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Carbohydrate esterase family 16 contains fungal hemicellulose acetyl esterases (HAEs) with varying specificity

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ABSTRACT

Acetyl esterases are an important component of the enzymatic machinery fungi use to degrade plant biomass and are classified in several Carbohydrate Esterase families of the CAZy classification system. Carbohydrate Esterase family 16 (CE16) is one of the more recently discovered CAZy families, but only a small number of its enzyme members have been characterized so far, revealing activity on xylan-derived oligosaccharides, as well as activity related to galactoglucomannan. The number of CE16 genes differs significantly in the genomes of filamentous fungi. In this study, four CE16 members were identified in the genome of *Aspergillus niger* NRRL3 and it was shown that they belong to three of the four phylogenetic Clades of CE16. Significant differences in expression profiles of the genes and substrate specificity of the enzymes were revealed, demonstrating the diversity within this family of enzymes. Detailed characterization of one of these four *A. niger* enzymes (HaeA) demonstrated activity on oligosaccharides obtained from acetylated glucuronoxylan, galactoglucomannan and xyloglucan, thus establishing this enzyme as a general hemicellulose acetyl esterase. Their broad substrate specificity makes these enzymes highly interesting for biotechnological applications in which deacetylation of polysaccharides is required.

Introduction

Hemicelluloses are the second most abundant polysaccharides in plant cell walls after cellulose and consist of three main structures, (arabino-)glucuronoxylan, xyloglucan and galactoglucomannan [1], that share as a common feature acetylation of some residues. In xylan and mannan, acetylation occurs mainly at xylopyranosyl and mannopyranosyl residues, respectively, at positions 2 or 3 [2]. Acetylation in xyloglucan occurs in both main chain glucopyranosyl residues and side chain galactopyranosyl and arabinofuranosyl residues [2]. Acetylation can inhibit enzymatic degradation of mannan [3] and xylan [4], and therefore most fungi that degrade plant biomass also contain genes encoding acetyl esterases in their genomes [5–7]. Deacetylation of polysaccharides is also relevant for biotechnological applications [8] and the production of oligosaccharides [9].

Acetyl esterases are part of the Carbohydrate Active Enzyme

Abbreviations: Ac, acetyl; CAZy, carbohydrate active enzyme; CE, carbohydrate esterase; diAc, monosaccharide residues with two acetyl residues attached to them; HAE, hemicellulose acetyl esterase; MAFFT, Multiple Alignment using Fast Fourier Transform; MeGlcA, methyl-glucuronic acid.

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Database (www.cazy.org) [10] in several Carbohydrate Esterase (CE) families. CE1 and CE5 have received the most attention, but studies on CE2, CE4, CE6, CE7 and some on CE16 have also been reported [2]. The CE16 CAZy family belongs to the GDSL family of serine esterases/lipases which is part of the SGNH hydrolase-type esterase domain scaffold [11, 12], and their relationship was to SGNH hydrolases was recently explored in a phylogenetic study [13].

The first comparison of two fungal CE16 enzymes on hemicellulose initially demonstrated that *T. reesei* and *M. thermophila* CE16s have similar modes of action on xylan oligosaccharides [14]. Both enzymes were described as *exo*-deacetylases due to their preference for acetyl groups on the non-reducing end of xylan oligosaccharides. Both enzymes showed a similar synergistic effect when a xylanase was also present [14]. *M. thermophila* CE16 was also shown to remove internal acetyl groups from xylan oligosaccharides and deacetylate 2-*O*-GlcA,3Ac residues to a limited extent [15]. Deacetylation of the non-reducing end by *T. reesei* CE16 was extensively studied [16], demonstrating that only xylan oligosaccharides acetylated at the non-reducing end are substrates for this enzyme, which also catalyzes 3-O -deacetylation of MeGlcA-substituted non-reducing-end xylopyranosyl residues [17]. Whether glucuronylated or not, xylan oligosaccharides acetylated at position 3 are deacetylated, but 2-acetyl (2-Ac) substituents are not hydrolyzed [18].

The xyloligosaccharide specificity of a CE16 from *A. niger* is similar to that of the *T. reesei* enzyme. Both enzymes appear only to attack the non-reducing end residue and deacetylate 3-Ac positions in 3-Ac, 2,3-Ac and 2-O-GlcA,3-Ac substituted residues, but the *A. niger* CE16 also deacetylates the 2 positions in 2-Ac and 2,3-Ac non-reducing end residues [19]. *P. anserina* CE16, on the other hand, deacetylates polymeric birchwood acetylglucuronoxylan. Its activity is similar to enzymes from CE1, CE4, CE5 and CE6, because it does not deacetylate glucuronylated positions, and prefers to remove acetyl groups most rapidly from di-acetylated (2,3-) xylose residues and more slowly from mono- 2-Ac and 3-Ac substituted residues [20].

Although activities and positional specificities of these four CE16 enzymes on xylan have been well described, information about deacetylation of mannans and xyloglucans is much more limited. *T. reesei* CE16 can transfer acetyl groups to mannooligosaccharides [21] and deacetylate mannooligosaccharides [22,23], while *M. thermophila* CE16 removes *O*-2 and *O*-3 acetyl groups from spruce galactoglucomannan to a limited extent [15]. A potential role for CE16 acetyl esterases in the de-acetylation of mannan in vivo was suggested by transcriptomics of *Aspergillus oryzae*, where a candidate CE16 encoding gene was controlled by the mannanolytic regulator ManR, [24]. There are no reports of CE16 enzymes acting on xyloglucans.

In this study, the substrate specificities of four CE16 enzymes from *A. niger* were compared. While only two CE16 genes were identified in the genome of *A. niger* CBS 513.88 [6], four CE16 genes were found in the gold-standard genome of *A. niger* NRRL3 [25]. Comparison of their activity on different model, poly- and oligomeric substrates showed different substrate specificities for these enzymes. One of them, HaeA, was purified and characterized in more detail, revealing that it is active on mannan, xylan and xyloglucan oligosaccharides.

Materials and methods

Phylogeny

Amino acid sequences of selected fungal CE16 candidates were obtained from the MycoCosm Webportal from the Joint Genome Institute (JGI, https://genome.jgi.doe.gov/mycocosm/home) and the CAZy website (http://www.cazy.org/CE16_characterized.html). SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) was used to identify and remove putative signal peptides. Unusually long and incomplete sequences were corrected manually based on BlastX alignment, whereas duplicate and ambiguous sequences were discarded. Sequence alignment was performed using MAFFT [26]. A phylogenetic tree was constructed using the Maximal Likelihood method of the MEGA7 program [27] with the Poisson correction distance of substitution rates, 1000 bootstrap re-samplings and complete deletion of gaps. The same settings were used for the Minimal Evolution and Neighbor-Joining trees, which were performed as comparison and bootstrap values ≥ 50 were added to the Maximal Likelihood tree. Four CE1 sequences were included as an outgroup.

Expression analysis

Expression of the four *A. niger* CE16 genes was analyzed in previously published transcriptome data [28–31]. The promoter regions of the genes (1000 bp) were analyzed for the presence of putative binding sites for XlnR ($GGCTAR_{AG}$) [32], AraR ($CGGD_{GAT}TAAW_{AT}$) [33], GaaR ($CCN_{ACGT}CCAA$) [28], RhaR ($TGV_{CAG}D_{GTA}CGG$) [30] and CreA ($S_{GC}Y_{CT}GGR_{AG}G$) [34].

Cloning and protein production

Genomic sequences encoding HaeA, HaeB, HaeC and HaeD were obtained from the *A. niger* NRRL3 genome (http://genome.fungalgenomics.ca/). Gene sequences were amplified by PCR using genomic DNA from *A. niger* NRRL3 as template [35]. The genes were cloned between the promoter and terminator of the *A. niger* glucoamylase gene of the ANIp7 integrative vector [36] using Ligation-Independent Cloning [37]. Primers are listed in Supplemental Table S1.

E. coli transformation, propagation and plasmid DNA isolation were performed using standard techniques [38]. The gene *haeC* was introduced by protoplast transformation [39,40] into strain CSFG_6002 (N593 Δ*glaA*) and the other three genes were introduced into CSFG_6012 (CSFG_6002 Δ*amyC* Δ*agdA* Δ*aamA*). Supernatants from transformants were screened for recombinant protein production in liquid MMJ medium [41] containing 15 g/L maltose.

Spores from positive transformants were inoculated in 200 mL MMJ medium at a concentration of 2×10^6 conidia/mL. Supernatants were harvested after incubating stationary cultures for 5 days at 30 °C. They were then desalted and concentrated using Vivaflow® cassettes based on the manufacturer's protocol (Sartorius).

Production and purification of HaeA enzyme

A recombinant *haeA*-expressing strain of *A. niger* was grown on MMJ. Following stationary cultivation for five days, the growth medium was clarified by centrifugation at 3700 x *g* for 30 min at 4 °C, and concentrated and buffer-exchanged using a Vivaflow 200 (Sartorius, Goettingen, Germany) device. The concentrated solution containing HaeA was buffer-exchanged (Macrosep devices, see below) using 45 mM Tris-HCl, pH 8.8, and then applied onto a 5 mL anion exchange column (HiTrap® DEAE-FF, GE Healthcare, Mississauga, Canada), washed with five volumes of buffer, and eluted using a linear gradient (flow rate, 6 mL/min) between 0 and 2 M NaCl in 45 mM Tris-HCl, pH 8.8. SDS-PAGE was used to evaluate the elution fractions. Fractions containing HaeA were pooled, protein concentrated using Macrosep® Advance Centrifugal Devices (10 K cutoff, Pall Corporation, Mississauga, Canada), and loaded onto a Superdex 75 10/300 GL (GE Life Sciences, Mississauga, Canada) gel filtration column equilibrated with 45 mM Tris-HCl, pH 8.8 containing 2 M NaCl. Chromatography was performed using a BioRad (Mississauga, Canada) DuoFlow System at room temperature. After SDS-PAGE inspection, the protein was concentrated to 1.56 mg/mL and stored at − 80 °C for further experiments.

Enzyme assays on pNP substrates and acetylated carbohydrates

All activity measurements were performed in 150 μL reaction volumes in 96 well microplates at 25 °C, using the cell supernatants

prepared as described above. Diluted protein solutions were prepared at 0.15 mg/mL (in 20 mM HEPES buffer containing 100 mM NaCl, pH 7.2); from these, 2 μL were used for enzyme activity measurement (final enzyme concentration was approx. 2 μg/mL).

Enzymes were tested for activity using *para*-nitrophenyl (*p*NP) esters with different chain lengths (acetate (2 C); butyrate (4 C) (Sigma Aldrich, St. Louis, MO, USA) performed in 20 mM HEPES, pH 7.2 with 100 mM NaCl, and 1 mM *p*NP-esters. *p*NP-acetate was used for activity assays with varying pH conditions using McIlvaine's buffer system (with varying concentrations of citric acid and Na_2HPO_4). Absorbance was registered at 348 nm every 30 s for 30 min using an Infinite 200 PRO Tecan (Morrisville, NC, USA) plate reader. Slopes (absorbance/min) were transformed into specific activity (μmol/min/mg of protein: U/mg) using an extinction coefficient of 18,000 M^{-1} cm^{-1} with the Lambert-Beer law [42] and subtracting non enzymatic auto-hydrolysis of the substrate at each pH.

Activity towards acetylated carbohydrates was tested using 96-well microplates, based on [43]. The reaction mixture contained 5 mM HEPES at pH 7.2, 100 mM NaCl and 0.05% bromothymol blue. Fully acetylated monosaccharides (Carbosynth, San Diego, CA, USA) were added to a final concentration of 0.1% (initially dissolved in 100% ethanol), whereas xylooligosaccharides (DP-2–7, 95% pure) (Cascade Analytical Reagents and Biochemicals, OR, USA) and konjac glucomannan (Megazyme, Bray, Ireland) were present at a concentration of 1%. The absorbance ratios between protonated (432 nm) and deprotonated (616 nm) forms of the bromothymol blue were registered daily using an Infinite 200 PRO Tecan plate reader and incubations occurred at room temperature using approx. 2 μg/mL of enzyme sample. Incubations were performed during 3 days at room temperature, in 5 mM HEPES buffer, pH 7.2, supplemented with 0.01% bromothymol blue.

An acetylated xylooligosaccharide mixture from hydrothermal treatment of *Eucalyptus globulus* wood [44] was kindly provided by Professor J. C. Parajó (University of Vigo, Spain). Spruce acetyl-galactoglucomannan isolated from the TMP mill process waters [45] was from Prof. Willför (Åbo Akademi University, Finland). Acetylated sugar beet pectin (Pectin Betapec RU301) was obtained from Herbstreith & Fox KG, Neuenbürg, Germany.

Acetylated xylooligosaccharide mixture and acetylated galactoglucomannan were hydrolyzed to shorter fragments with Shearzyme endo-1,4-β-xylanase (TM500L, 10,000 nkat/g substrate) (Novozymes, Bagsværd, Denmark) and *A. niger* endo-1,4-β-mannanase (E-BMANN, 2 000 nkat/g substrate) + *Cellulomonas fimi* β-mannosidase (E-BMOSCF 500 nkat/g substrate) (Megazyme), respectively, in 50 mM sodium citrate buffer, pH 5.0, at 40ºC for 24 h, after which the enzymes were inactivated in a boiling water bath for 10 min

Reaction conditions for intensive esterase treatments were: 2 mg/mL substrate concentration, 20 mM sodium citrate buffer, pH 5.0, 40ºC for 40 h. All esterases were dosed at 10 μg/mg substrate. For the determination of total Ac content in the substrates, NaOH was added to a final concentration of 0.1 M, the samples were incubated overnight, and then neutralized to pH 5–7 with HCl. Acetic acid released by the enzyme or alkali treatments was quantified with the K-ACET kit (Megazyme).

NMR spectroscopy

Samples (600 μL) were prepared at pH 7.4 with 100 mM phosphate buffer (K_2HPO_4/KH_2PO_4 diluted in D_2O (Sigma-Aldrich)) and supplemented with HaeA, or other enzymes mentioned below, at protein concentrations of 2 mg/L. Xylooligosaccharides (Cascade Analytical Reagents and Biochemicals,) and konjac glucomannan (Megazyme) were used at 2% and 0.67% concentration, respectively. Samples were prepared in duplicate (and single repetitions) and proton nuclear magnetic resonance spectra (^{1}H NMR) were measured on a Varian (Mississauga, Canada) VNMRS-500 MHz spectrometer in 5 mm NMR tubes at 298 K. Results shown represent a single measurement spectrum for each condition: the chemical shifts were calibrated using the HDO solvent peak at 4.8 ppm as reference.

In addition to HaeA, an in-house produced and purified CE5 esterase was used (*Humicola hyalothermophila* Humhy2p7_000603 (http://genome.fungalgenomics.ca/) for comparative purposes. Both purified enzymes were applied on xylan oligosaccharides (as described above) and five proton signals in the 2.0–2.3 ppm region (Fig. 6A, dashed box) were monitored. Three of these acetylation peaks are assigned based on previously published data of xylan oligosaccharides derived from aspen [46,47] and *Eucalyptus* [19]. In the case of konjac glucomannan, the ^{1}H NMR peak assignment was based on two recent papers [48,49], and also on in-house ^{2}D NMR data collected before the present work (unpublished data).

Viscometry of konjac glucomannan in the presence of HaeA

Viscosity was measured using a size 200 Cannon-Fenske 20–100 cSt routine viscometer (Thermo Fisher, Waltham, MA, USA). A substrate blank solution consisted of 5 mL 2% low viscosity konjac glucomannan (Megazyme) and 250 μL of 1 M phosphate buffer, pH 6. The solution was gently poured into the viscometer, pre-incubated in a water bath at 37 °C, and the flow time was measured until it remained constant. The reaction was started by adding 50 μL of purified enzyme (0.85 mg/mL) and the flow time and total reaction time were measured at various time points. Reciprocal specific viscosity was calculated as the quotient of flow time of water divided by the difference in the flow time of the solution minus the flow time of water. The dynamic viscosity was calculated as the product of the dynamic viscosity of water times the quotient of the flow time of solution divided by the flow time of water.

Mass spectrometry for oligosaccharide mass profiling

5–6 weeks old *Arabidopsis thaliana* plants (Columbia type) were washed with distilled water and 70% ethanol, after which leaves and stems were separated. Tissue material was stored at − 80 °C until use. Plant material was disrupted in 1.5 mL volumetric tubes. Two glass beads (2 mm diameter) were added to the material plus 500 μL 70% ethanol, then disrupted using a bead-beater device for 5 min in a 4 °C room. The solutions were centrifuged at 4000 *g* and alcohol insoluble residues collected, washed with 70% ethanol (final step with absolute ethanol), and stored at room temperature as powder.

Two spatula tips of *Arabidopsis*-derived alcohol insoluble residues (AIR) were placed into 1.5 mL volumetric tubes and 150 μL of 5 mM HEPES, pH 7.2, was added. In order to produce soluble xyloglucan oligosaccharides, 10 μL of GH74 enzyme (*Paenibacillus* sp. xyloglucanase, Megazyme) (final concentration of 6.7 U/mL) was added to these AIR suspensions. After an overnight incubation (approx. 16 h) at 37 °C, the suspensions were centrifuged at 6000 rpm for 10 min to separate soluble xyloglucans from the insoluble AIR material. After centrifugation, 140 μL of these supernatants were placed into fresh tubes, and HaeA was added (controls with no enzyme or other enzymes were also prepared). After overnight incubation, the solutions were rotavaporated to total dryness and re-suspended in 10 μL of distilled water. A tip of spatula of BioRad (Mississauga, Canada) cation exchange resin beads was also added to each tube. Equivalent volumes of diluted sample and 2,5 dihydroxybenzoic acid (Sigma-Aldrich, Oakville, Canada; 10 mg/mL in 50:50 mix of acetonitrile and 0.1% (v/v) trifluoroacetic acid) were co-crystallized and spotted onto 384-spot ground steel targets using the dried droplet method. MS spectra were acquired using an ultrafleXtreme™ MALDI-TOF/TOF system (Bruker Daltonics, Bremen, Germany) in positive ion reflector mode (calibrated mass range of 700–3500 *m/z*; FlexControl v3.4 software). Ion intensities for sample sets (replicate measurements, independent trials) were evaluated in FlexAnalysis (v3.4 software) by averaging four measurements of 500 shots each (i.e. 2000 shots total per sample).

Results

Fungal CE16 enzymes separate into four distinct Clades and have diverse expression profiles

Phylogenetic analysis of selected CE16 amino acid sequences resulted in four distinct Clades (Fig. 1). Currently, only two enzymes from Clade 1 [14,17,20,50,51], one from Clade 2 [17,19], and one from Clade 3 [52] have been (partially) characterized, demonstrating their ability to release acetyl groups from xylan or D-xylose. *A. niger* NRRL3 contains four CE16 candidates, one in Clade 1 (NRRL3_04916, *haeA*), one in Clade 2 (NRRL3_06053, *haeB*, the ortholog of CAK45102 [17]), and two in Clade 3 (NRRL3_08786, *haeC*, and NRRL3_06379, *haeD*). Orthologs for only *haeA* and *haeB* are detected in *A. niger* CBS513.88 [6]. Amino acid sequence similarity of the four *A. niger* genes is limited, and no distinct region of high homology can be identified (Suppl. Fig. S1).

Expression of *haeA-D* was analyzed in data from published transcriptome studies [28–31] (Suppl. Fig. 2) and summarized in Table 1. Despite encoding an active protein, no expression was observed for *haeC* under any of the tested conditions. The other genes were all expressed on plant biomass related substrates. Expression of *haeA* was highest on corn stover and soybean hulls, while on sugar beet pulp the highest expression was observed for *haeB* (Suppl. Fig. 2). Deletion of the major (hemi-) cellulolytic regulator in *A. niger* (XlnR) [53] or the pectinolytic regulator (GaaR) [28] resulted in strongly reduced expression of *haeA*, *haeB* and *haeD* (Suppl. Fig. 2), even though a putative XlnR binding site was only detected in the *haeA* promoter, and no putative GaaR binding sites were detected in the promoter of any of the genes. Deletion of the arabinanolytic regulator AraR [54] reduced the expression of all three genes at early time points on sugar beet pulp, but a putative AraR binding site was only detected in the *haeD* promoter. Similarly, deletion of the general carbon catabolite repressor CreA [55] resulted in increased expression of all three genes only at early time points on wheat bran and sugar beet pulp (Suppl. Fig. 2).

Recombinant production of the four A. niger enzymes reveals distinct differences in substrate specificity

HaeA-D were produced recombinantly in *A. niger* for biochemical characterization. No background activity was detected in the production strain, and therefore initial experiments were performed using the crude supernatants of these strains.

Chain-length preference and pH-activity profiles

Using *p*NP-acetate at pH 7.2, HaeC showed the lowest specific activity (4.8 U/mg), while the other enzymes had specific activities $>$ 40 U/mg, with HaeD being the most active at ~170 U/mg (Fig. 2A). In contrast, HaeC was the only enzyme active on *p*NP-butyrate, with a specific activity similar to that observed for *p*NP-acetate. It should be noted that the production level of HaeC was very low so while its data could not be fully used quantitatively, it was included for qualitative value.

Using *p*NP-acetate as a substrate, the enzymes, in general, preferred neutral-basic pH values, but with distinct differences (Fig. 2B). HaeC had the most acidic profile with a pH optimum between pH 5.6–7.5, while HaeB and HaeD had the highest activity at the most alkaline pH tested (pH 7.5). HaeA had a clear optimum at pH 6.0.

Monosaccharide deacetylation

Release of acetyl groups from different acetylated monosaccharides was analyzed at neutral pH (Fig. 3). All four enzymes were active on xylose tetraacetate, although the activity of HaeC is significantly lower than that of the others. Activity on glucose and mannose pentaacetate was only detected for HaeA and HaeD.

Activity on acetylated poly- and oligosaccharides

Activity of the enzymes was tested against polymeric acetylated spruce galactoglucomannan and oligosaccharides derived from it using *A. niger* endomannanase and *C. fimi* β-mannosidase, to ensure that some of the *O*-acetyl groups are located on the mannosyl residue at the non-reducing-end, and on acetylated eucalyptus xylooligosaccharides with and without pre-treatment with an *A. aculeatus* GH10 endoxylanase, which also leaves acetylation at the non-reducing end.

HaeA appears to be a general acetyl esterase that acts equally well on mannan, mannooligosaccharides and xylooligosaccharides, releasing in all cases 70–80% of the acetyl groups (Fig. 4A), and was the only enzyme releasing acetyl groups from konjac glucomannan (Fig. 4B). In contrast, HaeC is an acetyl xylan esterase. Although HaeD shares the highest sequence similarity with HaeC and also preferred xylan oligosaccharides as a substrate, it had a lower efficiency and was more sensitive to the length of the xylan oligosaccharides, similar to that reported for *T. reesei* CE16 [51], which is in the same Clade as HaeC and HaeD. HaeC and HaeD also deacetylated galactoglucomannan and mannooligosaccharides, but to a much lower degree than HaeA. HaeB performed poorly on all substrates. No significant activity was observed for any of the enzymes on acetylated pectin, as only 1% or less of acetyl groups were released (data not shown).

Detailed analysis of the specificity of HaeA

HaeA was selected and purified (Suppl. Fig. 3) for more detailed characterization as a broad-specificity hemicellulose acetyl esterase.

Deacetylation of konjac glucomannan

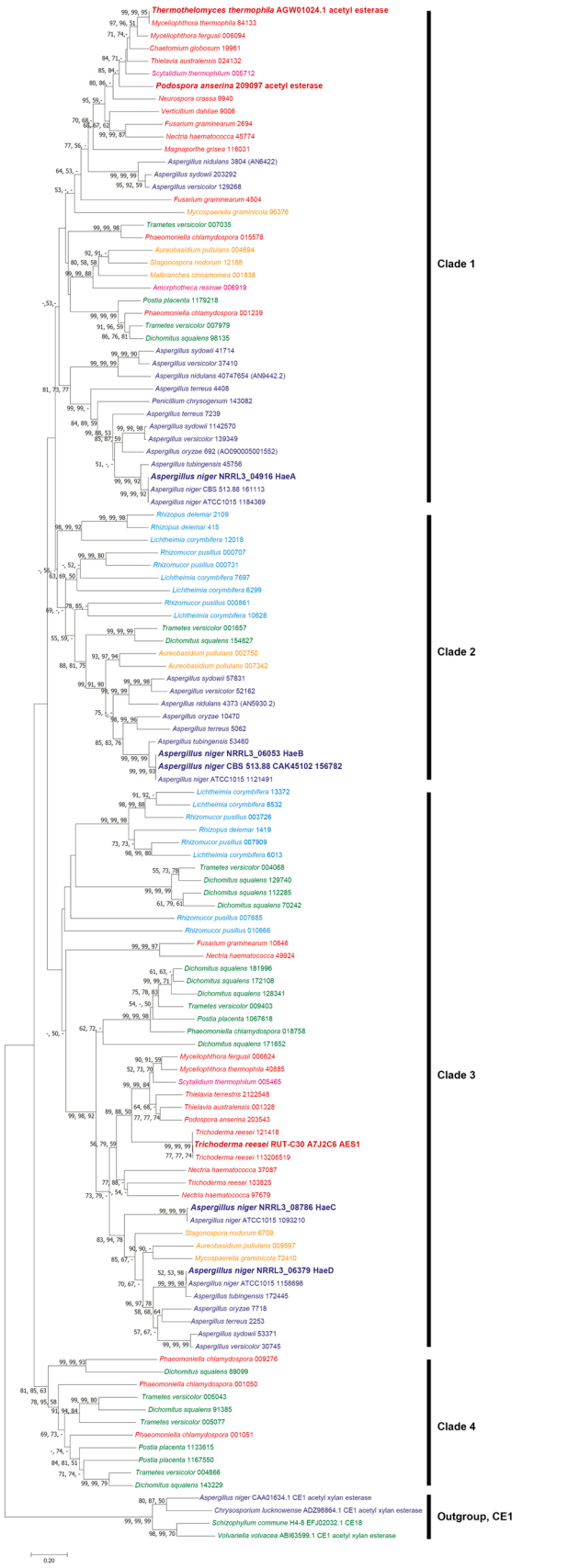
For many years, konjac glucomannan (KGM) has been reported to be acetylated, but with no consistent agreement about at which position. Recent studies indicated mannose and glucose as the main components of KGM in a molar ratio of 1.41 and the intact KGM shows low resolution ^{1}H NMR signals around 2 ppm attributable to acetylation [48,49]. Enzymatically hydrolyzed samples combined with NMR spectroscopy and mass spectrometry were used to examine further the fine structure. Almost equal amounts of O-acetyl groups were found at O-2 and O-3 positions of mannose residues, with no acetylation of glucose residues. A second paper also reported evidence of 2,3-*O*-diacetylation. However, signals were not assigned to the acetyl resonances in these publications.

Deacetylation of intact KGM was evaluated using ^{1}H NMR spectroscopy. The free acetate signal increased slowly in a reaction control with no enzyme added (Suppl. Fig. 4), but when HaeA was added the acetate peak increased rapidly, while simultaneously signals near 2 ppm (assigned to acetylation) were reduced to almost zero (Fig. 5A). After 0.5 h of incubation, acetate was released from KGM and after 24 h the acetylation signal was almost undetectable using proton ^{1}H NMR spectroscopy, correlating with an increase in free acetate. The increase in free acetate continued to 48 h, despite the absence of acetylation signals at 24 h: this may be attributable to some insoluble KGM in the NMR tube, from which additional acetate is released. Consistent with this observed deacetylation, an increase of viscosity over time was observed when konjac glucomannan was incubated with HaeA (Fig. 5B).

Deacetylation of xylooligosaccharides

Deacetylation of xylooligosaccharides was also characterized using NMR by monitoring the acetylation signals near 2 ppm (Fig. 6A) in the presence and absence of enzyme (Fig. 6B). Peak assignments for acetylation shown in Fig. 6A are based on previous studies [19,20]. The CE5 enzyme was included for comparison since it belongs to a different CAZy family for which the positional specificity for hemicellulose deacetylation has been well described previously [14,19].

Although HaeA did not fully de-acetylate some positions on the xylooligosaccharides, the intensities of all peaks decreased to some extent (Fig. 6B). Singly-substituted positions 2 (2-Ac) and 3 (3-Ac) were deacetylated slowly in comparison with the CE5 enzyme (Fig. 6B). After



(caption on next column)

Fig. 1. Phylogenetic tree of CE16 enzymes from a selection of fungi. The displayed tree is a representative tree of a Maximum Likelihood analysis with 500 bootstraps. Bootstrap values (≥50) at the nodes are values from Maximum Likelihood, Neighbor Joining and Minimum Evolution analysis, respectively. Four CE1 amino acid sequences were used as an outgroup. Characterized protein of this and other studies are in large bold-face font. Font-colors represent the different fungal taxonomic groups: red = Sordariomycetes, pink = Leotiomycetes, blue = Eurotiomycetes, orange = Dothidiomycetes, green = Agaricomycetes, light blue = Mucoromycetes.

about a week of incubation (6 days), the CE5 acetyl xylan esterase accumulated > 5–6-fold more free acetate due to much more extensive deacetylation at positions 2 and 3. HaeA deacetylates doubly-acetylated xylose units, at positions 2 and 3 (2,3-diAc), at a rate similar to the CE5 enzyme (Fig. 6B). Two unidentified peaks around 2.21 and 2.16 ppm, which could not be assigned to any structural feature (a and b), remain constant when the CE5 control is used but are removed when using HaeA protein.

Deacetylation of xyloglucan oligosaccharides

Since commercial xyloglucan is usually prepared using methods which deacetylate it, xyloglucan oligosaccharides (Fig. 7A) were prepared from *Arabidopsis thaliana* cell wall material using extraction methods coupled to oligosaccharide mass profiling. The alcohol insoluble residues were digested with a GH74 xyloglucanase from *Paenibacillus* sp. to generate xyloglucan oligosaccharides that were subsequently incubated with or without HaeA (Fig. 7B). As commonly encountered in Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS), both the acetylated and non-acetylated xyloglucan oligosaccharides ionized as sodium or potassium adducts (i.e. addition of 23 (Na^+) or 39 (K^+) mass units), and were detected using positive ion mode. When HaeA was present, xyloglucan oligosaccharides deacetylation by HaeA was clearly observed (Fig. 7C). The three acetylated peaks at 1597 (XLFG), 1451 (XLLG, XXJG and/or XXFG+) and 1435 (XXFG) *m/z* disappeared while corresponding deacetylated peaks (e.g. at 1409 and 1393 *m/z*) increased in abundance. In addition, the ion related to XXLG/XLXG increased highly in intensity in the presence of the higher enzyme amount (data shows only the highest concentration tested).

Discussion

Acetyl esterases are involved in degradation of various plant biomass polymers [56] and belong to various CAZy families [10]. While most studies describe members of CE1-CE5 and CE16 as acetyl xylan esterases, indications exist for a broader role of CE16 acetyl esterases, especially with respect to galactoglucomannan deacetylation [15,24]. Four CE16 genes were identified in the gold-standard genome of *A. niger* [25]. One of them (*haeB*) is the ortholog of a previously published CE16 esterase from *A. niger*, which had only been tested on xylan-related substrates [17,19]. In the present study, this enzyme performed poorly on poly- and oligosaccharide substrates. The distribution of the four *A. niger* CE16 enzymes across three of the four Clades in the phylogenetic tree demonstrated the diversity within this species. Two of the *A. niger* proteins (HaeC and HaeD) are members of Clade 3, which also contains the published CE16 of *T. reesei* [14]. Significant expression levels for *haeC* were not detected in any of the tested conditions [28–31], suggesting that this could be a pseudogene. However, heterologous production of HaeC resulted in an active enzyme, and while the activity on *p*NP-acetate was lower than that of the other three enzymes, it was comparably active on several other substrates. The other genes were expressed during growth on various plant biomass substrates and their expression profiles were affected by deletion of transcriptional regulators involved in plant biomass degradation by *A. niger*, such as XlnR [53], GaaR [28] and AraR [54], although promoter analysis suggested that some of these effects may be indirect. Deletion of CreA [55] resulted in increased expression of *haeA*, *haeB* and *haeD* at early time points of

Table 1
Summary of expression profiles of the four *A. niger* CE16 genes and identification of putative regulator binding sites in their promoters.

Gene	Expressed on plant biomass substrates	Regulated by XlnR	XlnR binding site (s)	Regulated by AraR	AraR binding site (s)	Regulated by GaaR	GaaR binding site (s)	Regulated by RhaR	RhaR binding site (s)	Regulated by CreA	CreA binding site (s)
haeA	yes	yes	yes	yes/no	no	yes	no	no	no	yes	yes
haeB	yes	yes	no	yes	no	yes	no	no	no	yes	yes
haeC	no	n/a	yes	n/a	no	n/a	yes	n/a	yes	n/a	yes
haeD	yes	yes	no	yes/no	yes	yes	no	no	yes	yes	yes

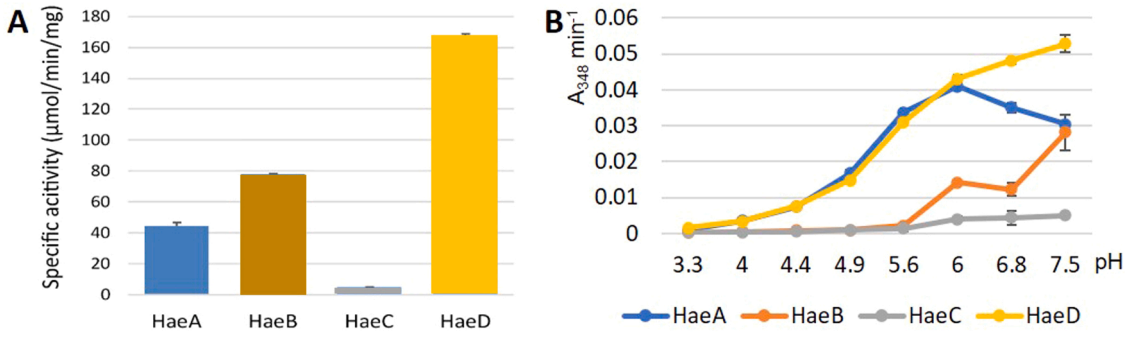


Fig. 2. Characterization of the activity of the four HAEs from *A. niger*. A. Specific activities of the enzymes on *p*NP-acetate determined in 20 mM HEPES with 100 mM NaCl at pH 7.2. Protein concentration of all enzyme preparations was ~0.2 µg/mL. Data was obtained from triplicate averages ± SEM. B. pH-activity profiles on *p*NP-acetate using McIlvain's buffer with no salt. Data was obtained from duplicate averages.

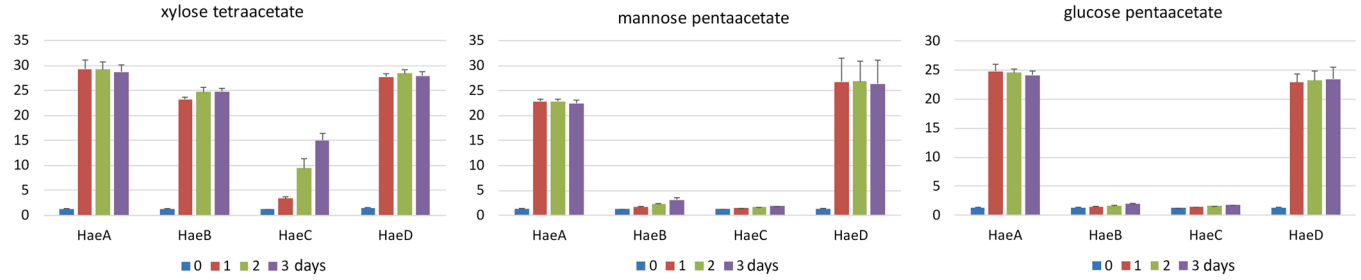


Fig. 3. Deacetylation of monosaccharides. Three acetylated single sugars were tested as substrates for the *A. niger* HAEs. The values at the y-axis represent the absorbance ratio at 432/616 nm that was determined (triplicate ± SEM) for each HAE over three days with daily measurements (indicated by the different color bars). Error bars indicate SEM of triplicate reactions.

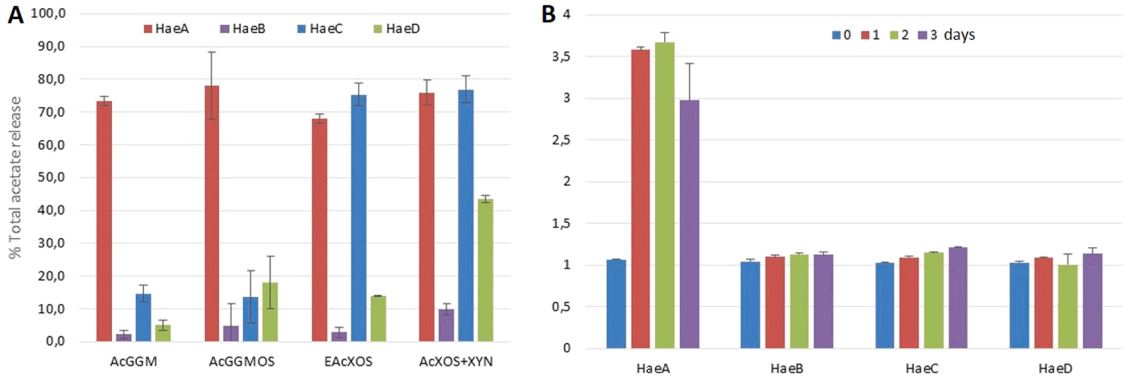


Fig. 4. Deacetylation of poly- and oligosaccharides. A. Galactoglucomannan (AcGGM) and AcGGM derived oligosaccharides (AcGGMOS), acetylated Eucalyptus xylooligosaccharides (EAcXOS) and EAcXOS pre-treated with a GH10 endoxylanase (AcXOS+XYN) were tested as substrates for the different *A. niger* supernatants. The percentage of acetate released (triplicate ± SEM) for each supernatant is given. B. Absorbance ratio at 432/616 nm was registered (triplicate ± SEM) for each supernatant and 1% konjac glucomannan for three days with daily measurements (indicated by the different color bars).

growth on wheat bran and sugar beet pulp, which matches the general pattern of CreA effects during growth on plant biomass, where the largest influence was revealed at early time points [31].

Recombinant production of the *A. niger* CE16 enzymes revealed distinct characteristics, such as variation in their pH profile and substrate specificity. While all enzymes are active on *p*NP-acetate and

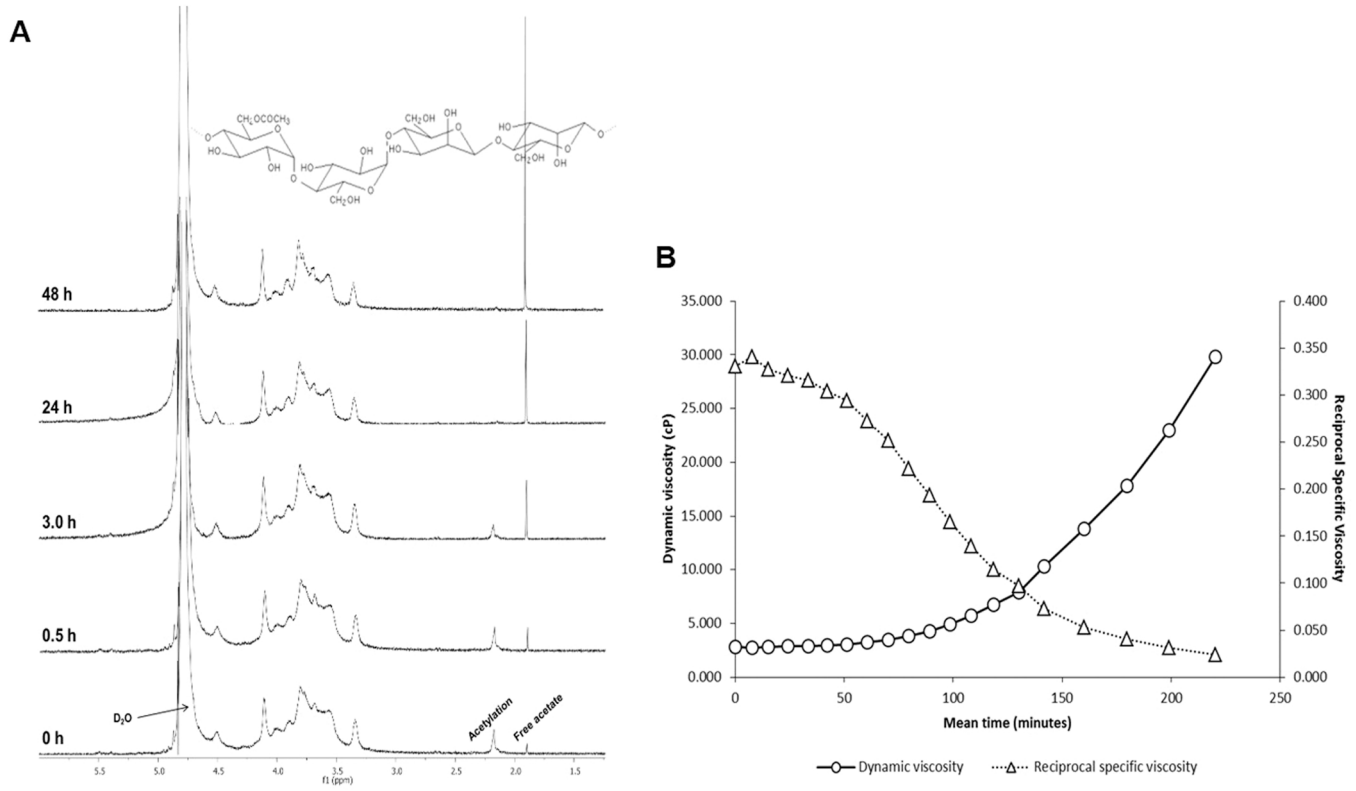


Fig. 5. Activity of HaeA on konjac glucomannan. A. Nuclear magnetic resonance analysis of konjac glucomannan deacetylation by *A. niger* HaeA. Reactions were conducted in duplicate and single plots represent the same tube in time. Peaks assigned to D_2O, acetyl groups and acetate are shown with labels. The structure of konjac glucomannan shown at the top of the figure was obtained from http://www.cybercolloids.net/. Substrate in buffer with no enzyme (used as a control) and another HaeA independent condition are shown in Suppl. Fig. 4B. Viscometry analysis of the effect of HaeA on konjac glucomannan.

xylose tetraacetate, activity on glucose pentaacetate and mannose pentaacetate was only observed for HaeA and HaeD. HaeA had the highest activity on galactoglucomannan and oligosaccharides derived from this polymer, while it shared the highest activity on xylooligosaccharides with HaeC. The activity of CE16 enzymes on xylooligosaccharides has been described before [2] and has been the main focus in more recent studies [14,15,17,20]. Activity on galactoglucomannan was reported previously for a CE16 enzyme of *M. thermophila* [15], but esterase activity on galactoglucomannan was already reported much earlier for a purified enzyme from *Aspergillus* [57]. Comparison of the N-terminal amino acid sequence of this enzyme (XEXTTTNPTYFFTD) [58] to the *A. oryzae* genome, corresponded to a gene that was suggested to be an galactomannan acetyl esterase in a transcriptomic study [24], and which interestingly is the ortholog of *A. niger* HaeA. Both previous studies reported that Clade 1 CE16s are more active towards acetylated galactoglucomannans than glucuronoxylan, as was also noted for Clade 1 HaeA in the present study. HaeD activity on acetylated mannose, glucose as well as on short glucomannooligosaccharides matches the *T. reesei* CE16 from the same Clade 3 [14].

Surprisingly, no activity was detected on acetylated pectin, despite indications from the expression profile that the genes may be part of the pectinolytic system of *A. niger*. A recent study, in which machine learning was used as a tool to identify novel pectinases in *A. niger*, suggested that there is high probability for *haeA* and *haeB* of being pectinase encoding genes [59]. A previous study reported a pectin acetyl esterase from *Aspergillus aculeatus* with a MW of around 40 kDa that is active on partially degraded pectin, but not on intact pectin and was specific for the rhamnified regions of pectin [60]. The predicted MW of HaeA and HaeB are 34 and 40 kDa, respectively, and the *A. aculeatus* genome contains orthologs for *haeA*, *haeB* and *haeD*. It would therefore be interesting to explore further the activity of HaeA and HaeB on pectin-oligosaccharides, but unfortunately the substrate used in the *A. aculeatus* study [60] was not available.

Considering the high activity and broad specificity, and because this enzyme had not been characterized before, the specificity and product profile of *A. niger* HaeA were analyzed in more detail. Overall, the activity of purified HaeA with xylooligosaccharides is comparable to published CE16s, particularly Clade 1 enzymes from *P. anserina* and *M. thermophila*, which both are active on polymeric xylan [15,20]. *T. reesei* CE16 (Clade 3) is not active on polymeric or oligomeric xylan [51], but removes primarily one acetyl group from the non-reducing end of xylooligosaccharides, acting as exo-deacetylase [14,16]. HaeA does not show preference for positions 2 or 3, while preference for position 3 over position 2 was reported for *T. reesei* CE16 [17,51]. This enzyme is more similar to *A. niger* HaeC and HaeD as they all belong to Clade 3, suggesting a different preference for these two Clades. ^{1}H NMR showed that HaeA was also active on doubly acetylated xylose residues (2, 3-diAc), like the CE16 enzymes from *P. anserina, M. thermophila* and *A. niger* [17,20]. This feature, under the tested experimental conditions, was also observed with the CE5 control enzyme, as expected from the literature [19].

Incubations of HaeA with (galacto)glucomannans resulted in effective deacetylation of spruce galactoglucomannans and konjac glucomannan. KGM has been shown previously to be acetylated predominantly at position 2-O and 3-O of mannose residues, with some 2,3 Ac-diacetylation, so the positional specificity of the enzymes appears to be similar between the two substrates, although rates of decetylation of individual positions could not be determined in KGM.

Xyloglucan oligosaccharides were also generated and detected here using the previously-described oligosaccharides mass profiling technique [61], by a biochemical digestion of *Arabidopsis* cell walls with xyloglucanase. The resulting mixture was then used as substrate for HaeA and analyzed by MALDI-MS. Results showed that four Na^+-carbohydrate ions were frequently detected in *Arabidopsis*

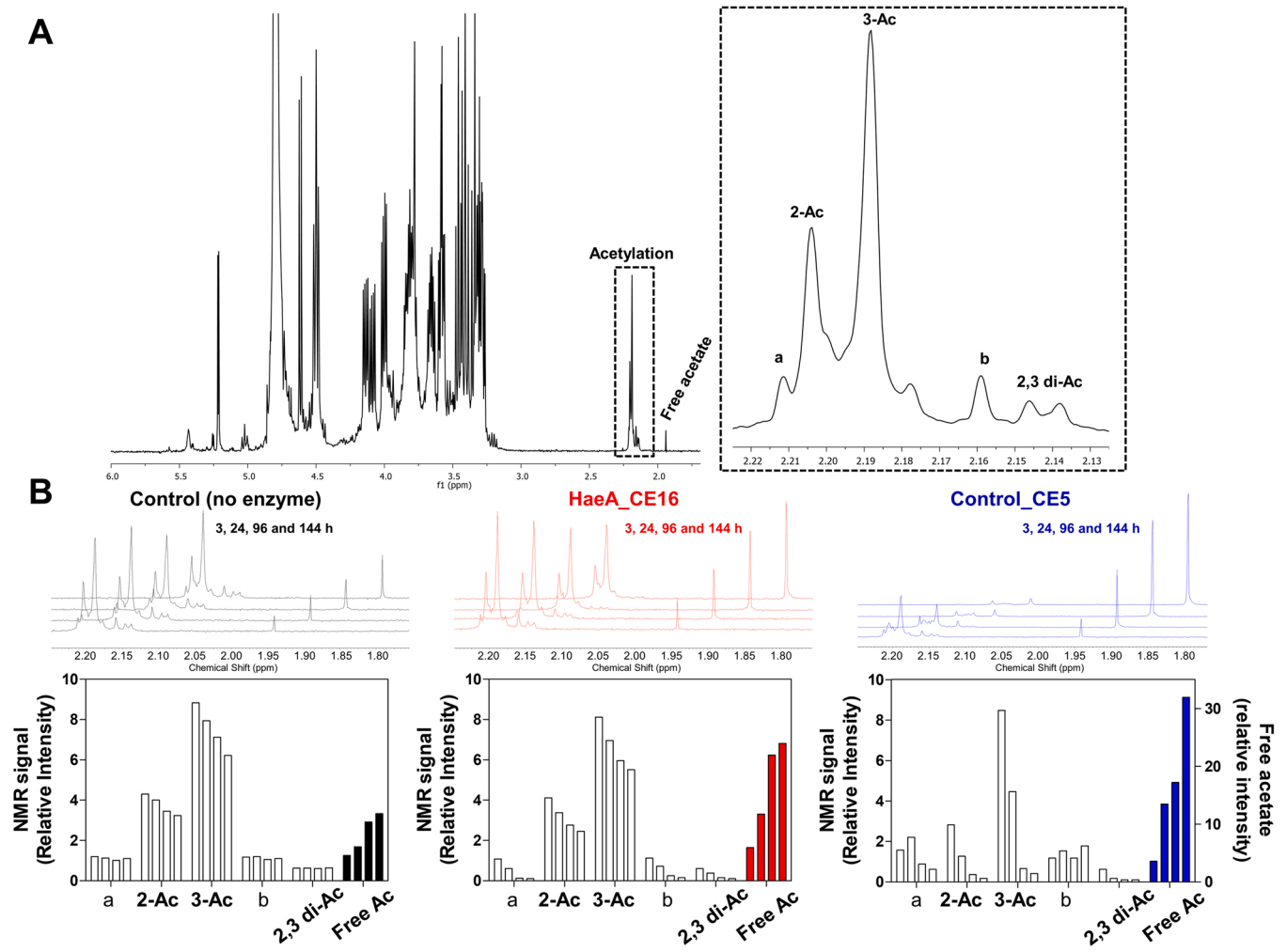


Fig. 6. Nuclear magnetic resonance analysis of xylan oligosaccharide deacetylation by two different esterases. A. ^{1}H NMR spectrum of xylan oligosaccharides, zooming in on the acetyl groups region and peaks assigned to each substitution. That region is also zoomed and depicted in the panel of **B** for each enzyme after 3, 24, 96 and 144 h at room temperature. Bar graphs corresponding to the quantification of each signal in time. White bars represent acetylated positions and black/red/blue bars the acetate release from the reaction. A purified CE5 enzyme, from *Humicola hyalothermophila*, was used as a de-acetylation control; the right Y-axis (free acetate) of its graph has different magnitude for this condition (> 30).

oligosaccharides mass profiling: XXXG; XXLG/XLXG; XXFG and XLFG. The last three are usually present (and detected) in their acetylated forms in *Arabidopsis* oligosaccharides mass profiling [62], but under the conditions used here the acetyl-substituted forms of XXLG/XLXG (XXLG/XLXG) were not detected, and only XXFG and XLFG were identified. Another ion, the mass of which fits with oligosaccharides XLLG/XXJG (1409 *m/z*, M+Na^{+}) was also detected. This ion was previously observed in root xyloglucan oligosaccharides mass profiles [63, 64]. Acetylated forms of XLLG/XXJG were also detected (peak 1451 *m/z*). When this mixture of XGs was treated with HaeA, the acetylated forms of XLFG, XLLG/XXJG and XXFG disappeared, and although the acetylated forms of XXLG/XLXG were not detected, the relative peak intensity attributed to their de-acetylated forms increased in a concentration-dependent manner. However, it is important to point out that the peak intensities cannot be used in a quantitative way under the conditions of these experiments, so more rigorous techniques will be needed in future to quantify and compare this process between different enzymes. As far as we are aware, this is the first report showing xyloglucan oligosaccharide deacetylation activity in fungal or bacterial CAZy CE families.

Conclusions

CE16 acetyl esterases are a relatively new CAZy enzyme family, which have been mainly evaluated for their activity on xylan-related substrates. Here the four CE16 members of *A. niger* NRRL3 were characterized and differences in their sequence, expression profile and biochemical properties were demonstrated. Clade 1 enzymes have a broad substrate specificity, acting on xylan, galactomannan and xyloglucan, while the enzymes from Clade 2 and 3 are active on xylan, but have only low activity on galactomannan. When the present data is combined with that of other characterized CE16 enzymes, it is also clear that the Clades differ in the positions on xylo-oligosaccharides from which they can remove acetyls. Despite these differences, all four enzymes were defined as hemicellulose acetyl esterases, to differentiate them from the previously described acetylxylan esterases in other CAZy families. The broad specificity of HaeA makes this a highly attractive enzyme for various biotechnological applications.

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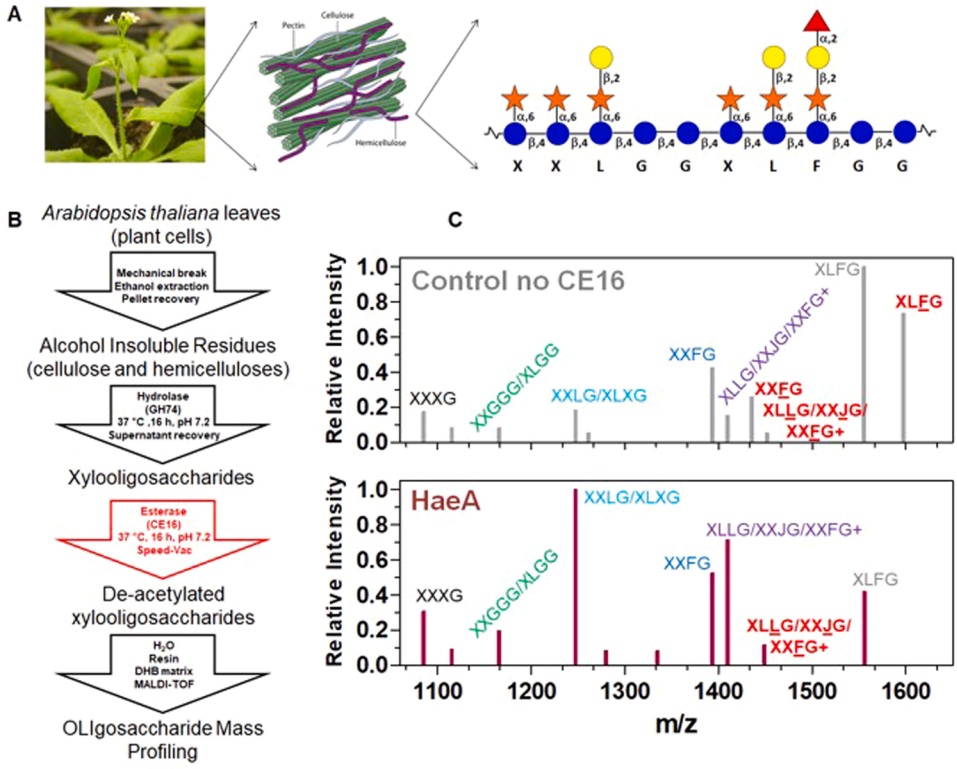

Fig. 7. Detection of xyloglucan oligosaccharide deacetylation using MALDI mass spectrometry. A. Schematic presentation of xyloglucan. **B.** Summary of the process of xyloglucan oligosaccharide (XGO) generation from *Arabidopsis thaliana* plant material. Alcohol Insoluble Residues (extracted from plant leaves were treated with a xyloglucanase (GH74 hydrolase) to generate xyloglucan oligosaccharides. After hydrolysis, *A. niger* HaeA (CE16) was added and oligosaccharide changes were evaluated using oligosaccharide mass profiling. **C.** The ions generated after treatment. Each peak represents the singly charged species of the respective XGO plus Na^+ or K^+ (+); for example, XXFG and XXFG+ corresponds to the same XGO plus Na^+ (1370 + 23; 1393 adduct) or plus K^+ (1370 + 39; 1409 adduct). The representative spectra depict the XGOs generated after xyloglucanase treatment (upper panel) as compared to the successive treatment with HaeA (lower panel). The standard XGO labeling is as follows: G, glucose; X, glucose decorated with xylose; L, glucose decorated with xylose and galactose; F, glucose decorated with xylose, galactose and fucose; and J, glucose decorated with xylose and double galactose. The cell wall figure at the top is based on [7], and the XXLGGXLFGG representation from https://www.glycopedia.eu/e--chapters/the-plant-cell-walls/article/secondary-wall-hemicelluloses.

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CRediT authorship contribution statement

FAV: Investigation, Writing – original draft, Writing – review & editing. **SK, SLC, JFLI**: Investigation. **SMJL, MP**: Formal analysis. **AB**: Experimentation, Writing – original draft. **AD**: Methodology, Validation. **MJL**: Investigation, Data collection. **KSH**: Writing – review & editing, Funding acquisition. **MRM, MAH**: Investigation, Writing – review & editing. **AT, MT, JP**: Conceptualization, Supervision, Visualization, Funding acquisition, Project administration, Resources, Writing – review & editing. **RPdV**: Conceptualization, Supervision, Funding acquisition, Project administration, Resources, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Statement

All data is included in the manuscript.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2022.04.003.

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