Two novel GH11 endo-xylanases from *Myceliophthora thermophila* C1 act differently toward soluble and insoluble xylans

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ABSTRACT

Two novel GH11 endo-xylanases from *Myceliophthora thermophila* C1 (C1), Xyl7 and Xyl8, were purified and the influence of solubility and molecular structure of various xylans on their efficiency was investigated. Both endo-xylanases were hindered by a high degree of substitution of a xylan. The two GH11 xylanases released different products from the xylans, in which Xyl7 displayed a degradation product composition closer to GH10 xylanases. A correlation of the degradation product composition with a specific residue at position 163 in the amino acid sequence of Xyl8 is suggested: tyrosine in Xyl8; valine in Xyl7. This is confirmed with examples of various endo-xylanases reported in literature.

The C1 GH11 xylanases were more efficient on self-associated xylan compared to C1 GH10 endo-xylanases and they released more small xylooligomers from these xylans. This is contrary to the general assumption that GH10 xylanases degrade xylans to a higher degree than GH11 xylanases.

1. Introduction

Xylans represent the main hemicellulose component of secondary plant cell walls. They comprise (1,4)-β-D-xylopyranose backbone, which can be substituted with short carbohydrate chains based on L-arabinofuranosyl units, 4-*O*-methyl-D-glucuronopyranosyl acids, D- or L-galactopyranosyl or D-glucuronopyranosyl acids and may also contain acetyl, feruloyl or *p*-coumaroyl groups [1,2]. Depending on the type and degree of substitution, the polymers differ in solubility [3,4]. The biodegradation of these xylans requires endo-1,4-β-D-xylanases (EC 3.2.1.8), β-D-xylosidases (EC 3.2.1.37) and several accessory enzymes, such as α-L-arabinofuranosidase, α-glucuronidase, acetylxylan esterase,

ferulic acid esterase, and *p*-coumaric acid esterase [5,6]. The principal commercial sources for these enzymes are filamentous fungi, of which strains of *Aspergillus* and *Trichoderma* are the most common ones used [7]. Also, the genome of the Ascomycetes fungus *Myceliophthora thermophila* C1 (C1) has been shown to contain many genes encoding carbohydrate-active enzymes, including several xylan degrading activities [8,9].

Most endo-xylanases belong to glycoside hydrolase (GH) family 10 or 11 based on amino acid similarities and structural features [6,10]. GH10 endo-xylanases are highly active toward short xylooligomers. They exhibit less substrate specificity than GH11 endo-xylanases and can hydrolyze different types of decorated xylans [6]. GH11 endo-xylanases are highly specific and do not tolerate many decorations on the xylan backbone [11]. GH11 endo-xylanases are composed of three antiparallel β-sheets and one α-helix. The three-dimensional structure of these endo-xylanases has been described as a "partly closed right hand". This "hand" contains a thumb-like structure, which is the most flexible region of the xylanase. The movement of this thumb could be essential for the function of the enzyme as it determines the width of the cleft and accompanies the binding of substrates in the catalytic cleft [12].

Although classification of the glycoside hydrolase enzymes is aimed at providing a convenient tool to derive mechanistic information, it has been shown that the GH family classification alone cannot predict enzyme breakdown products and enzyme efficiency toward soluble and insoluble substrates ([9] and references

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therein). Furthermore, differences in amino acid sequence and conformation of the enzymes was shown to explain the formation of different breakdown products by GH10 enzymes [9].

Only three C1 GH11 xylanases have been subjected to preliminary xylanase characterization tests before [13]. In this study, two novel C1 GH11 endo-xylanases, Xyl7 and Xyl8, were subjected to detailed analysis using the same effective approach as described previously for C1 GH10 endo-xylanases [9]. The GH11 xylanases were purified and characterized biochemically in order to determine their action pattern toward different xylans. These xylans varied in type and degree of substitution as well as in solubility. Their functionality was combined with predicted structural features of the protein. Furthermore, their performance was compared with the results obtained for GH10 endo-xylanases of *M. thermophila* C1 as described previously [9].

2. Materials and methods

2.1. Substrates

Wheat arabinoxylan (WAX), medium viscosity was obtained from Megazyme (Wicklow, Ireland). Beech wood xylan (BeWX), birch wood xylan (BiWX) and oat spelt xylan (OSX) were from Sigma-Aldrich (St. Louis, MO, USA). These substrates contain different types and degrees of substituents, resulting in different solubility in aqueous solutions [9]. Since BiWX and BeWX were obtained through alkali extraction, acetyl groups were not present as substituents. The solubility of BiWX and BeWX in water was evaluated according to Hespell and Cotta [14]. Xylans (quadruplicates per sample) were stirred in water (45 °C, final concentration 5%, w/v), incubated for 120 min and centrifuged (19,000 × g). The supernatant was removed and the pellet freezedried to determine the remaining insoluble fraction. The solubility of BeWX was found to be 88% (±0.7, w/w), and for BiWX 54% (±0.4, w/w).

Wheat straw water unextractable solids (WS WUS) (wheat straw, harvested in Groningen, The Netherlands, May 2003), wheat straw alkaline soluble solids (WS KOHss), wheat straw alkaline residue (WS KOHres) and a mixture of the WS KOHss and WS KOHres in the same weight ratios as obtained during extraction (WS KOHmix) were prepared in our laboratory [9,15]. The neutral monosaccharides were analyzed as their alditol acetates using gas chromatography after prehydrolysis with 72% (w/w) sulfuric acid followed by 1 M H_2SO_4 hydrolysis. A part of the hydrolysate was used for the determination of the uronic acid content by the colorimetric m-HDP assay [16]. The constituent monosaccharide composition of the substrates as already presented in previous research [9] is added as supplementary table (table A1).

2.2. Enzyme production

Xyl7 and Xyl8 from *Myceliophthora thermophila* C1 (C1) were recognized from the genome. They were overproduced in a specially designed C1-expression host (LC strain) [17]. In addition, a construct of the *xyl7* gene, which lacked the carbohydrate binding module (CBM), was used to transform the LC strain. The strains were made as described in previous research [17] and grown aerobically in 2-L fermenters in a mineral medium containing glucose, ammonium sulfate and trace elements for the essential salts [18]. After biomass formation, the enzyme was produced in a fed-batch process under glucose limitation at pH 6.0 and 32 °C. The production resulted in: Xyl7 (representing a xylanase with CBM), Xyl7dCBM (representing a xylanase without CBM) and Xyl8 (representing a xylanase, naturally present without CBM).

2.3. Enzyme purification

The first two purification steps of Xyl7dCBM included ion exchange chromatography using a Source30S column (GE Healthcare, Uppsala, Sweden) with 15 mM potassium phosphate buffer, pH 6.0 and a gradient of 0–0.5 M potassium chloride (KCl) in five column volumes (CV). The active fraction was loaded onto a Source30Q column (GE Healthcare) with the same buffer with a gradient of 0–0.5 M KCl in six CV. Next, a phenyl sepharose 6 HS FF column (GE Healthcare) with 10 mM potassium phosphate buffer, pH 6.0 and a gradient of 1–0 M ammonium sulfate in eight CV was used. A Superdex 75 column (GE Healthcare) with 10 mM sodium acetate buffer, pH 5.0 as eluent was used for final purification.

The purification of Xyl7 started with ion exchange chromatography using a Source30S column with 10 mM sodium acetate buffer, pH 5.0 and a gradient of 0–0.5 M sodium chloride (NaCl) in five CV. Secondly, a Source30Q column was used with the same buffer with a gradient 0–0.5 NaCl in five CV. Finally, the active fractions were applied to a Superdex 75 column using a 10 mM sodium acetate buffer, pH 5.0.

The overexpressed Xyl8 was purified in three steps. The first step included gel-filtration using a BioGel P4 column (Biorad, Hercules, CA, USA) with a 10 mM Bis–Tris/HCl buffer, pH 6.8. Secondly, anion exchange chromatography was applied using a Source30Q column (GE Healthcare) with 10 mM Bis–Tris/HCl buffer of pH

6.8 and a gradient of 0–0.5 M NaCl in six CV. Finally, separation on a SourceISO column (GE Healthcare) was carried out in 50 mM sodium acetate buffer with 1.7 M ammonium sulfate, pH 5.0 and a gradient of 1.7–0 M ammonium sulfate in six CV. A Millipore Pellicon XL system was used for final desalting and concentrating the Xyl8 fraction.

After ultrafiltration and freeze drying, each active fraction was characterized by SDS-PAGE (as described in Section 2.5) and tested for xylan degrading activity toward wheat arabinoxylan (WAX, medium viscosity, megazyme: 2 mg/mL sodium acetate buffer, 50 mM, pH 5.0; 30 min) with the PAHBAH reducing sugar assay [19,20]. The assay uses *p*-hydroxy benzoic acid hydrazide in aqueous alkali for the coloring reaction with reducing sugars. 30 min incubation was in the linear range of activity. The concentration of released reducing ends was determined using a xylose calibration curve.

The protein bands of SDS-PAGE corresponding to Xyl7 were isolated from the gel and sent to The Scripps Research Institute (Florida, USA) for ESI-MS/MS analysis to identify the extra proteins; the identification of Xyl8 was done by use of Maldi-TOF MS [13]. The preparations were tested for 1,4-β-D-xylosidase activity. This was assayed as described by Herr et al. [21]. The liberated 4-nitrophenol was measured at 400 nm.

2.4. Temperature and pH optimum

The temperature optima were determined within the range of 20–90 °C using 1% (w/v) BiWX (Sigma) with 0.01% (w/w on protein basis) enzyme in 0.2 M sodium acetate buffer (pH 5.0). The reaction was stopped after 10 min by boiling the digests.

The pH optima of the endo-xylanases were determined at the temperature optimum of each enzyme, using 1% (w/v) birch wood xylan with 0.01% (w/w on protein basis) enzyme. McIlvaine buffers were prepared by mixing 0.1 M citric acid and 0.2 M disodium hydrogen phosphate to obtain buffers in a pH range of 3.0–8.0. Activity was measured using the PAHBAH reducing sugar assay [19,20].

2.5. Protein analysis

For protein characterization, SDS-PAGE was performed by using precast 8–16% gradient gels (Lonza Group Ltd, Basel, Switzerland) in running buffer containing 25 mM Tris/HCl, 192 mM glycine and 0.1% (w/v) SDS (pH 8.3) at 125 V. The samples were denatured before being loaded on to the gel at 95 °C for 5 min in loading buffer containing 0.35 M Tris/HCl, 10.3% (w/v) SDS, 36% (v/v) glycerol, 5% (v/v) 2-mercapto-ethanol and 0.012% (w/v) bromo-phenol-blue (pH 6.8). The gels were stained using Coomassie Brilliant Blue. Protein concentration was measured using the Bradford method [22] and Bradford reagents (Sigma–Aldrich).

2.6. Enzyme degradation of the substrates

Substrates were suspended in 50 mM sodium acetate buffer, pH 5.0 (5 mg/mL). Enzyme was added (0.063 units/mg; one unit of activity corresponds to the quantity of enzyme hydrolyzing 1 μmol of substrate or releasing 1 μmol of reducing sugars (in sugar equivalents) per minute at 50 °C) and the mixture was incubated at 50 °C for 30 min, 1, 2, 4, 8 and 24 h. An equal dose of fresh enzyme was added at 24 h and the reaction was stopped by boiling the samples after 48 h. Results and discussion of WAX, BeWX, BiWX and OSX are based on samples of 30 min, 2, 8 and 24 h (using the response factor of xylotetraose), as the 1 and 4 h samples were fully in line with the other samples and there were no significant differences observed after 48 h. The enzyme dosing in the experiments was activity-based instead of molar-based. This might result in an underestimated performance of Xyl8. However, the enzymes were overdosed and did not display differences after addition of fresh enzyme, which means that an end-point situation is used for data interpretation of the total hydrolyzing capacity of the endo-xylanases.

For the insoluble substrates WS WUS, WS KOHmix and WS KOHres (5 mg/mL 50 mM sodium acetate buffer, pH 5.0) ten times the amount of enzyme was added compared to the soluble substrates. WS KOHss was the only substrate that was also dissolved in 50 mM sodium acetate buffer, pH 5.0 containing 20% (v/v) dimethyl-sulfoxide (DMSO). Aliquots were taken at 8 and 24 h. Substrate solutions without enzyme addition were used as blanks. The results for the different time points were fully in line with each other; only the 24 h samples are discussed.

Substrate degradation was monitored by high performance anion exchange chromatography (HPAEC) [16]. Quantification of the results was based on the response factor of xylotetraose. Relative amounts of monomers and oligomers were calculated either as percentage of the particular sugar present in the initial substrate, or as percentage of the total amount of carbohydrates in the initial substrate.

2.7. Sequence analysis

The DNA sequence data are available at the GenBank database under the accession numbers, *xyl7* JF508855 and *xyl8* JF508856. The alignment of the amino acid sequences was made with the ClustalW multiple alignment program [23]. The CPH-models 3.0 program (available at www.cbs.dtu.dk/services/CPHmodels/; Nielsen et al. [24]) was used for homology modeling.

Table 1
Overview of the characteristics of the GH11 endo-xylanases from *Myceliophthora thermophila* C1 in this study.

Sample	Activity (U/mg protein)[a]	Molecular mass (kDa)[b]	p*I*[c]	pH optimum	Temperature optimum (°C)
Xyl7dCBM	19.5	22	7.3	5.5–6.5	50–60
Xyl7	27.2	30	7.6	5.5–6.5	50–60
Xyl8	170.6	22	6.2	5.5–6.0	50–65

[a] Measuring reducing sugars using wheat arabinoxylan as substrate, Section 2.3.
[b] Measured by SDS-PAGE.
[c] Calculated value.

3. Results and discussion

3.1. GH11 endo-xylanases from Myceliophthora thermophila C1

The genes *xyl7* and *xyl8* that have been found in the *M. thermophila* C1 genome were encoding glycoside hydrolase family 11 (GH11) endo-xylanases. Xyl7 occurs naturally with a family 1 (cellulose) carbohydrate binding module (CBM) at the C-terminus of the protein.

3.1.1. Purification of the endo-xylanases

The purification of Xyl7 yielded in total 3 fractions containing active forms of Xyl7. ESI-MS/MS analysis on the fraction with the highest molecular mass confirmed that this fraction contained the full length Xyl7 (30 kDa) with the intact CBM. The study was continued with the pure fraction of full length Xyl7.

After four sequential purification steps, the pool of Xyl7dCBM was still not completely pure. Fractions containing only the additional proteins did not show significant xylanase activity (data not shown). In addition, the extra proteins found in the SDS-PAGE gel were analyzed by ESI-MS/MS. These proteins were identified as proteins not involved in plant cell wall degradation. It was decided to continue with this not completely pure preparation.

Xyl8 occurs naturally in C1 as a xylanase without CBM. After the three purification steps, the Xyl8 fraction contained next to the purified enzyme, similarly to Xyl7dCBM, some proteins that were not involved in cell wall degradation and not showing any xylanase activity. Also for Xyl8 it was decided to continue with this not completely pure preparation. Table 1 gives an overview of the characteristics per enzyme.

None of the endo-xylanases displayed β-xylosidase activity toward pNPh-β-xyloside (no further data shown).

3.1.2. Temperature and pH optima

Under the conditions used, the enzymes displayed their highest activity between 50 and 60 °C. All enzymes retained ≥50% of their maximum activity between 20 and 65 °C. Xyl8 even retained 80% of its maximum activity at 20 °C. The pH optima showed that the enzymes were mostly active between pH 5.0 and 6.5, exhibiting at least 80% of their maximum activity. The maximum activity under the conditions used was found at pH 6.0 for Xyl7dCBM and Xyl7 and at pH 5.5 for Xyl8 (no further data shown).

3.2. Enzyme performance on commercial xylans

3.2.1. Degradation products

The degradation of WAX, BeWX, BiWX and OSX was followed by high performance anion exchange chromatography (HPAEC). An example (Xyl7) of each chromatogram with peak annotation [9] is added as supplementary Fig. A1. For all substrates the amounts of monomers and oligomers were plotted against time. As an example, Fig. 1 shows the data for each of the enzymes toward BiWX. The results of the WAX, BeWX and OSX degradation products can be found in Figs. A2–A4.

The main components released by the endo-xylanases from the best soluble and highest substituted xylan WAX, were multiple arabinofuranosyl substituted xylooligomers, followed by single arabinofuranosyl substituted oligomers. The ratio of the different products released from WAX was comparable for all the endo-xylanases (Fig. A2); only small differences were observed in the amount of xylose and xylobiose released by the enzymes.

From BeWX, BiWX and OSX, both Xyl7 with and Xyl7 without CBM released xylobiose, xylotriose (Fig. 1 and Figs. A3, A4) and substituted oligomers (mainly 4-*O* methyl glucuronopyranosyl substituted oligomers of which the precise structure has been revealed in similar studies by Biely et al. [11]; Fig. A1). Remarkably, the C1 xylanase Xyl7dCBM displayed relatively high xylose release. The release of xylose is not often observed for endo-xylanases belonging to GH11 [13,25]. Xyl8 released mainly substituted oligomers and xylotriose, and to a lesser extent xylobiose, xylotetraose and xylopentaose. Xyl8 displayed a different release of components compared to the Xyl7 endo-xylanases. In particular, more of the larger oligosaccharides and less of the small oligosaccharides were released by Xyl8 from these commercial xylans.

3.2.2. Hydrolyzing capacity of the endo-xylanases on commercial xylans

The total hydrolyzing capacity (the w/w% of the substrate that is degraded by the enzymes to monomers and oligomers) after 24 h per enzyme per substrate is displayed in Fig. 2A.

The performance of the C1 GH11 endo-xylanases increased with a decreasing degree of substitution despite the fact that a less substituted xylan also shows lower solubility [4]. It is known that GH11 endo-xylanases do not tolerate high substitutions on the xylan backbone [11], although the correlation to the consequential insolubility has not been made before.

For each of the C1 GH11 endo-xylanases, the total hydrolyzing capacity on BeWX and BiWX was higher than that on WAX. The lower degrees of substitution of BeWX and BiWX in comparison to WAX (table A1) might cause the better performance of the enzymes on these substrates. Xyl7 (with CBM) and Xyl8 performed worse on BiWX compared to BeWX, while Xyl7dCBM did not exhibit significant differences in performance toward these 4-*O* methyl glucuronopyranosyl substituted xylans. The differences found for the enzyme performance toward BeWX and BiWX are assumed to be caused by the distribution pattern of the 4-*O* methyl glucuronopyranosyl substituents on the xylan backbone. The sugar composition (table A1) shows that BeWX contains equal amounts of 4-*O* methyl glucuronopyranosyl substituents as BiWX, but has a higher solubility: 88% (w/w) for BeWX and 54% (w/w) for BiWX. Partial insolubility of xylans is suggested by Ebringerova and Heinze [3] to be caused by interchain aggregation of unsubstituted regions (self-association), which is observed for BiWX and OSX [26]. Xyl7 (with CBM) performed very well on OSX compared to the other substrates, which indicates that Xyl7 is not hindered by self-association of xylan. Consequently, the lower hydrolyzing capacity of Xyl7 on BiWX must be a result of the presence of 4-*O* methyl glucuronopyranosyl substituents and not of the partial self-association. Xyl7dCBM was the most efficient xylanase in the degradation of OSX. Xyl8 performed equally on OSX and BiWX. This suggests that
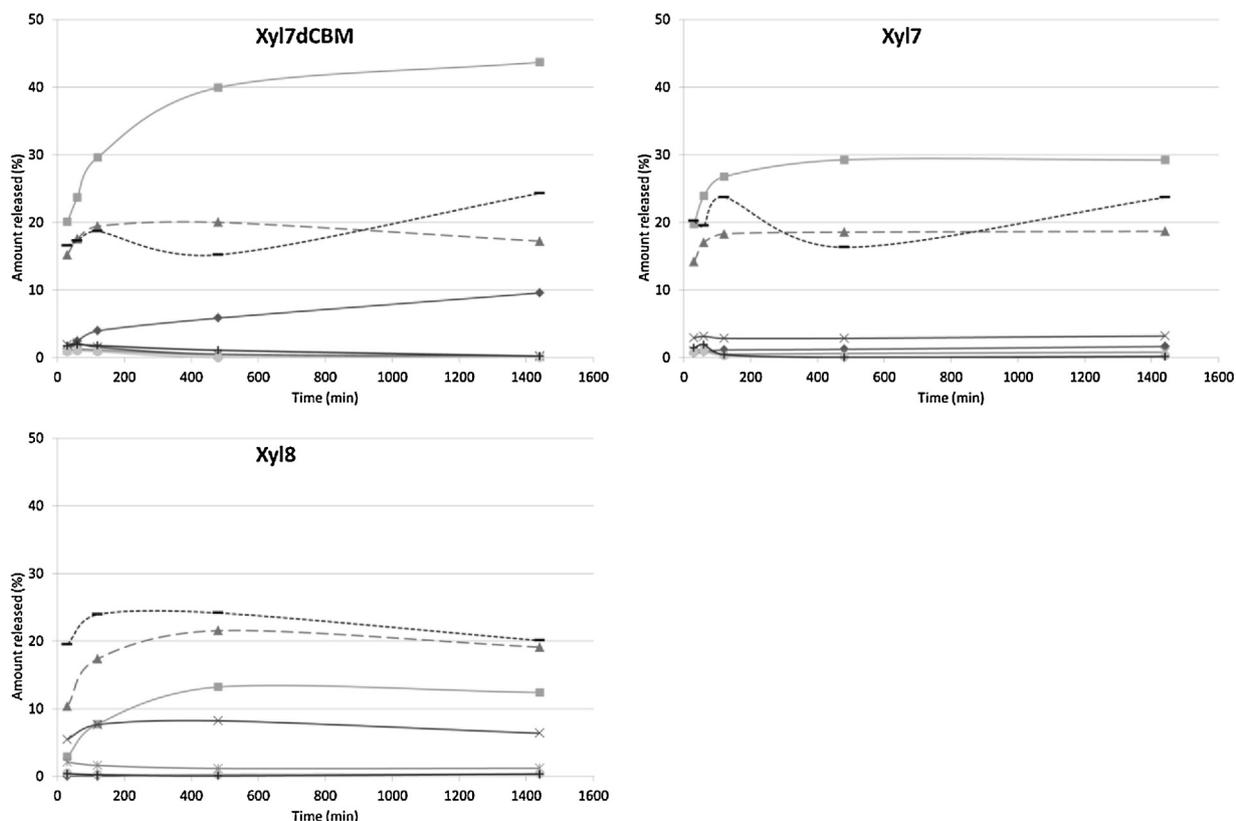
Fig. 1. Release of xylose and oligomers after birch wood xylan (BiWX) digestion by different C1 GH11 endo-xylanases. The amount of xylose components is displayed as weight percentage of the total amount of xylose. The amount of substituted oligomers is displayed as weight percentage of the total amount of carbohydrates in BiWX. ◆, xylose; ■, xylobiose; ▲ - ■, xylotriose; ✕, xylotetraose; ✳, xylopentaose; ●, xylohexaose; ╋, linear xylooligomers DP > 6; ▪▬▪, other (enzyme activity: 0.063 U/mg substrate, pH 5.0, 50 °C).

Xyl8 indeed is hindered by self-association of the linear parts of xylan.

Xyl7dCBM was most efficient in total hydrolyzing capacity of all substrates, followed by Xyl7 and Xyl8. Xyl7dCBM is smaller compared to Xyl7 (with CBM). It is therefore assumed that Xyl7dCBM is able to interact with self-associated xylan more easily than the xylanase with CBM, resulting in a higher release of monomers and oligomers. Xyl8 clearly performed differently from Xyl7, even though they are both GH11 endo-xylanases of the same origin. Xyl7dCBM performed similarly to the C1 GH11 endo-xylanase Xyl5 (Xyn11C) reported previously [13]. None of the xylanases reported by these authors was comparable to the characteristics found for Xyl8.

3.3. Enzyme performance on xylan rich wheat straw fractions

Fig. 2B shows that the solubilization of WS WUS after 24 h incubation was highest for Xyl7 (6% mono- and oligomers released). Xyl8 released 3% of the total substrate as monomers and oligomers, even though it does not contain a CBM. WS WUS consists of the water unextractable solids of wheat straw and the carbohydrates are still interlinked within the complex cell wall matrix.

The alkaline treatment of the WS WUS increased the amount of monomers and oligomers released by the enzymes by up to 20–30% (WS KOHmix) which is due to the destroyed alkaline-labile interactions in the cell wall. After alkali treatment and separation of the soluble xylan fraction and residue, the residue still contained 6% (w/w) of xylose (table A1), of which 60% could be solubilized by the GH11 endo-xylanases.

The xylans extracted from WS WUS with alkali were partly soluble in buffer and were very well degraded by both Xyl7 xylanases. Xyl7dCBM was most efficient and Xyl7 least efficient as indicated by 68% and 48% mono- and oligomers released, respectively. Xyl8 degraded 37% of this extracted xylan fraction to mono- and oligomers. The behavior of the GH11 endo-xylanases on WS KOHss in buffer resembled that of OSX, which can be ascribed to self-association of the low substituted xylans.

By use of DMSO, the possible effect of self-association of xylan was ruled out by the exclusion of hydrogen bond formation between the xylan polymers [27]. Hence, in this solvent the only factors determining the enzyme performance were the degree and type of substitution of the xylan. The enzymes were not influenced by the more apolar environment, as WAX in 20% (v/v) DMSO incubated with all GH11 xylanases showed similar degradation patterns compared to buffer alone (no further data shown).

The effect of WS KOHss solubilization in 20% (v/v) DMSO decreased the amount of released monomers and oligomers for Xyl7dCBM (decrease of ~10%, Fig. 2B). Xyl7 performed equally well in the more apolar environment, whereas Xyl8 benefits from xylan solubilization in DMSO (Fig. 2B).

The results show that Xyl7 (with CBM) was not hindered by self-association of xylans and is only hindered by the type and degree of substitution of a xylan. Xyl7dCBM was very efficient in the degradation of self-associated xylan. When xylans were self-associated and formed insoluble aggregates Xyl8 displayed limited activity. In addition, it was concluded that the CBM was not beneficial for the action of Xyl7 on insoluble substrates.
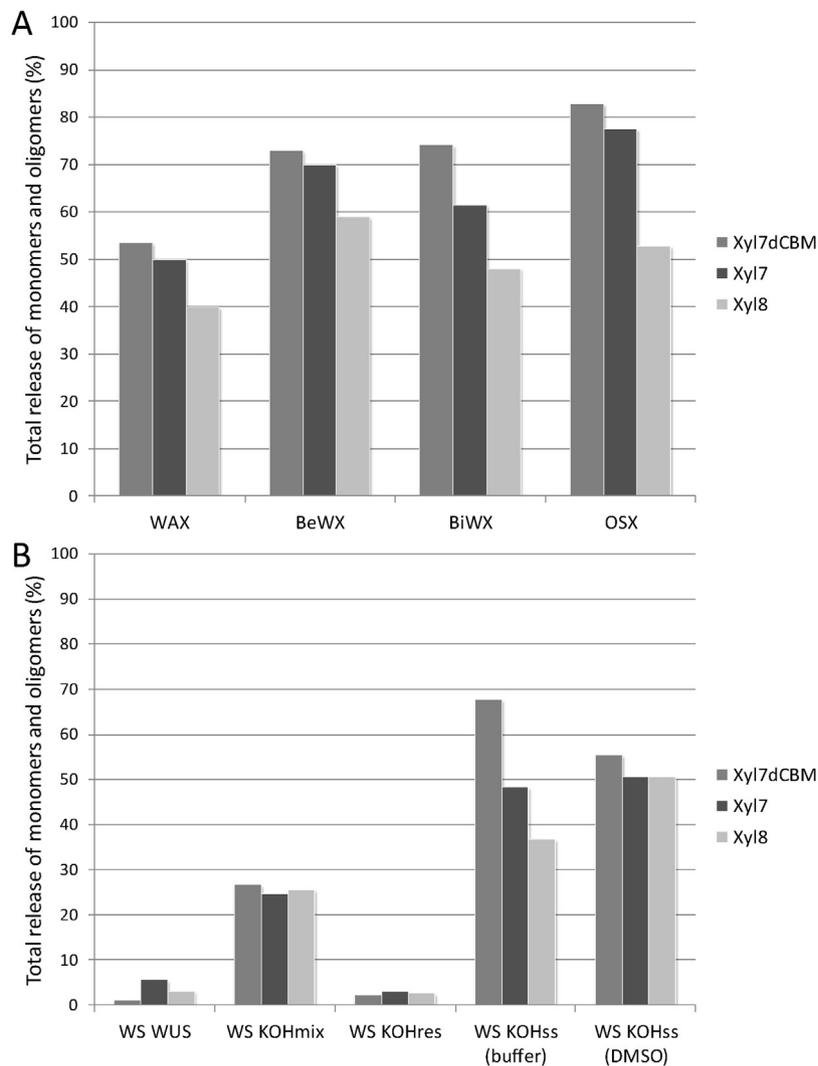
Fig. 2. Release (w/w%) of monomers and oligomers from the carbohydrate fraction of wheat arabinoxylan (WAX), beech wood xylan (BeWX), birch wood xylan (BiWX) and oat spelt xylan (OSX) (A) and from the carbohydrate fraction of wheat straw water unextractable solids (WS WUS), wheat straw after alkaline treatment (WS KOHmix), wheat straw alkaline residue (WS KOHres), wheat straw alkaline soluble solids (WS KOHss) suspended in buffer and WS KOHss suspended in 20% DMSO (B), by the C1 endo-xylanases with and without carbohydrate binding module (dCBM) after 24 h of incubation (enzyme activity: 0.063 U/mg substrate, pH 5.0, 50 °C) based on high performance anion exchange chromatography results.

3.4. Differences in enzyme behavior correlate with protein structure

3.4.1. Alignment with heterologous GH11 endo-xylanases

In order to relate the differences in enzyme performance of the C1 GH11 endo-xylanases with their protein structures, the alignments of Xyl7 and Xyl8 with heterologous GH11 endo-xylanases are indicated in Fig. 3. These alignments show the conserved catalytic residues (black residues) and the residues involved in binding the substrate (grey residues). Based on these alignments, the homology between the catalytic modules (CBM not included) of Xyl7 and Xyl8 is 63%. Compared to other heterologous GH11 endo-xylanases from *Trichoderma reesei*, *Aspergillus niger*, *Penicillium funicolosum* and *Chaetomium thermophilum*, Xyl7 and Xyl8 showed 40–60% homology with respect to residues involved in substrate binding and the structure of the catalytic cleft. Compared to the other xylanases, Xyl8 had additional amino acids at the N-terminal part of the protein sequence, but these residues did not seem to be involved in catalysis neither were they recognized as a CBM (indicated with x in Fig. 3).

Construction of model structures of both C1 GH11 endo-xylanases showed that the secondary structure of these enzymes was very similar (Fig. 4). This corresponds with literature stating that the partially closed right hand structure as well as many residues and residue clusters are conserved in GH11 endo-xylanases [28].

3.4.2. Difference in one amino acid responsible for different enzyme behavior?

One of the differences between the amino acid sequence of Xyl7 and Xyl8 was residue Tyr163 in Xyl8, which was a valine in Xyl7 (Val152), indicated with * in the alignment in Fig. 3. It has recently been reported that the Tyr163 is variable in 68% of the GH11 endo-xylanases and that it is involved in the stacking interaction at the non-reducing subsite position (−2) [28]. It was hypothesized that the difference in subsite (−2) caused flexibility of this region, thereby becoming more tolerant toward substituents on the xylan backbone near the cleavage site of the enzyme; or by a change in relative binding affinity at the other subsites due to a larger distance to this (−2) subsite. This flexibility in the catalytic cleft can also explain the behavior reported for other GH11 xylanases: The presence of a valine instead of tyrosine is also found for Xyl5 (Xyn11C) which displayed, after glucuronoxylan and arabinoxylan digestion, a product composition closer to the GH10 family

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                                                                          ×××××××××××
Xyl7           1 ---MVALSS- LLVAASAAAV AVAAPS---- ----EALQKR ---------- QTLTSSQTGF HDGFYYSFWT
Xyl18          1 ---MVSFKA- LVLGAVG-AL SFPFNVTE-- ---LSEAHAR GENVTELLMS RAGTSPQTGW HGGYYFSFWT
Xyn2 Trees     1 ---MVSFTS- LLAGVAAISG VLAAPAAEV- --ESVAVEKR ---------- QTIQP-GTGY NNGYFYSYWN
XynB Anige     1 ---MLTKNLL LCFAAAKAVL AVPHDSVVE- --RSDALHKL S--------- ERSTPSSTGE NNGFYYSFWT
XynB Pfuni     1 ---MGISSIL LSALIAGGAL ALPAAEPVS- ----FDIRDE ---------- -NITLARRA- EAINYNQDYI
XynC Pfuni     1 ----MKLFLA AIVLCATAAT AFPSELAQR- --AAGDLSKR ---------- QSITTSQTGT NNGYYYSFWT
Chaetomium     1 ---MVNFST- LFLAASTAAL AAAAP----- -----SIEKR ---------- QTLTSSATGT HNGYYYSFWT

Xyl7          49 DGAGNVRYTN E-AGGRYSVT WSGNNGNWVG GKGWNPG-AA RNISFTGQYN ----PNGNSY LAVYGWTRNP
Xyl18         61 DNGGTVNYWN G-DNGRYGVQ WQNC-GNFVG GKGWNPG-AA RTINFSGSFN ---PSG-NGY LAVYGWTQNP
Xyn2 Trees    53 DGHGGVTYTN G-PGGQFSVN WSNS-GNFVG GKGWQPGTKN KVINFSGSYN ----PNGNSY LSVYGWSRNP
XynB Anige    56 DGGGDVTYTN G-DAGSYTVE WSNV-GNFVG GKGWNPG-SA QDITYSGTFT ----PSGNGY LSVYGWTTDP
XynB Pfuni    51 ASGANVQYSP NMAAGSFSIN YNTQ-GDFVV GLGWQPG-DA NPITYSGSFS ----ASGVGI LAVYGWSTNP
XynC Pfuni    54 NGGGEVTYTN G-DNGEYSVT WVDC-GDFTS GKGWNPA-NA QTVTYSGEFN ----PSGNAY LAVYGWTTDP
Chaetomium    47 DGQGNIRFNL E-SGGQYSVT WSGN-GNWVG GKGWNPGTDN RVINYTADYR ----PNGNSY LAVYGWTRNP

                                                                      *
Xyl7         113 LIEYYIVENF GTYDPSTGAQ RLGSITVDGS TYNILKTTRV NQPSIEGTST FDQFWSVRTN KR--SSGSVN
Xyl18        124 LIEYYIVESF GTYDPSSQAQ VLGTFYQDGS NYKIAKTTRY NQPSIEGTST FDQFWSVREN HR--TSGSVN
Xyn2 Trees   117 LIEYYIVENF GTYNPSTGAT KLGEVTSDGS VYDIYRTQRV NQPSIIGTAT FYQYWSVRRN HR--SSGSVN
XynB Anige   119 LIEYYIVESY GDYNPGSGGT YKGTVTSDGS VYDIYTATRT NAASIQGTAT FTQYWSVRQN KR--VGGTVT
XynB Pfuni   115 LVEYYVMEVH DG--YQTAGT HKGTVTTDGG TYDIWEHQQV NQPSILGTST FNQYISIRQS PR--TSGTVT
XynC Pfuni   117 LVEYYILESY GTYNPSSGLT SLGQVTSDGG TYDIYSTQRV NQPSIEGTST FNQYWSVRTE KR--VGGTVT
Chaetomium   111 LIEYYVESF GTYDPSTGAT RMGSVTTDGG TYNIYRTQRV NAPSIEGTKT FYQYWSVRTS KR--TGGTVT

Xyl7         181 VKAHFDAWAQ AG-LRLGTHD YQIMATEGYF S--------- ----SGSATI TVGEGTSSGG GGDNGGGNNG
Xyl18        192 VGAHFARWQQ AG-LRLGTHN YQIMATEGYQ S--------- ----SGSSDI TVW------- ----------
Xyn2 Trees   185 TANHFNAWAQ QG-LTLGTMD YQIVAVEGYF S--------- ----SGSASI TVS------- ----------
XynB Anige   187 TSNHFNAWAK LG-MNLGTHN YQIVATEGYQ S--------- ----SGSSSI TVQ------- ----------
XynB Pfuni   181 VQNHFNAWAQ AG-MNLGTLN YQVMAVESWS GSGSGQISLS KGTGGGSTTT TPTGPTSTST APSSGGTGAA
XynC Pfuni   185 TANHFAAWKA LG-LEMGTYN YMIVSTEGYE S--------- ----SGSSTI TVS------- ----------
Chaetomium   179 MANHFNAWRQ AG-LQLGSHD YQIVATEGYY S--------- ----SGSATV NVGGSTTGGN NGGNNGGNNG

                            CBM1
Xyl7         237 G--GGNTGTC SALYGQCGGQ GWTGPTCCSQ GTCRVSNQWY SQCL 278
Xyl18            ---------- ---------- ---------- ---------- ---- 230
Xyn2 Trees       ---------- ---------- ---------- ---------- ---- 223
XynB Anige       ---------- ---------- ---------- ---------- ---- 225
XynB Pfuni   282 QWGQCGGIGW TGPTTCVAPY TCKYENAYYS QCQ------- ---- 282
XynC Pfuni       ---------- ---------- ---------- ---------- ---- 223
Chaetomium   260 GNNGGNTGSN VSIS------ --RPRKMGSL ASTR------ ---- 260
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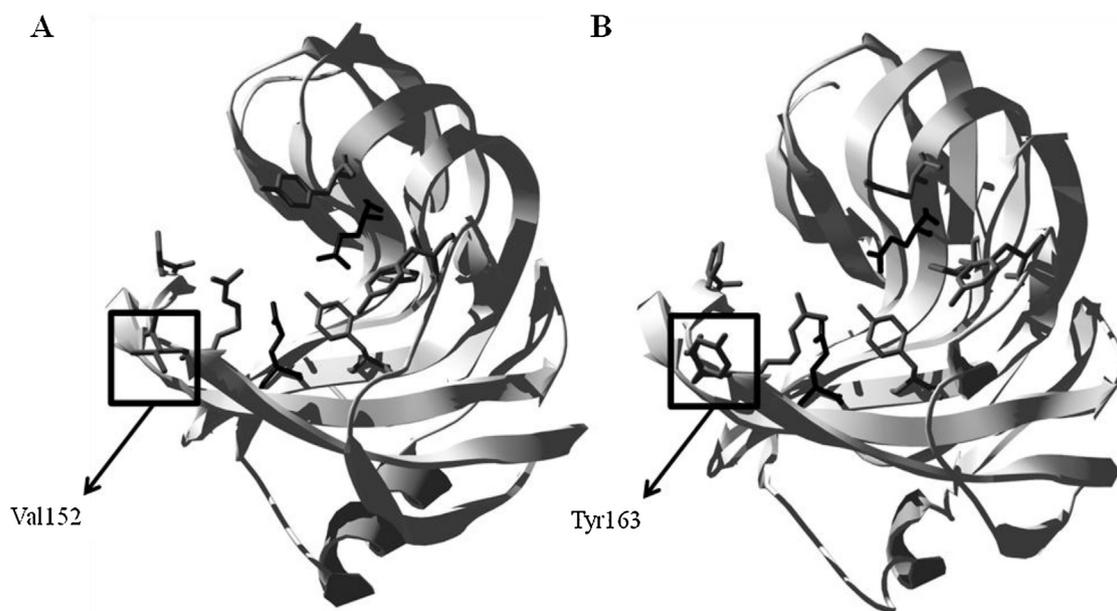
Fig. 3. Alignment of C1 GH11 endo-xylanases with different heterologous GH11 endo-xylanases. The black colored residues are the catalytic residues, the gray colored residues are involved in binding the substrate and the residues in the box are the CBM1 residues in the enzymes (based on Hakulinen et al. [33]. Xyn2 Trees: Xylanase Xyn2 from *Trichoderma reesei*, Genbank accession nr. AAB29346; XynB Anige: Xylanase XynB from *Aspergillus niger*, Genbank accession nr. AAM95167; XynB Pfuni: Xylanase XynB from *Penicillium funiculosum*, Genbank accession nr. CAD33900; XynC Pfuni: Xylanase XynC from *Penicillium funiculosum*, Genbank accession nr. CAC15487; Chaetomium: xylanase Xyn11A from *Chaetomium thermophilum*, Genbank accession nr. CAD48749; x extra amino acids in N-terminal part of Xyl8, * Tyr163 in Xyl8 and Val152 in Xyl7.

enzymes [13]. The other two C1 GH11 xylanases reported by these authors did not display GH10-like behavior and it transpired that both contain a tyrosine at position 163 [13]. For GH11 endo-xylanases of *Trichoderma reesei*, *Aspergillus niger* and *Tricho-derma viride* release of xylose [29] can be linked to the valine on this position, whereas the lack of xylose release for *Bacillus subtilis* [29] was correlated to the tyrosine at this position. The only exception in this case is the high pI-endo-xylanase of *Trichoderma reesei*, which released only minor amounts of xylose [29], but does contain a valine. Endo-xylanases of *Penicillium funiculosum* both contain a valine (* in Fig. 3), and also showed xylose release [30]. However, the different performance of this xylanase compared to other GH11 endo-xylanases has been attributed to different amino acid residues in the loop forming "thumb" [31] and was previously not correlated to the valine.

The structural difference and sequence variation of the tyrosine at position 163 seems a possible explanation for the different hydrolytic performances toward various xylans by the GH11 endo-xylanases and also seems to be related to xylose release. However, this hypothesis needs to be experimentally confirmed with site-directed mutagenesis.

3.5. Interfamily differences for GH10 and GH11 endo-xylanases from Myceliophthora thermophila C1

Both GH11 (this study) and GH10 [9] endo-xylanases of *M. thermophila* C1 display intrafamily differences. Next to these intrafamily differences, interfamily differences were also evaluated. Table 2 displays a schematical representation of the factors that were studied. Toward more linear and thus less soluble xylan, the GH11 endo-xylanases were more efficient than the GH10 xylanases releasing more small degradation products (Fig. A5). Especially Xyl7dCBM (GH11) was very efficient in the release of small products including xylose, occasionally even releasing higher amounts of xylose than the GH10 enzymes. The general assumption that GH10 endo-xylanases hydrolyze xylans to a higher degree than GH11 endo-xylanases [11,13,32], is only valid for the degradation products from WAX (Fig. A5). The structural difference and sequence variation of the tyrosine was more efficient toward less soluble BiWX as compared to BeWX due to the distribution pattern of the 4-*O* methyl glucuronopyranosyl substituents (Table 2). Toward the various insoluble fractions of wheat straw, the GH11 endo-xylanases were as efficient as the GH10 endo-xylanases (no further data shown). Alkaline treatment improved the action of all xylanases. The prevention of self-association of

Fig. 4. Model structure of the catalytic domains of Xyl7 (A) and Xyl8 (B). The model of Xyl7 is obtained using xylanase Xyn11A from *Chaetomium thermophilum* (Genbank accession nr. CAD48749ncbi-p:CAD48749) as template. The model of Xyl8 is obtained using xylanase XynC from Penicillium funicolosum (Genbank accession nr. CAC15487ncbi-p:CAC15487) as template. The catalytic residues are indicated in black and the residues involved in binding are indicated in grey (based on Hakulinen et al. [33]).

Table 2
Performance of the GH10 and GH11 C1 endo-xylanases toward various substrate characteristics.

Family	Enzyme	Lower degree of substitution	More random distribution of 4-O-methylglucuronic acid substituents	Alkaline treatment of wheat straw	Absence of cellulose	Prevention of xylan self-association
GH10	Xyl1dCBM	**0**	+	++	++	++
	Xyl1	−	+	++	−	++
	Xyl3dCBM	++	+	++	−	++
	Xyl3	+	+	++	++	+
GH11	Xyl7dCBM	++	0	++	++	−
	Xyl7	+	−	++	++	0
	Xyl8	+	−	++	+	+

−, lower efficiency.
0, no effect.
+, higher efficiency.
++, very high efficiency.

xylan by DMSO was only effective for the endo-xylanases of GH10 (Table 2).

For the endo-xylanases of *M. thermophila* C1 studied, the CBM1 did not enhance the degradation of soluble or insoluble material (this study; [9]). No correlation was found for the performance of the xylanases and the N- or C-terminal position of their CBM.

A difference in the primary protein structure of the GH11 xylanases was suggested to result in major differences in enzyme performance toward the various substrates, even though the secondary protein structure was comparable. For C1 GH10 xylanases, differences in the secondary protein structure resulted in differences in enzyme performance toward various soluble and insoluble substrates [9].

4. Conclusions

The type and degree of substitution of xylan as well as self-association of linear xylans displayed intrafamily differences within two GH11 endo-xylanases of *Myceliophthora thermophila* C1. The different residue at position 163 in the amino acid sequence of the xylanases (valine instead of tyrosine), could explain the differences in xylanase performance, especially concerning xylose

release. A carbohydrate binding module did not enhance xylan degradation. It can be concluded that the family division for endo-xylanases does not provide information on degradation products and that a detailed characterization and protein structure elucidation is necessary to display intra- and interfamily differences.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enzmictec.2013.03.019.

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