does not catalyse condensation reactions of xylose.

Substrate requirements of the enzyme

The evidence presented so far shows that *T. reesei* β -D-xylan xylohydrolase liberates D-xylopyranose residues present on non-reducing terminals. In natural substrates, however, such residues can be bound not only β -1,4-glycosidically and, furthermore, may have a variable chemical environment. Some xylans were

Table 5 Initial rates of liberation of D-xylose from methyl β -D-glycosides of xylooligosaccharides and two aldouronic acids by *T. reesei* β -D-xylan xylohydrolase

Reaction conditions: 1 mM substrate solutions in 0.1 M sodium citrate buffer, pH 4.0, incubated with the enzyme (final concentration 2.5 munits/ml) at 50 °C.

Substrate	Initial rate of hydrolysis	
	($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$)	($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{unit}^{-1} \cdot \text{ml}^{-1}$)
Xyl-4Xyl-Me	0.020	8.16
Xyl-4Xyl-3Xyl-Me	0.039	15.5
Xyl-4Xyl-2Xyl-Me	0.044	17.6
Xyl-4[Xyl-2]Xyl-Me	0.044	17.6
Xyl-4[MeGlcUA-2]Xyl-4Xyl	0.020	8.00
Xyl-4Xyl-4[MeGlcUA-2]Xyl-4Xyl-4Xyl	0.021	8.40
Xyl-4[Ara-3]-Xyl-OMe	< 0.001	< 0.1
Xyl-3Xyl-Me	0.013	5.28
Xyl-3Xyl-4Xyl-Me	0.015	5.92
Xyl-2Xyl-Me	0.0004	0.16
Xyl-2Xyl-4Xyl-Me	< 0.0001	< 0.1
Xyl-4[Xyl-3]Xyl-Me	< 0.0001	< 0.1
Xyl-4[Xyl-3]Xyl-4Xyl-Me	< 0.0001	< 0.1

Figure 3 Structural arrangements with non-reducing terminal D-xylopyranose residues accessible (A) and resistant (B) to hydrolysis by *T. reesei* β -D-xylan xylohydrolase

shown to contain small numbers of xylopyranose residues also in their side chains, linked to the main chain by β -1,3-linkages [1]. To learn more about the catalytic properties of the enzyme, particularly about its requirement for the environment of the terminal xylopyranose residues, the activity of the enzyme was examined on variety of methyl β -glycosides of linear and branched xylo-oligosaccharides containing various types of linkages, including a compound containing an α -1,3-L-arabinofuranosyl branch (Table 5). Two aldouronic acids, con-

taining a 4-O-methylglucuronose residue α -1,2-linked to a xylopyranose residue other than the non-reducing terminal one, were also used as substrates.

The results indicate that the enzyme is capable of releasing terminal β -1,4- and β -1,3-linked xylopyranose residues linked either to a non-substituted D-xylopyranose residue (compounds containing two consecutive unsubstituted xylopyranose residues on the non-reducing terminal), or to an α -1,2- or β -1,2-substituted xylopyranose residue (compounds of the type Xyl-4-[Xyl-2]Xyl-

or Xyl-4[4MeGlcUA-2]Xyl-). The rates of liberation of xylose from these two groups of compounds did not differ very much. The rate of hydrolysis of the β -1,3-linkage is only a third of that of the β -1,4-linkage. A surprising finding is that the enzyme does not liberate readily D-xylopyranose residues β -1,4-linked to an α -1,3- or β -1,3-substituted xylopyranosyl residue (compounds of the type Xyl-4[Ara-3]Xyl- or Xyl-4[Xyl-3]Xyl-). In such compounds the substituent is apparently so close to the site of the enzyme attack (i.e. the glycosidic oxygen) that the formation of the enzyme-substrate complex is sterically impaired. Only if the substituent is linked to a more distant C-2 position does the β -1,4-linkage becomes susceptible to the enzyme attack. In contrast with several classical β -xylosidases [11,13,17,19], the enzyme shows very low rates of hydrolysis of β -1,2-linked xylopyranose residues. The structural requirements of the enzyme for the environment of a targetted non-reducing terminal D-xylopyranose residue are illustrated in Figure 3. In accordance with the finding that the rate of hydrolysis of β -1,4-xylo-oligosaccharides increases in line with increasing chain length, the observed substrate structure requirements of the enzyme further support the conclusion that the enzyme investigated here is actually a β -D-xylan xylohydrolase. Thus we are re-defining a previously described enzyme. We also provide clear evidence for a combination of both β -D-xylosidase and α -L-arabinofuranosidase activity in the same enzyme. While similar findings have already been reported for some hemicellulases from bacteria [41,44,45], the present enzyme is the first case where clear evidence for these two distinct activities in a fungal enzyme has been obtained.

Note added in proof (received 15 November 1996)

While this paper was being proofed out, we became aware of the results of Margolles-Clark et al. [46] describing the cloning of a β -xylosidase gene from *T. reesei*. The deduced amino acid sequence, the α -L-arabinofuranosidase activity and the ability to hydrolyse xylan are all in accordance with the protein described in this study.

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REFERENCES

- Timell, T. E. (1967) Wood. Sci. Technol. **1**, 45–70
- Buchert, J., Ranua, M., Kantelinen, A. and Viikari, L. (1992) Appl. Microbiol. Biotechnol. **37**, 825–829
- Wong, K. K. Y. and Saddler, J. N. (1992) CRC Crit. Rev. Biotechnol. **12**, 413–435
- Tenkanen, M., Puls, J. and Poutanen, K. (1992) Enzyme Microb. Technol. **14**, 566–574
- Törrönen, A., Mach, R. L., Messner, R., Gonzalez, R., Kalkkinen, N., Harkki, A. and Kubicek, C. P. (1992) Bio/Technology **10**, 1461–1465
- Biely, P., Kremnický, L., Alföldi, J. and Tenkanen, M. (1994) FEBS Lett. **356**, 137–140
- Biely, P., Vrsanska, M., Kremnický, L., Tenkanen, M., Poutanen, K. and Hayn, M. (1993) in Proceedings of the TRICEL Symposium on *Trichoderma reesei* Cellulases and Other Hydrolases, Espoo 1993 (Souminen, P. and Reinikainen, T., eds.) (Found. Biotech. Ind. Ferment. Res. **8**), pp. 125–135, Fagapaino Oy, Helsinki
- Saarelainen, R., Paloheimo, M., Fagerström, R., Suominen, P. and Nevalainen, H. (1993) Mol. Gen. Genet. **241**, 497–503
- Törrönen, A., Harkki, A. M. and Rouvinen, J. (1994) EMBO J. **13**, 2493–2501
- Törrönen, A. and Rouvinen, J. (1995) Biochem. **34**, 847–856
- Matsuo, M. and Yasui, T. (1984) Agric. Biol. Chem. **48**, 1845–1852
- Poutanen, K. and Puls, J. (1988) Appl. Microbiol. Biotechnol. **28**, 425–432
- Deleyn, F., Claeysens, M. and De Bruyne, C. K. (1982) Methods Enzymol. **83**, 639–644
- Kitpreechavanich, V., Hayashi, M. and Nagai, S. (1986) Agric. Biol. Chem. **50**, 1703–1711
- Matsuo, M. and Yasui, T. (1984) Agric. Biol. Chem. **48**, 1853–1860
- Oguntimein, G. B. and Reilly, P. J. (1980) Biotechnol. Bioeng. **22**, 1143–1154
- Rodionova, N. A., Tavobilo, I. M. and Bezborodov, A. M. (1983) J. Appl. Biochem. **5**, 300–312
- Uziie, M., Matsuo, M. and Yasui, T. (1985) Agric. Biol. Chem. **49**, 1159–1166
- Kerstens-Hilderson, H., Loontjens, F. G., Claeysens, M. and De Bruyne, E. K. (1969) Eur. J. Biochem. **7**, 434–441
- Lee, S. F. and Forsberg, C. W. (1987) Appl. Environ. Microbiol. **53**, 651–654
- Nawamori, T., Watanabe, T., Shinke, R., Kohno, A. and Kawamura, Y. (1990) J. Bacteriol. **172**, 6669–6672
- Shao, W. and Wiegell, J. (1992) J. Bacteriol. **174**, 5848–5853
- Kristufek, D., Zeilinger, S. and Kubicek, C. P. (1995) Appl. Microbiol. Biotechnol. **42**, 713–717
- Gamerith, G. and Strutzenberger, H. (1992) in Xylans and Xylanases (Visser, J., Beldman, G., Kusters-van Someren, M. A. and Voragen, A. G. J., eds.), pp. 339–347, Elsevier, Amsterdam, London, New York and Tokyo
- Kovac, P. (1979) Collect. Czech. Chem. Commun. **45**, 892–900
- Hirsch, J. and Petrakova, E. (1984) Chem. Pap. **38**, 409–417
- Hirsch, J. and Kovac, P. (1979) Carbohydr. Res. **77**, 241–244
- Kovac, P. and Hirsch, J. (1980) Carbohydr. Res. **79**, 303–307
- Kovac, P. (1979) Collect. Czech. Chem. Commun. **44**, 928–932
- Kovac, P., Hirsch, J. and Kovacic, V. (1979) Carbohydr. Res. **75**, 109–116
- Hirsch, J., Petrakova, E. and Schraml, J. (1984) Carbohydr. Res. **131**, 219–226
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) Anal. Chem. **28**, 350–356
- Sternberg, D. and Mandels, G. R. (1979) J. Bacteriol. **139**, 761–767
- Paleg, L. G. (1959) Anal. Chem. **31**, 1902–1904
- Kristufek, D., Hodits, R. and Kubicek, C. P. (1994) FEMS Microbiol. Lett. **115**, 259–264
- Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
- Laemmli, U. K. (1970) Nature (London) **227**, 680–685
- Morrissey, J. H. (1981) Anal. Biochem. **117**, 307–310
- Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
- Mischak, H., Hofer, F., Weisinger, E., Messner, R., Hayn, M., Tomme, P., Küchler, E., Esterbauer, H., Claeysens, M. and Kubicek, C. P. (1989) Biochim. Biophys. Acta **990**, 1–7
- Herrmann, M. C. (1995) Ph.D. Thesis, University of Vienna
- Utt, E. A., Eddy, C. K., Keshav, K. F. F. and Ingram, L. O. (1991) Appl. Environ. Microbiol. **57**, 1227–1234
- Sinnott, M. L. (1990) Chem. Rev. **90**, 1171–1202
- Drouet, P., Zhang, M. and Legoy, M. D. (1994) Biotechnol. Bioeng. **43**, 1075–1080
- Shao, W. and Wiegell, J. (1992) J. Bacteriol. **174**, 5848–5853
- Gasparic, A., Marinsek-Logar, R., Martin, J., Wallace, R. J., Nekrep, F. V. and Flint, H. J. (1995) FEMS Microbiol. Lett. **125**, 135–142
- Margolles-Clark, E., Tenkanen, M., Nakari-Setälä, T. and Penttilä, M. (1996) Appl. Environ. Microbiol. **62**, 3840–3846