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Stereochemistry of the hydrolysis of glycosidic linkage by endo-β-1,4-xylanases of *Trichoderma reesei*

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Abstract Methyl β-D-xylotrioside was used as a non-reducing substrate to investigate the stereochemistry of hydrolysis of β-1,4-xylopyranosidic linkage by purified endo-β-1,4-xylanases (EC 3.2.1.8) of *Trichoderma reesei*, employing ^{1}H NMR spectroscopy. The fungus produces one acidic species (pI 4.8–5.5), designated as EXI, and one alkaline species (pI 8.5–9.0), designated as EXII. Both enzymes were found to cleave the xylotrioside predominantly to methyl β-D-xyloside and xylobiose. Monitoring of the intensity of the H-1 signals of α- and β-xylobiose during the time course of hydrolysis clearly showed that both enzymes liberate the β-anomer of xylobiose, i.e. a product with anomeric configuration identical with that of the cleaved glycosidic linkage. This means that both EXI and EXII belong to the so-called retaining glycanases that utilize the double displacement reaction mechanism of hydrolysis.

Key words: Xylanase; Reaction mechanism; Hydrolysis; Enzyme family; Nuclear magnetic resonance; *Trichoderma reesei*

1. Introduction

Intense research of microbial endo-β-1,4-xylanases (EC 3.2.1.8) (further Xln) in recent years, connected with their great biotechnical potential, led to considerable progress in understanding their production, multiplicity and gene organization [1–3]. Grouping of described enzyme species on the basis of their physico-chemical properties led to the discovery of two groups of Xln: (i) high molecular mass/low pI value species; (ii) low molecular mass/high pI value species [4]. This grouping correlated well with classification of Xln into families F and G on the basis of hydrophobic cluster analysis and amino acid sequence homologies [5–7]. Recent studies focussed on catalytic properties of the two Xln families have indicated that there are significant differences in substrate specificity and catalytic properties between the enzyme species of the two families [8]. Xln of family F exhibit greater catalytic versatility, in other words, they are less specific than Xln of family G. As far as the stereochemistry of the hydrolysis of the glycosidic linkage by enzymes of the two families is concerned, information is rather limited. Two representatives of family G (*Bacillus subtilis* and *Schizophyllum commune* Xln A) and one of family F (*Clostridium thermocellum* Xyn Z) were shown to utilize the same mechanism of hydrolysis, associated with the retention of the configuration of the glycosidic linkage [9]. The idea that Xln of the two families utilizes the double displacement mechanism of hydrolysis found further support in the study of the catalytic properties of Xln of *Streptomyces lividans*, a producer of Xln belonging to both families [8].

In this paper we report on the stereochemistry of hydrolysis of glycosidic linkage by two specific Xln of the cellulolytic fungus *Trichoderma reesei*. During growth on both cellulose and xylan the fungus produces two Xln, designated as EXI and EXII [10–12], which, according to their primary structure, belong to the G family of Xln [13]. They show very similar molecular mass (EXI 19 kDa; EXII 20, 21 kDa [12,13]), but differ significantly in catalytic properties [12,14] and pI values (EXI, pI 4.8–5.5; EXII, pI 8.5–9.0) [10,12,13]. In addition to the striking difference in the pI value, the sequence homology of EXI with other Xln of family G is not as great as that of EXII [13], which suggested that EXI may represent an intermediate species between Xln of family F and G. In fact, a hydrophobic cluster analysis of family G Xln has confirmed that EXI and EXII of *T. reesei* are the enzyme species most distant on a phylogenetic tree for family G Xln [15]. Regarding the occurrence in the *T. reesei* cellulolytic system of the pair of cellobiohydrolases (CBHI and CBHII) from which one is retaining (CBHI) and the other inverting glycanase (CBHII) [16,17], we considered it important to examine the stereochemistry of the hydrolysis of glycosidic linkage by the two Xln.

2. Materials and methods

2.1. Enzymes

The two specific Xln of *T. reesei* C-30, EXI and EXII, were purified to homogeneity by ion-exchange chromatography and gel filtration as described by Tenkanen et al. [12]. Specific activity of EXI (acidic) was 5.53 U/mg, specific activity of EXII (alkaline) was 37.6 U/mg. The enzyme activity was determined as described [18] and one unit is defined as the amount of enzyme liberating from beechwood 4-*O*-methyl-D-glucurono-D-xylan 1 μmol equivalents of D-xylose at 30°C. For NMR spectroscopy experiments the enzymes were subjected to hydrogen/deuterium exchange by several successive ultrafiltrations in D_2O buffer (0.05 M sodium acetate, pD 5.0) using Centricon-10 concentrators (Amicon) with a 10 kDa cut-off.

2.2. Substrates

Methyl β-D-xylotrioside [19] was a generous gift from Dr. P. Kováč (NIH, Bethesda, USA). Xylobiose was synthetized from 4-nitrophenyl β-D-xylopyranoside enzymically [20]. For NMR spectroscopy experiments the xylotrioside was three times lyophilized from D_2O.

2.3. Preliminary hydrolysis experiments

Methyl β-D-xylotrioside (20 mM) in the D_2O buffer was mixed with various amounts of the enzymes at 25°C and at time intervals from a few minutes up to an hour the reaction mixtures were analyzed by thin-layer chromatography on cellulose (alluminium-coated sheets, Merck, Germany) in ethylacetate/acetic acid/water (3:2:2, by vol.) to identify the products and to find the enzyme concentration that would

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ensure rapid hydrolysis of the substrate, the rate of which would be substantially greater that the rate of mutarotation of xylobiose.

2.4. ^1H NMR experiments

0.5 ml of a 20 mM solution of methyl β-D-xylotrioside in the D_2O buffer was mixed with 20–50 μl of the solution of EXI (final concentration 4.5 U/ml) or EXII (final concentration 100 U/ml), immediately transferred to a NMR tube and ^1H NMR spectra were recorded (Brucker AM 300, Germany) at 25°C after various times of incubation.

3. Results and discussion

In preliminary experiments in which the cleavage of methyl β-D-xylotrioside with *T. reesei* EXI and EXII was followed by thin-layer chromatography, it was established that both enzymes cleave the substrate preferentially at the middle glycosidic linkage to give methyl β-D-xyloside and xylobiose. Minor cleavage modes corresponding to the formation of D-xylose and methyl β-D-xylobioside, and xylotriose and methanol, were apparent at later stages of the reaction. Degradation of methyl β-D-xylotrioside with both enzymes was also accompanied by the formation of trace amounts of oligosaccharides larger than xylotriose, evidence for the glycosyl transfer reactions. After these findings, the task to establish the mechanism of the cleavage of the glycosidic linkage remained limited to the determination of the anomeric configuration of xylobiose as the main reducing product. In agreement with previous data on the rate

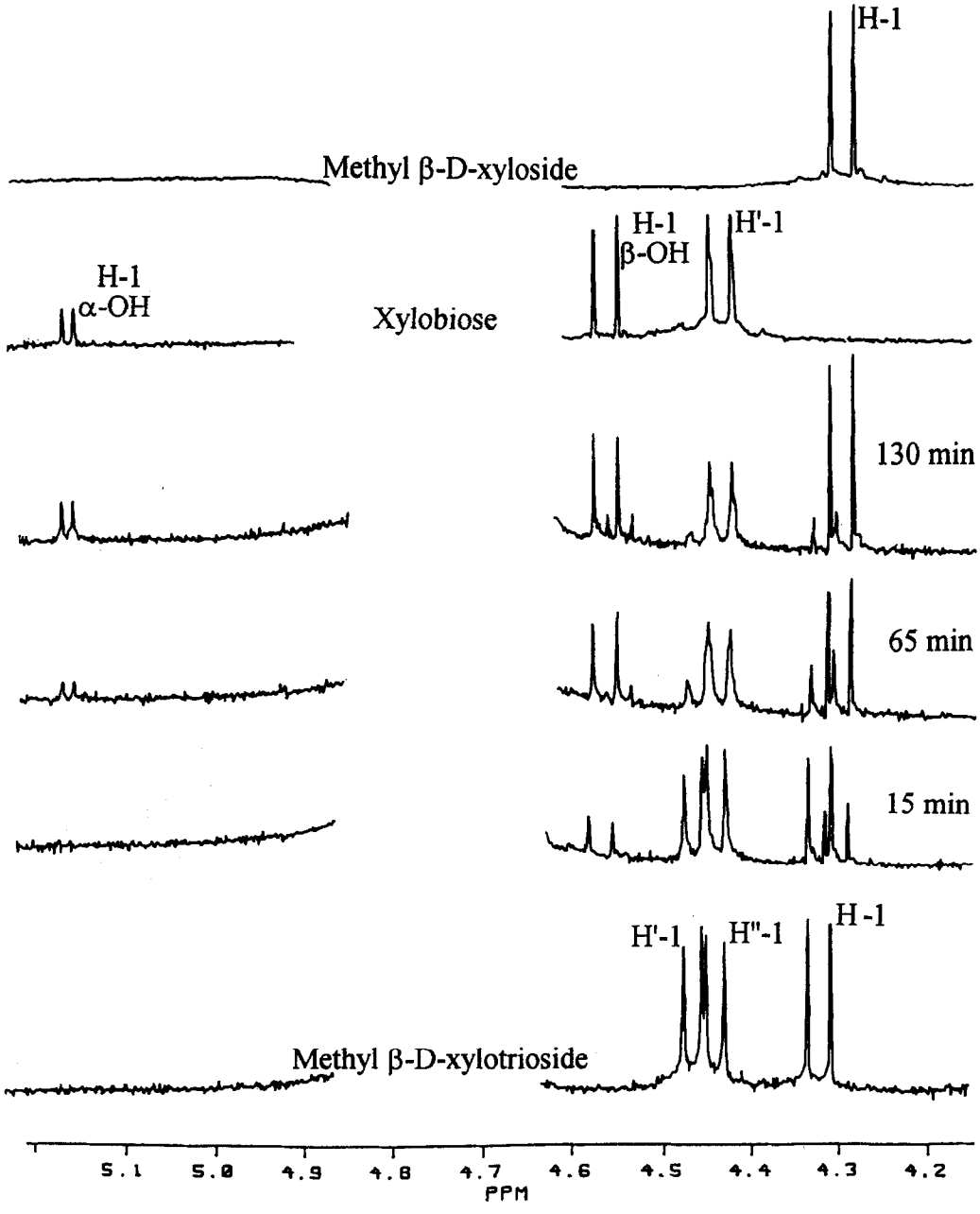

Fig. 1. Anomeric regions of the ^1H NMR spectra of methyl β-D-xylotrioside, xylobiose, methyl β-D-xylopyranoside and the enzymic digest of the trioside with EXI of *T. reesei* recorded at the time of incubation given in min. Concentration of methyl β-xylotrioside 20 mM, concentration of EXI 4.5 U/ml.

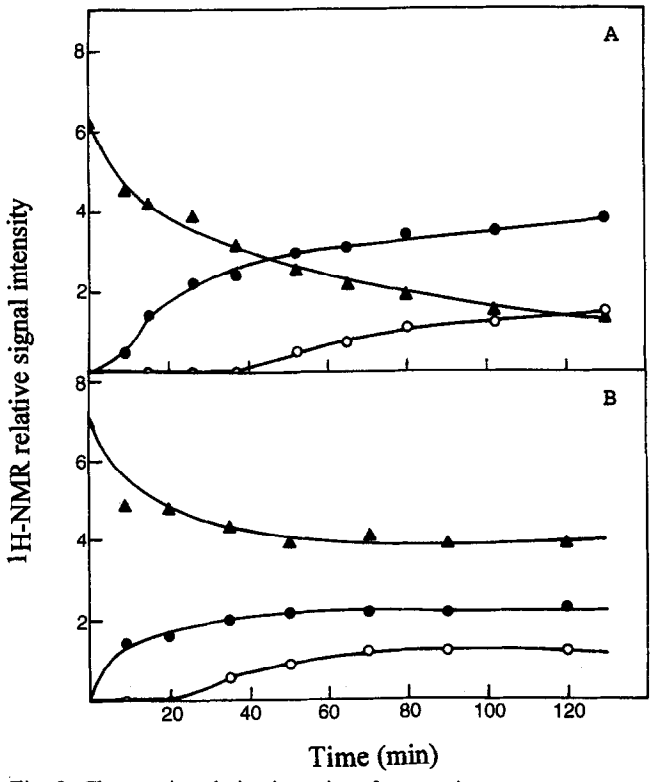

Fig. 2. Changes in relative intensity of anomeric proton resonances during hydrolysis of methyl β-D-xylotrioside with EXI (part A) and EXII (part B): H-1 signal of the substrate (▲); H-1 signal of β-xylobiose (●) and H-1 signal of α-xylobiose (○).

of hydrolysis of xylotriose and xylotetraose with EXI and EXII [14], EXII showed much lower affinity towards methyl β-D-xylotrioside, so its concentration had to be much higher than that of EXI (see section 2).

Fig. 1 shows the anomeric region of the ^{1}H NMR spectra of methyl β-D-xylotrioside (the substrate), methyl β-D-xyloside and xylobiose (the main products), and several recordings of the spectra of the reaction with EXI in the time course. This assignment of the signals is given in Table 1. The addition of EXI results in disappearance of the signals of H-1, H′-1 and H″-1 of the substrate and appearance of new signals that are identical with the H-1 signal of methyl β-D-xyloside (doublet centered at 4.29 ppm) and initially two and later three H-1 signals of xylobiose centered at 4.43, 4.55 and 5.16 ppm. The signal at 4.55 ppm, corresponding to the newly formed reducing end, is clearly that of the β-anomer of xylobiose ($J_{1,2}$ 7.7 Hz). The signal of the α-anomer of xylobiose (5.16 ppm, $J_{1,2}$ 3.7 Hz) appears much later as shown in Fig. 2, obviously due to mutarotation. Fig. 1 also shows appearance of one low intensity doublet centered at 4.54 ppm, which may correspond to the β-anomer of D-xylose, a product of a minor cleavage mode. The signals of anomeric hydrogens of the corresponding methyl β-D-xylobioside are not visible, perhaps due to overlapping by the signals of the substrate and major products.

Table 1
^{1}H NMR data for the anomeric protons of methyl β-D-xylotrioside, xylobiose and methyl β-D-xylopyranoside (300 MHz, D_2O)

Chemical shift σ ppm*	Coupling constants $J_{1,2}$ (Hz)	Assignment
4.32	7.5	H-1 in methyl β-D-xylotrioside
4.44	7.5	H″-1 in methyl β-D-xylotrioside
4.47	7.6	H′-1 in methyl β-D-xylotrioside
5.16	3.7	H-1 in α-D-xylobiose
4.55	7.7	H-1 in β-D-xylobiose
4.43	7.5	H′-1 in xylobiose
4.29	7.5	H-1 in methyl β-D-xyloside

*Values are centered for doublets and relative to 3-(trimethylsilyl)propane sulfonic acid Na salt.

An analogous experiment was carried out with EXII. Larger concentrations of the enzyme in the mixture with methyl β-D-xylotrioside resulted in a faster rate of its hydrolysis. From Fig. 2 it is clear that again the β-anomer of xylobiose is the principal form of the dimer liberated in the reaction.

The results lead to a conclusion that both specific Xln of *T. reesei*, the distant members of the G family of Xln belong, similarly as the xylan-hydrolyzing endo-β-1,4-glucanases of *T. reesei*, EGI [9], to the retaining glycanases, utilizing the double displacement mechanism of the hydrolysis of glycoside linkage [21]. This statement is in consonance with glycosyl transfer reactions of both enzymes observed at high concentrations of reducing β-1,4-xylooligosaccharides [14] and confirmed in this work. The results support the view that the entire family of G Xln are retaining hydrolases.

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