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Cloning, expression, and characterization of an oligoxyloglucan reducing end-specific xyloglucanobiohydrolase from *Aspergillus nidulans*

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Abstract—An oligoxyloglucan reducing end-specific xyloglucanobiohydrolase from the filamentous fungus *Aspergillus nidulans* was cloned and expressed in *Pichia pastoris* as a secreted histidine-tagged protein and purified by affinity chromatography. The enzyme acts on xyloglucan oligomers and releases the first two glycosyl residue segments from the reducing end, provided that neither the first glucose nor the xylose attached to the third glucose residue from the reducing end is not further substituted. The enzyme has a specific activity of 7 U/mg at the pH optimum of 3 and at the temperature optimum of 42 °C.

Keywords: Xyloglucan; Cell wall; Enzyme; *Pichia pastoris*

1. Introduction

Xyloglucan is a major structural polysaccharide found in the primary cell wall of higher plants. It consists of a backbone of β-(1→4)-linked D-Glc*p* residues, most of which are substituted at C-6 with α-D-Xyl*p* to which D-Gal*p*, L-Fuc*p*, and L-Ara*f* can be attached. An unambiguous letter code is used for the nomenclature of each segment depending on the side chain:[1] An unsubstituted D-Glc*p* is assigned **G**, α-D-Xyl*p*-(1→6)-β-D-Glc*p* segment is named **X**, β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p* is **L** and **F** refers to α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p* (Fig. 1). Most xyloglucans are composed of repeats of a cellotetraose backbone bearing either two or three xylose side chains referred to as poly-XXGG and poly-XXXG, respectively.[2]

Whereas many *endo*-glucanases cleave both xyloglucan and cellulose or carboxymethyl cellulose (CMC), some xyloglucan-specific *endo*-glucanases have been found with no or very low activity on other β-glucans.[3–5] Recently, a novel glycosidase (oligoxyloglucan reducing end-specific cellobiohydrolase, OXG-RCBH, glycoside hydrolase family 74, EC 3.2.1.150) that releases two glycosyl subunits from the reducing end of xyloglucan oligosaccharides (depending on the branching pattern) was cloned from the yeast *Geotrichum* sp. M128 and expressed in *E. coli*.[6]

Polysaccharide-degrading enzymes are useful tools for analyzing the structure of high-molecular-weight polysaccharides from cell walls.[7] A prerequisite for such applications is high purity and specificity. This report describes the cloning of an oligoxyloglucan reducing end-specific xyloglucanobiohydrolase (OREX) from *Aspergillus nidulans*, its expression in *Pichia pastoris*, purification via affinity chromatography, and the characterization of the recombinant enzyme using different oligoxyloglucan substrates.

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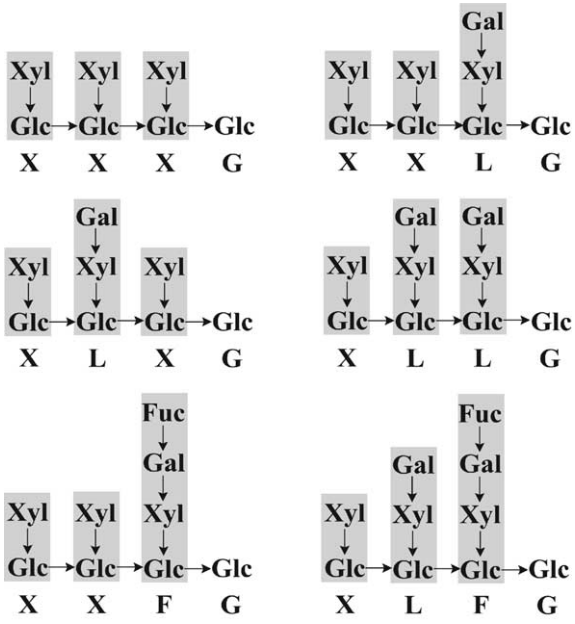

Figure 1. Structure of xyloglucan tetrameric repeating subunits $[XG]_1$ and letter code used for the nomenclature of the individual segments according to Ref. 1. Glc: glucose; Xyl: xylose; Gal: galactose; Fuc: fucose.

2. Results

A BLASTp search of *Geotrichum* sp. M128 OXG-RCBH amino acid sequence against the translated *A. nidulans* genome sequence revealed that the locus AN1542.2 encoded a very similar enzyme.[8,9] The sequences of the two proteins were 52.6% identical (422 out of an 803 amino acids overlap).[6] Therefore, the enzyme can be classified in the same family (GH74). Since *A. nidulans* gene lacked apparent introns, we obtained the gene by PCR of genomic DNA. The recombinant *A. nidulans* enzyme has a predicted molecular mass of 90.4 kDa, which was similar to that of OXG-RCBH (Fig. 2). The coding sequence was modified by addition of yeast α-factor signal peptide at the N-terminus and a myc tag and a 6x histidine tag at the C-terminus. The recombinant gene was placed under control of a methanol-inducible promoter and introduced into *P. pastoris* genome.

Maximum expression of *A. nidulans* gene was achieved in MM (minimal methanol) medium. Three days after induction, approximately 20 mg of purified enzyme per liter of culture medium could be recovered by affinity chromatography. The purified protein exhibited a higher molecular mass than expected (Fig. 3, lane 1), most likely due to glycosylation at one or more of the 12 putative sites for N-linked glycosylation (Fig. 2). Many proteins are glycosylated in *P. pastoris*.[10] Removal of the N-linked sugars by PNGase treatment reduced smearing, and the size of the resulting band shifted to around 90 kDa (Fig. 3, lane 2), which is in good agreement with the expected mass.

The effect of temperature and pH on enzyme activity was measured using tamarind xyloglucan tetrameric subunits $[XG]_1$ as a substrate. The pH optimum was approximately pH 3 (Fig. 4a), and the temperature optimum was approximately 42 °C (Fig. 4b). The purified enzyme had an activity of 7 U/mg protein.

2.1. Enzyme characterization using tamarind xyloglucan tetrameric subunits $[XG]_1$

Fragments obtained by digestion of xyloglucans by Novozyme xyloglucanase were analyzed by capillary electrophoresis (CE). Tamarind xyloglucan mainly consists of four different repeating subunits $[XG]_1$: XXXG, XXLG, XLXG, and XLLG (Fig. 5a).[11,12] The peaks were assigned by comparison with the matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrum (Fig. 6a). In general, the ions were observed as a sodium adduct. All molecular ions $[M+Na]^+$ and post-source decay (PSD) fragment ions such as $[M-\text{hexosyl}+Na]^+$ are subsequently described by abbreviations like M^+ and $M^+-\text{hexosyl}$. Therefore, peak *m/z* 1085 is derived from XXXG, *m/z* 1247 from the isomers XXLG and XLXG, and peak *m/z* 1409 from XLLG. In addition, we also isolated the four subunits by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD, data not shown) and analyzed the fractions by CE and MALDI-TOF. This confirmed that CE is capable of separating the isomers XLXG and XXLG (Fig. 5a).

After incubation of tamarind xyloglucan tetrameric subunits $[XG]_1$ with purified OREX, the peaks of XXXG (*m/z* 1085) and XXLG (*m/z* 1247) disappeared (Fig. 6b) but XLLG (*m/z* 1409) and XLXG (*m/z* 1247) remained. The fact that the small peak *m/z* 1247 is derived only from XLXG and not from XXLG was confirmed by CE of the digest where no XXLG was detected (data not shown). Furthermore, three additional peaks appeared in the lower mass region, and they are assigned to XG (*m/z* 497), XX (*m/z* 629), and LG (*m/z* 659), evidently originating from XXXG and XXLG. No XL (*m/z* 791) was detected, indicating that XLXG or XLLG are apparently not substrates for the enzyme.

The enzyme also acts on reduced xyloglucan oligosaccharides but significantly slower. In the case of the reduced heptasaccharide XXXGol (*m/z* 1087), the corresponding fragments XX (*m/z* 629) and XGol (*m/z* 499) were observed in the MALDI-TOF spectrum (data not shown), but the activity was approximately 150 times lower than toward the unreduced oligomers.

2.2. Enzyme characterization using cotton xyloglucan tetrameric subunits $[XG]_1$

To further analyze this specificity, cotton xyloglucan, which contains additional subunit structures, was also

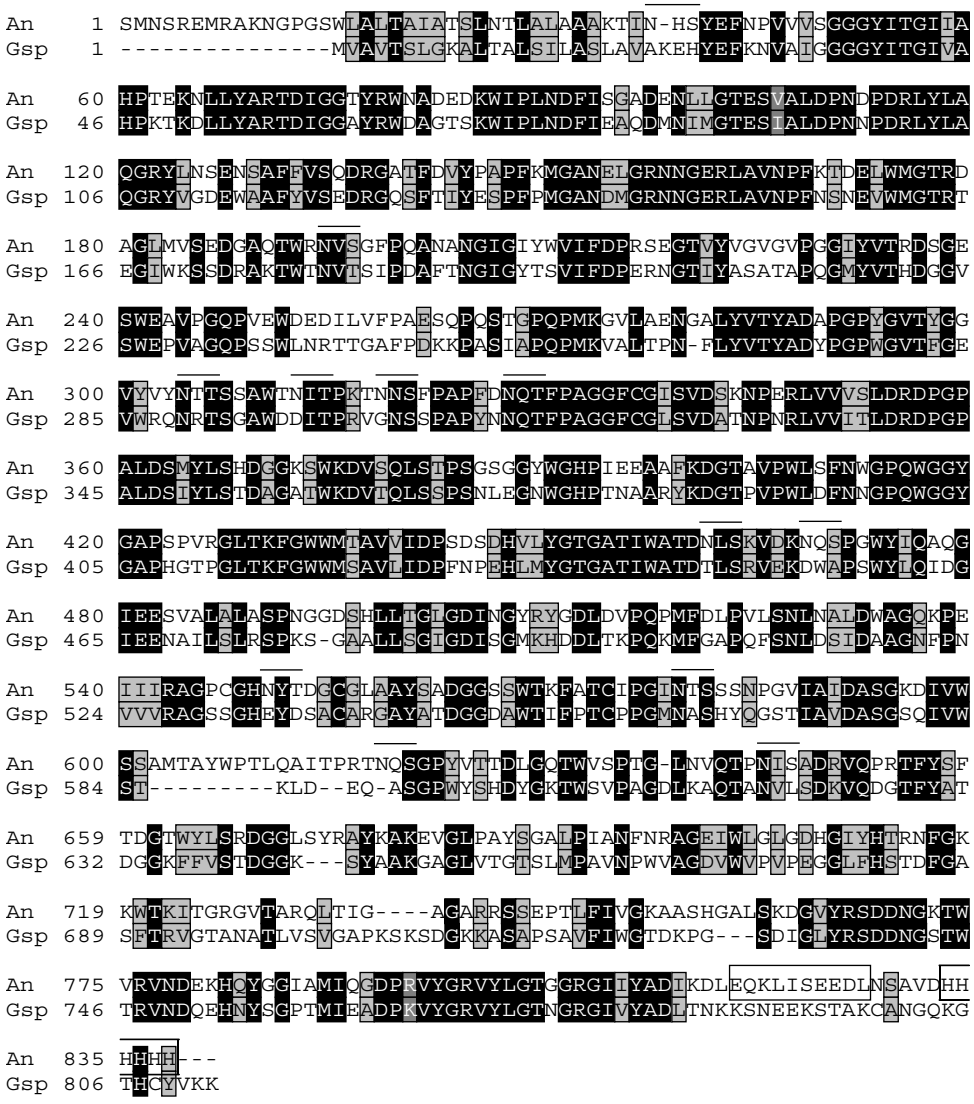

Figure 2. Alignment of the deduced amino acid sequence of the recombinant *A. nidulans* OREX (An) and the native *Geotrichum* sp. M128 OXG-RCBH (Gsp), assuming the α-factor was cut off during the secretion pathway by the Kex2 dibasic endoprotease and the intracellular Ste13 aminopeptidase. Identical and similar regions are highlighted in black and grey, respectively. Possible N-glycosylation sites are marked with bars and boxes indicate the c-myc epitope and the 6x histidine tag.

analyzed.[13] Figure 5b shows the CE electropherogram and Figure 7a the corresponding MALDI-TOF trace of the subunits from cotton xyloglucan. In addition to the subunits already described, XXXG, XXLG, XLXG, and XLLG two further peaks were detected, namely XXFG and XLFG.[13] The fucose content of these structures was evident in the PSD spectra. Since a fucosyl linkage is cleaved much more easily than other glycosidic linkages, the loss of a fucosyl residue (M^+-146) dominated the fragment pattern (data not shown).[14] No XFXG or XFLG subunits have been described in cotton xyloglucan and the presence of these isomers would have been visible as additional peaks in CE analysis similar to the observation for the isomers XLXG and XXLG (Fig. 5a).[13] After incubation of cotton xyloglucan subunits with OREX the peaks XXXG, XXLG, and XXFG vanished but XLXG, XLLG, and XLFG were still present (Fig. 7b). Now, the peaks *m/z* 497, *m/z* 629, *m/z* 659, and *m/z* 805 can be explained by the fragments XG, XX, LG, and FG, respectively. Similar to the results with tamarind, no XL (*m/z* 791) was observed in the digest.

2.3. Enzyme characterization using tamarind xyloglucan octameric subunits $[XG]_2$

In order to confirm that the enzyme has *exo*-activity only from the reducing end, we analyzed the fragments produced by reaction with tamarind xyloglucan octameric subunits $[XG]_2$ derived from a partial degradation and high-performance liquid chromatography (HPLC) isolation. Theoretically, 16 structures are possible, and

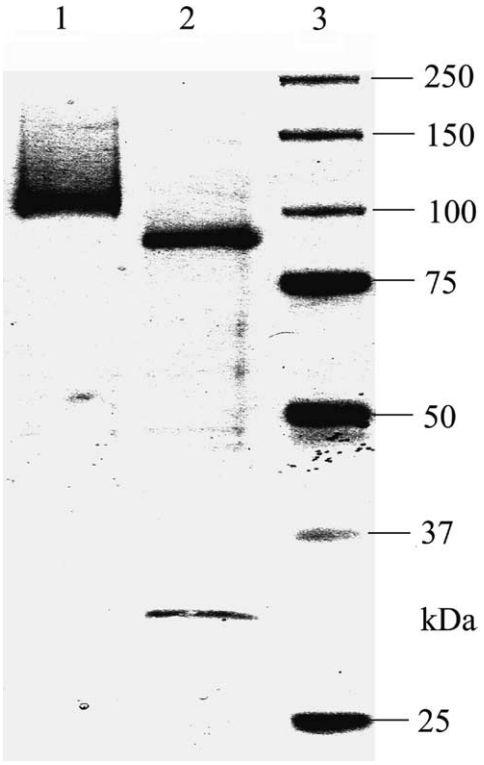

Figure 3. SDS-PAGE of affinity chromatography-purified *A. nidulans* OREX stained with gel code® blue. Lane 1: purified enzyme; lane 2: purified enzyme after PNGase F digestion (the lower band is derived from PNGase); lane 3: molecular mass marker.

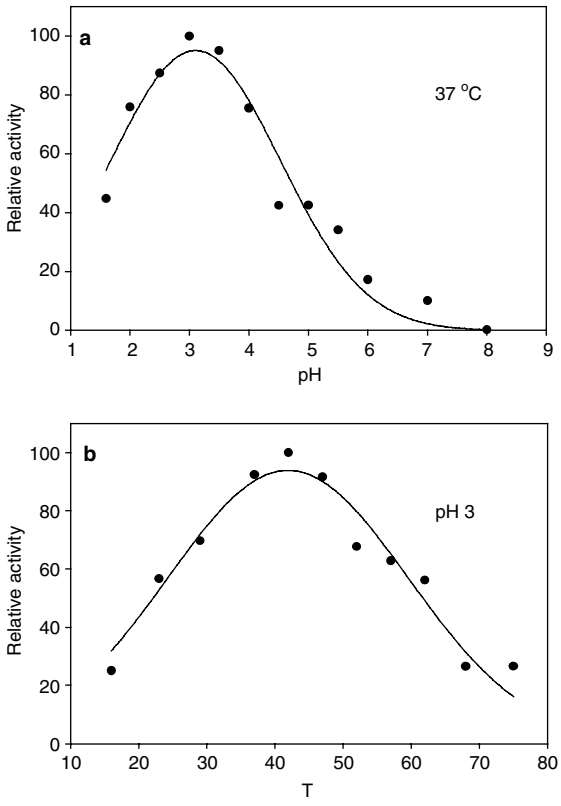

Figure 4. (a) pH and (b) temperature dependence of *A. nidulans* OREX.

the combinations are listed in Table 1. Since many structures are isomeric, the number of the calculated possible molecular masses are reduced to 5, namely 2129, 2291, 2453, 2615, and 2777. Except for 2129 (XXXGXXXG), these peaks are also observed in the corresponding MALDI-TOF spectrum (Fig. 8a). It was not possible to separate the isomers by CE, so it was not possible to determine the ratio in which they are present. Therefore, we first tried to predict the generated fragments from all possible structures, provided that the enzyme will only cut if the xylose at the third glucose from the reducing end is not further substituted. All structures that were subjected to such a degradation are marked in bold (Table 1), and generated fragments are listed together with their masses. Interestingly, only XG (*m*/*z* 497) and LG (*m*/*z* 659) are released. XX (*m*/*z* 629), which would only occur if the enzyme also acts from the non-reducing end, is not formed. In the MALDI-TOF mass spectrum of the digest (Fig. 8b) all calculated fragment peaks (*m*/*z* 491, 659, 1835, 1997) were observed except for *m*/*z* 1673 (XXXGXX). This is not unexpected, for its possible precursor structures XXXGXXXG (*m*/*z* 2129) and XXXGXXLG (part of the *m*/*z* 2291 peak) were only present in very low amounts or not at all present. Additional peaks (*m*/*z* 2291, 2453, 2615, and 2777) representing the molecular ions of structures, that are not degradable by the enzyme

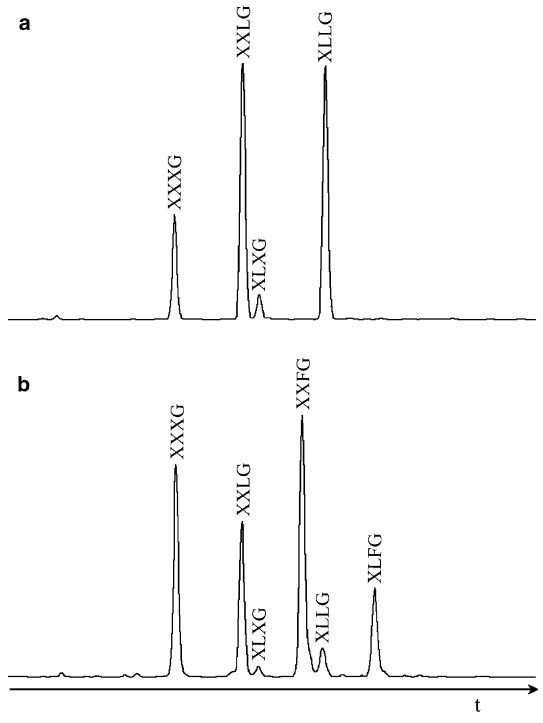

Figure 5. Capillary electrophoresis of APTS-labeled tetrameric subunits $[XG]_1$ obtained by xyloglucanase digestion of xyloglucan from (a) tamarind and (b) cotton.

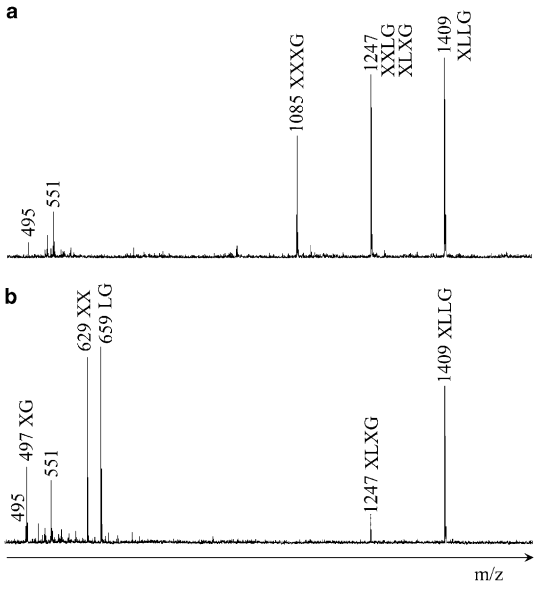

Figure 6. MALDI-TOF mass spectra of tamarind xyloglucan tetrameric subunits $[XG]_1$ (a) before and (b) after incubation with *A. nidulans* OREX.

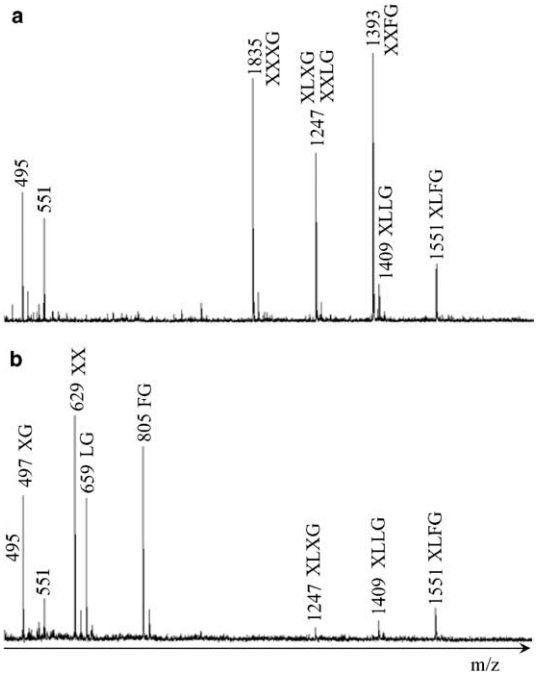

Figure 7. MALDI-TOF mass spectra of cotton xyloglucan tetrameric subunits $[XG]_1$ (a) before and (b) after incubation with *A. nidulans* OREX.

are shown in Table 1. The same study was performed using tamarind xyloglucan octameric subunits $[XG]_2$ where the reducing end was substituted with APTS. This time, no fragments were observed using CE, showing that such pre-labeled substrates are not suitable for studies with this enzyme.

2.4. Enzyme characterization using cellotetraose and xylotetraose

We analyzed the ability of OREX to cleave the cellotetraose backbone with no side chains attached. In addition, because some enzymes capable of cleaving β-(1→4)-glucan linkages are also capable of cleaving the β-(1→4)-xylan backbone, we incubated OREX with β-(1→4)-xylotetraose.[15,16] In both cases, no degradation or generation of products was observed by CE after APTS labeling.

3. Discussion

A. nidulans OREX releases the first two glycosyl segments from oligoxyloglucans. In this respect, it has a similar mode of action as *Geotrichum* sp. M128 OXG-RCBH but shows different pH and temperature optima (pH 3 and 42 °C, respectively) in contrast to pH 3.5–5 and 50–60 °C.[6] In addition, we extended the substrate analysis to fucosylated structures. The enzyme recognizes the reducing end of xyloglucan oligosaccharides and hydrolyzes the first two glycosyl segments only if the xylose at the third glucose from the reducing end is not further substituted (X**X**XG, X**X**LG, X**X**FG). None of the subunits having a substituent at this particular xylose (X**L**XG, X**L**LG, X**L**FG) were degraded. Since the rule also applies to octameric xyloglucans $[XG]_2$, we

Table 1. Possible structures of tamarind xyloglucan octameric subunits $[XG]_2$ and expected fragments after *A. nidulans* OREX digestion

Mass (Na^+ adduct)	Possible structure	Fragments	Fragment mass (Na^+ adduct)
2129	**XXXGXXXG**	XXXGXX + XG	1673 + 497
2291	XXXGXLXG, **XXXGXXLG**, **XLXGXXXG**, **XXLGXXXG**	XXXGXX + LG, XLXGXX + XG, XXLGXX + XG	1673 + 659, 1835 + 497, 1835 + 497
2453	XXXGXLLG, **XLLGXXXG**, XLXGXLXG, **XLXGXXLG**, **XXLGXXLG**, XXLGXLXG	XLLGXX + XG, XLXGXX + LG, XXLGXX + LG	1997 + 497, 1835 + 659, 1835 + 659
2615	XLLGXLXG, **XLLGXXLG**, XLXGXLLG, XXLGXLLG	XLLGXX + LG	1997 + 659
2777	XLLGXLLG	—	—

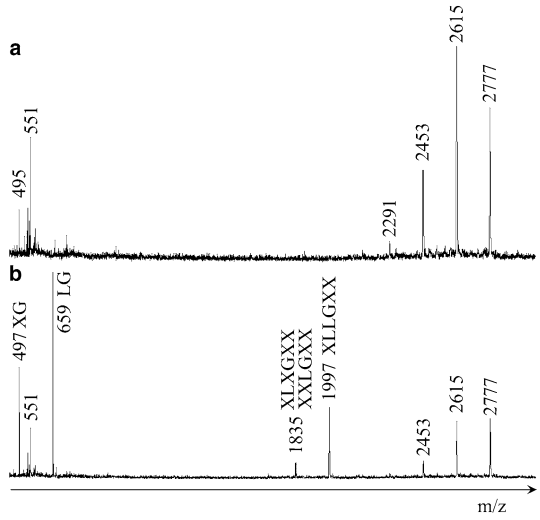

Figure 8. MALDI-TOF mass spectra of tamarind octameric repeating subunits $[XG]_2$ (a) before and (b) after incubation with *A. nidulans* OREX.

can extrapolate that it will act in the same way on larger xyloglucan repeating subunits $[XG]_3, [XG]_4, [XG]_5, \ldots, [XG]_\infty$ or on xyloglucans with additional sugars like arabinose or a second xylose in the side chains.

Due to the 6x histidine tag, the enzyme can easily be purified making it a useful tool for researchers to analyze the fine structure of xyloglucans. We have deposited the clone in the Fungal Genetics Stock Center (FGSC number 9925) where the clone is freely available to the research community.

In order to prevent confusion with cellobiohydrolases, which truly release only cellobiose from cellulose and other β-glucans, we propose to name the enzyme and those with similar activity, 'oligoxyloglucan reducing end-specific xyloglucanobiohydrolase' (OREX). This nomenclature more accurately reflects the different possible structures of the xyloglucan segments released by this enzyme.

4. Experimental

4.1. Construction of expression plasmid

A. nidulans FGSC A4 (Glasgow wild type) was obtained from the Fungal Genetic Stock Center (FGSC, University of Kansas Medical Center) and grown in complete medium (minimal medium supplemented with 0.5% yeast extract, 1% peptone, 2% glucose, and Hutner's trace elements, pH 4.5) at 37 °C.[17,18] Chromosomal DNA was extracted according to protocol 12 in Ref. 19.

The DNA encoding AN1542.2, including the native signal peptide sequence, was amplified by polymerase chain reaction (PCR) using the primer pair 5′-CACG-**ACACGTG**AGATGAGGGCGAAGAAC-3′ (forward) and 5′-TTGTGC**TCTAGA**TCCTTGATATCGGCA-TAG-3′ (reverse). Bold characters in the oligomers indicate the restriction sites *Pml*I and *Xba*I, respectively. The amplified fragment was cloned at the corresponding sites in vector pPICZαC (Invitrogen) so that it was in frame with the yeast α-secretion factor and under transcriptional control of the alcohol oxidase promoter (AOX1). The recombinant protein also contained a C-terminal c-myc epitope and a 6x histidine tag. The product was transformed into chemically competent TOP10 *E. coli* (Invitrogen) and zeocin resistant colonies were sequenced (ABI PRISM™ Big Dye™ dideoxy nucleotide termination sequencing, Applied Biosystems) to identify an error-free clone pSBAN1.

4.2. Screening of recombinant colonies and expression optimization

pSBAN1 was linearized with *Pme*I and transformed into *P. pastoris* X-33 (Invitrogen) by electroporation. Zeocin-resistant *P. pastoris* clones were isolated and screened for protein expression by a small-scale protein expression study. BMGY/BMMY/BMM/MMY/MM (buffered complex glycerol medium/buffered complex MeOH medium/buffered minimal MeOH medium/complex MeOH medium/minimal MeOH medium) were prepared as follows: BMGY contained 10 g/L yeast extract (Becton Dickinson), 20 g/L peptone (Becton Dickinson), 100 mM phosphate buffer (pH 6.0), 3.4 g/L yeast nitrogen base without $(NH_4)_2SO_4$ and without amino acids (Sigma), 10 g/L $(NH_4)_2SO_4$, 10 mL/L glycerol (Sigma), and 0.4 mg/L histidine (Research Organics). In BMMY, BMM, MMY, and MM the glycerol was substituted by 5 mL/L MeOH. For BMM, yeast extract and peptone were omitted, and for MMY, phosphate buffer was omitted. MM medium did not contain yeast extract, peptone, and phosphate buffer. Clones were grown in 5 mL of BMGY in 50-mL tubes at 28 °C in an orbital shaker (250 rpm) for 24 h, and expression was induced by transferring cells into 5-mL BMMY and grown for another 24 h. Supernatant was then analyzed by dot-blots on nitrocellulose membrane (Schleicher & Schuell) using anti-c-myc (Sigma), anti-rabbit IgG peroxidase conjugated (Pierce) antibodies, and the SuperSignal® West Pico Chemiluminescence kit (Pierce). The transformant showing the highest enzyme concentration using this assay was named PPAN1 and was grown in 100 mL BMGY in a 500-mL flask at 28 °C in an orbital shaker (250 rpm) to an OD_{600} of 3–6. Cells were diluted to an OD_{600} of 1.0 with 50 mL of each of BMMY, BMM, MMY, and MM medium in 250 mL flasks and incubated for 5 days. Each day, 0.5 mL of the supernatant was collected, and the media were supplemented with 0.3 mL of MeOH. Dot-blots for time points (1–5 days) were performed as described above.

4.3. Purification of enzyme

Cells were grown in 50 mL of BMGY in a 250-mL flask in an orbital shaker (250 rpm) at 28 °C to an OD_{600} of 3–6, and aliquot cells were diluted to an OD_{600} of 1.0 with 50 mL of MM and incubated for 3 days with daily addition of 0.3 mL of MeOH. The suspension was centrifuged (10 min, $5000 \times g$) and the supernatant was 10-fold concentrated using Amicon Ultra-15 centrifugal units (10 kDa cut-off, $5000 \times g$, Millipore). A nickel chelate His-Bind Resin (Novagen) column (0.7×5 cm) was extensively washed with water, flushed with 10 mL of 400 mM $NiSO_4$, washed with 10 mL of buffer A (500 mM NaCl, 20 mM Tris–Cl, pH 7.9) containing 10 mM imidazole and stored at 4 °C. The concentrated supernatant was adjusted to pH 7.5 with NaOH and loaded onto the column at 4 °C. The column was rinsed with 10 mL of buffer A (pH 7.9) containing 50 mM imidazole, and the enzyme was eluted with 10 mL of 250 mM imidazole in buffer A (pH 7.9). The eluate was concentrated in the centrifugal units and repeatedly washed with water in order to remove imidazole. Protein content was measured using the BCA Protein Assay (Pierce). SDS-PAGE was performed using a BioRad 10% polyacrylamide criterion™ gel and stained with gel code® blue (Pierce). For *N*-glycosyl removal the protein was treated with PNGase F (New England Biolabs) according to the supplied protocol.

4.4. Generation of cotton xyloglucan

Cell walls of cotton suspension cultures were prepared as described earlier and then digested with endopolygalacturonase (Megazyme).[20] After washing with water the wall residue was extracted with 24% KOH containing 0.1% $NaBH_4$. After neutralization with HOAc, dialysis, and lyophilization, the extract was passed through a 150×10-mm column packed with Poros 50 DEAE (Perseptive Biosystems) running in NH_4OAc buffer (30 mM, pH 5.2). The xyloglucan that did not adsorb to the column was used for the experiments reported here.

4.5. Generation of xyloglucan tetrameric and octameric repeating subunits

Tamarind xyloglucan (Megazyme, 10 mg/mL water) and cotton xyloglucan (10 mg/mL water) were each incubated with 10 μg/mL of xyloglucanase (Novozyme) at 37 °C for 24 h. The solution was centrifuged (5 min, $16{,}000 \times g$), the supernatant was boiled for 3 min, and centrifuged again. The final supernatant contained fragments of various tetrameric repeating subunits $[XG]_1$.

For the generation of fragments consisting of octameric repeating subunits $[XG]_2$, 127 mg of tamarind xyloglucan was dissolved in 10 mL of 50 mM NH_4OAc buffer (pH 4.5) and digested with 1 U of cellulase (Megazyme) at 37 °C for 1 h. The digest was boiled for 3 min to stop the reaction, and 2-mL aliquots were fractionated on a 500×22.5-mm stainless steel column packed with Toyopearl HW-50S (Supelco, 50 mM NH_4OAc, flow rate 2 mL/min). A Shodex RI-71 refractive index detector was used to monitor the eluant from the column. Pooled fractions containing $[XG]_2$ were freeze dried, dissolved in water, and freeze dried again.

APTS-labeled repeating subunits $[XG]_2$ were obtained by derivatizing 1 mg of tamarind octameric repeating subunits with 10 μL of 9-aminopyrene-1,4,6-trisulfonate (APTS, 100 mM in 15% acetic acid) and 25 μL of $NaBH_3CN$ (1 M in Me_2SO) and kept at 55 °C.[21] After 2 h, 250 μL of water was added, and the solution was passed through a 150×10-mm Toyopearl HW-40S (Supelco) gel-filtration column using a mixture of 25% CH_3CN and 75% 50 mM NH_4OAc (pH 5.2, 1 mL/min) as eluant. Fractions (1 mL) were collected and monitored by CE. Fractions containing labeled $[XG]_2$ were combined and dried in a speed-vac.

4.6. Enzyme assay

Enzyme assays contained 30 μL of buffer and 10 μL of substrate either generated tamarind xyloglucan tetrameric subunits $[XG]_1$ or the reduced heptasaccharide XXXGol (Megazyme, 10 mg/mL) and 10 μL of enzyme solution (10 μg/mL in water). After 15 min, 500 μL of borate buffer (0.1 M, pH 9), 250 μL of 1% 2-cyanoacetamide reagent (Aldrich), and 200 μL of water were added, and the mixture was boiled for 10 min and cooled in tap water.[22] The enzyme blank and substrate blank were prepared in the same way replacing substrate and enzyme solutions with water, respectively. The absorbance at 276 nm was read on a Beckman-Coulter DU 640 spectrophotometer against a reagent blank. A glucose reference curve (0–125 nmol) was prepared accordingly.

A pH-dependent assay was performed at 37 °C with the following buffers (0.1 M): pH 1.6 (HCl/KCl), pH 2.0, pH 2.5, pH 3.0, and pH 3.4 (HCl/glycine), pH 4.0, pH 4.5, pH 5.0, and pH 5.5 (acetate), pH 6.0, pH 7.0, and pH 8.0 (phosphate).[23]

For temperature optimum determination the assay was performed at pH 3. The increase of 1 μmol reducing ends per min was defined as 1 unit (U).

4.7. Capillary electrophoresis (CE)

Cellotetraose (0.5 mg/mL in water, Sigma) and xylotetraose (0.4 mg/mL in water, Megazyme), each 10 μL, were incubated each with 30 μL of HCl/glycine buffer (0.1 M, pH 3) and 10 μL of enzyme solution (10 μg/mL in water) at 42 °C for 24 h. The solution was centrifuged (5 min, $16{,}000 \times g$), the supernatant was boiled

for 3 min and centrifuged again. Aliquots of samples (tamarind $[XG]_1$, cotton $[XG]_1$, cellotetraose, or xylotetraose digest) were dried in a speed-vac and resuspended in 2 μL of APTS (100 mM in 15% acetic acid) and 5 μL of $NaBH_3CN$ (1 M in Me_2SO) and kept at 55 °C.[21] After 2 h, 50 μL of water was added, and 10 μL of the mixture was diluted with 190 μL water and analyzed using CE (P/ACE™ MDQ Molecular Characterization System, Beckman-Coulter) equipped with a 50-cm eCap™ capillary (75 μm I.D., 375 μm o.d., Beckman-Coulter) and laser detection (488 nm excitation, 520 nm emission) at an applied voltage of 25 kV using Carbohydrate Separation Buffer (Beckman-Coulter).

4.8. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF)

Spectra were recorded in the positive-ion mode using a Voyager DE-PRO (Applied Biosystems) MALDI-TOF mass spectrometer operated at an accelerating voltage of 20 kV. Samples were desorbed/ionized with a 3-Hz pulse repetition rate nitrogen laser ($\lambda = 337$ nm). Delay time was set to 100 ns, grid voltage 76% and guide wire 0.05%. For post-source decay (PSD) analysis, the precursor ion was selected by a Bradbury–Nielsen ion gate. Xyloglucan repeating subunits (100 μL tamarind $[XG]_1$, cotton $[XG]_1$, or tamarind $[XG]_2$), 100 μL of buffer (pH 4) and 10 μL of enzyme (200 μg/mL) were incubated at 42 °C for 24 h. Each solution (1 μL) and 24 μL of 2,5-dihydroxybenzoic acid (Aldrich, 10 mg/mL in MeOH/water, 65+35 v/v) were premixed, and 1 μL was crystallized on the sample plate under vacuum. Calibration was done using a mixture of glucose oligomers (degree of polymerization 1–20, Beckman-Coulter).

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