



Trichoderma reesei CE16 acetyl esterase and its role in enzymatic degradation of acetylated hemicellulose

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ABSTRACT

Background: *Trichoderma reesei* CE16 acetyl esterase (AcE) is a component of the plant cell wall degrading system of the fungus. The enzyme behaves as an exo-acting deacetylase removing acetyl groups from non-reducing end sugar residues.

Methods: In this work we demonstrate this exo-deacetylating activity on natural acetylated xylooligosaccharides using MALDI ToF MS.

Results: The combined action of GH10 xylanase and acetylxyylan esterases (AcXEs) leads to formation of neutral and acidic xylooligosaccharides with a few resistant acetyl groups mainly at their non-reducing ends. We show here that these acetyl groups serve as targets for TrCE16 AcE. The most prominent target is the 3-O-acetyl group at the non-reducing terminal Xylp residues of linear neutral xylooligosaccharides or on aldouronic acids carrying MeGlcA at the non-reducing terminus. Deacetylation of the non-reducing end sugar may involve migration of acetyl groups to position 4, which also serves as substrate of the TrCE16 esterase.

Conclusion: Concerted action of CtrGH10 xylanase, an AcXE and TrCE16 AcE resulted in close to complete deacetylation of neutral xylooligosaccharides, whereas substitution with MeGlcA prevents removal of acetyl groups from only a small fraction of the aldouronic acids. Experiments with diacetyl derivatives of methyl β-D-xylopyranoside confirmed that the best substrate of TrCE16 AcE is 3-O-acetylated Xylp residue followed by 4-O-acetylated Xylp residue with a free vicinal hydroxyl group.

General significance: This study shows that CE16 acetyl esterases are crucial enzymes to achieve complete deacetylation and, consequently, complete the saccharification of acetylated xylans by xylanases, which is an important task of current biotechnology.

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1. Introduction

Development of efficient processes for bioconversion of plant biomass depends on detailed knowledge of both plant cell structure and the microbial enzymes involved in the degradation of plant cell wall components. One group of such enzymes is the acetylxyylan

esterases (AcXEs), whose role is to deacetylate xylopyranosyl (Xylp) residues in the xylan main chain or in xylooligosaccharides to create new sites for further hydrolysis by endo-β-1,4-xylanases and β-xylosidases. These enzymes are needed in biorefinery processes which do not involve alkaline pretreatments that lead to saponification of all ester linkages present in plant cell walls. An example of partially acetylated plant hemicellulose is hardwood glucuronoxylan. The polysaccharide contains four different types of acetylated Xylp residues in the main chain: singly 2- or 3-O-acetylated Xylp residues, doubly 2,3-di-O-acetylated Xylp residues and 3-O-acetylated Xylp residues α-1,2-substituted with 4-O-methyl-D-glucuronic acid (MeGlcA) [1–3].

In a recent study [4] we investigated the mode of action of members of four AcXE families (CE1, CE4, CE5 and CE6 [5]) on aspen acetylglucuronoxylan isolated by steam explosion. ¹H NMR was used to establish the positional specificity of the enzymes on

Abbreviations: AcE, acetyl esterase; AcXE, acetylxyylan esterase; CE, carbohydrate esterase; Xylp, D-xylopyranose or D-xylopyranosyl; MeXylp, methyl β-D-xylopyranoside; Xyl₂-Xyl₇, β-1,4-xylobiose-β-1,4-xyloheptaose; MeGlcA, 4-O-methyl-D-glucuronic acid or 4-O-methyl-D-glucuronosyl; Xyl_nAc_y, acetylated β-1,4-xylooligosaccharide containing x xylose residues and y acetyl groups; MeGlcAXyl_nAc_y, acetylated aldouronic acid containing one MeGlcA, x xylose residues and y acetyl groups; HexXyl_nAc_y, oligosaccharide containing one hexopyranose residue of unknown nature, x xylose residues and y acetyl groups

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polymeric substrate and MALDI ToF MS was used for analysis of the action of AcXEs on neutral and acidic xylooligosaccharides generated from aspen acetylglucuronoxylan by *Clostridium thermocellum* GH10 endo- β -1,4-xylanase (CtGH10) [4,6]. Serine-type AcXEs, which occur in CE families 1, 5 and 6, were capable of liberating acetic acid from singly and doubly acetylated Xylp residues. Aspartate metalloenzymes, which are members of the CE4 family, deacetylated effectively only singly 2- or 3-O-acetylated Xylp residues. The CE4 AcXEs require the neighboring hydroxyl group at positions 3 or 2 non-acetylated or otherwise unsubstituted [7–9]. Neither the serine-type nor the aspartate-type AcXEs were capable of liberation of the 3-O-acetyl groups on MeGlcA-substituted Xylp residues. Studies with oligosaccharides further showed that a limited number of additional acetyl groups are resistant to AcXEs in both neutral and acidic xylooligosaccharides, in particular acetyl groups located on non-reducing-end Xylp residues that may become non-hydrolyzable by spontaneous migration from position 2 and 3 towards position 4 [6]. Studies using monoacetyl and diacetyl derivatives of 4-nitrophenyl β -D-xylopyranoside have convincingly demonstrated the occurrence of this type of migration and showed that the 4-monoacetylated and the 3,4-di-O-acetylated derivatives are the most abundant in equilibrium [10]. A similar situation could occur on the non-reducing end Xylp residues of xylooligosaccharides, both for non-substituted Xylp or Xylp substituted with MeGlcA [11,12].

There is currently only one known microbial esterase that deacetylates oligosaccharides at the non-reducing end [11]. This enzyme is the acetyl esterase (AcE) from *Trichoderma reesei* belonging to the CE16 family (TrCE16) which is a component of the cellulolytic system of the fungus [13–16]. Earlier studies of catalytic properties of the enzyme showed that it does not deacetylate polymeric substrates like acetylglucuronoxylan but acted on acetylated xylobiose [14]. The enzyme also catalyzes the deacetylation of 4-nitrophenyl β -xylopyranoside monoacetates and oligosaccharides acetylated at the non-reducing sugar residue, acting at positions 3 and 4 and, less effectively, also at position 2 [11,17]. The enzyme also efficiently catalyzes transacetylation in aqueous medium saturated with vinyl acetate to position 3 of the non-reducing residues of cello-, manno- and xylooligosaccharides [11,18]. The acetyl group could be removed from acetylated saccharide acceptors by the same enzyme in the absence of an acetyl group donor [11]. In contrast to the formation of single 3-O-acetylated products in the case of cellooligosaccharides, transacetylation to Xyl or xylooligosaccharide acceptors afforded a mixture of acetyl derivatives because the acetyl group migrated to other positions [10], including position 4. This is in accordance with the proposed occurrence of the acetyl group at position 4 of the non-reducing end of neutral and acidic xylooligosaccharides resistant to AcXEs [6,19]. On the basis of these properties the enzyme was assigned as an exo-acting deacetylase [19]. Thus, the TrCE16 enzyme appears to be a good candidate for deacetylation of non-reducing end Xylp residues in both neutral and acidic xylooligosaccharides resistant to further deacetylation by AcXEs in families CE1, CE4, CE5 and CE6 [6].

In this study we have analyzed the action of TrCE16 on natural substrates and the interplay of this enzyme with various AcXEs. The exo-type action of TrCE16 on the non-reducing end sugar residues was demonstrated on xylooligosaccharides generated from aspen acetyl glucuronoxylan by the CtGH10 xylanase alone or in the presence of four different AcXEs (CE1, 4, 5 and 6). Nearly complete deacetylation of oligosaccharides could be achieved by combining several AcXEs with TrCE16 AcE.

2. Materials and methods

2.1. Polysaccharides, oligosaccharides, glycosides and enzyme preparations

Aspen acetyl glucuronoxylan was isolated from aspen sawdust by hot water extraction as described elsewhere [6]. Di-O-acetates (2,3-, 2,4- and 3,4-) of Me- β -Xylp were generous gifts from Dr. P. Kovac

(National Institute of Health, Bethesda, MD, USA), Dr. J. Hirsch (Institute of Chemistry, Slovak Academy of Sciences) and Dr. A. Fernandes-Mayorales (Instituto de Química Orgánica General, CSIC, Madrid, Spain). 2,3,4-Tri-O-acetate of MeXylp was prepared by a standard acetylation procedure [20].

Endo- β -1,4-xylanase of GH10 family from *C. thermocellum* (CtGH10) (xylanase catalytic module of Xyn Z) was a recombinant enzyme prepared as described previously [6]. A CE1 family AcXE from *Schizophyllum commune* (ScCE1) was purified as described [20]. A CE4 family AcXE from *Streptomyces lividans* (StCE4, Ref. [21]) was supplied by Drs. Claude Dupont and Dieter Kluepfel (Institute of Armand Frappier, Laval, Canada). A similar enzyme from *C. thermocellum* (CtCE4) [8] was provided by Profs. Carlos M.G.A. Fontes (Universidade Técnica de Lisboa, Portugal) and Gideon J. Davies (University of York, UK). A CE5 family AcXE from *T. reesei* (TrCE5) [14] was kindly provided by Prof. Maija Tenkanen (University of Helsinki, Finland). A family CE6 AcXE from *Orpinomyces* sp. (OCE6) was from Megazyme Int. (Ireland). The recombinant CE16 AcE from *T. reesei* (TrCE16) was produced and purified as reported by Li et al. [16].

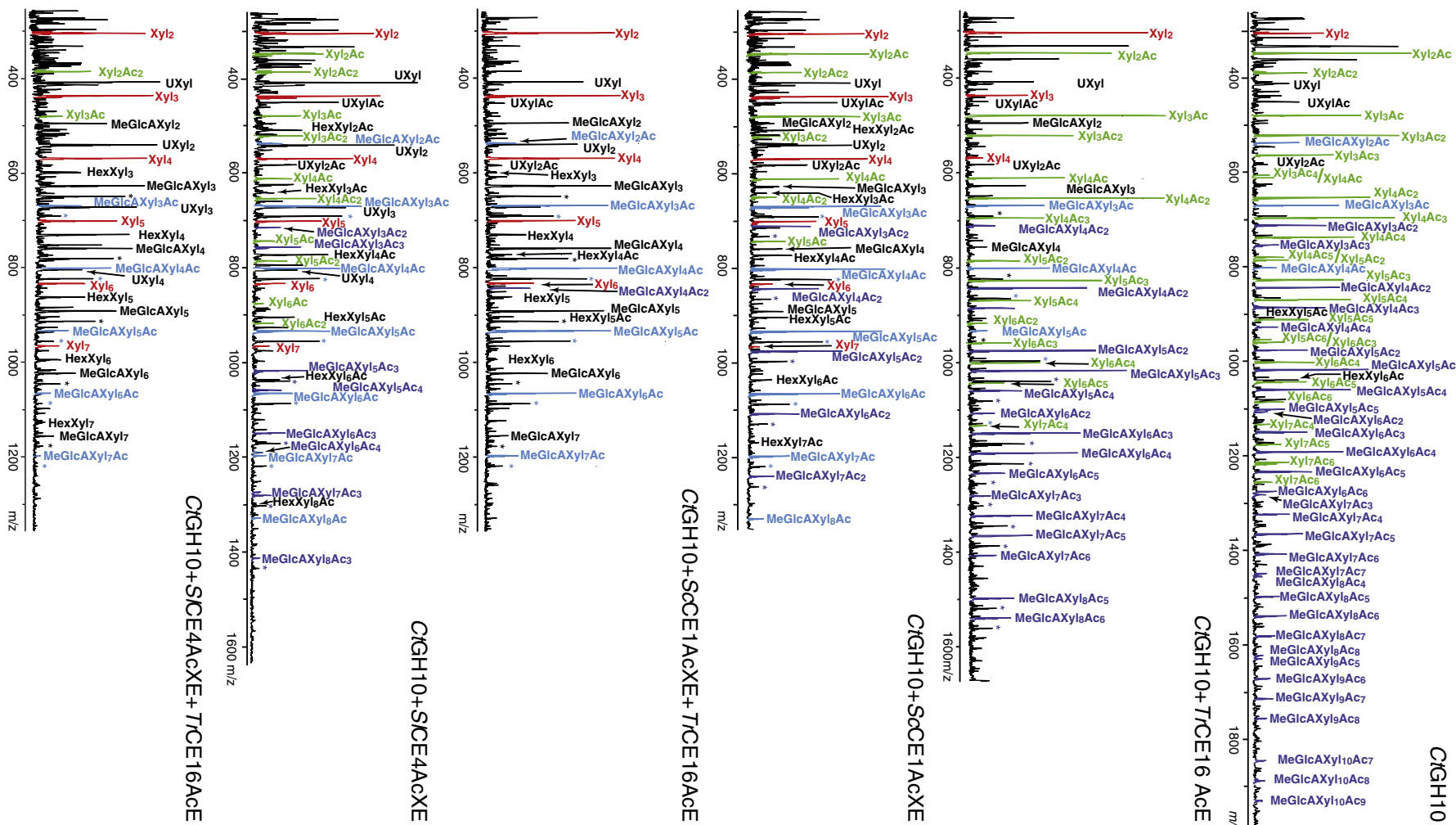
2.2. Hydrolysis of aspen acetyl glucuronoxylan with enzymes

A solution of aspen acetyl glucuronoxylan (0.5%, w/v) in 0.05 M Tris/HCl buffer (pH 6.5) was incubated with an endoxylanase (CtGH10; 0.07 mg/ml) at 40 °C. After 24 h the mixture was heated at 100 °C for 5 min to denature the enzyme. 50 μ l aliquots of the mixture with heat-inactivated xylanase were mixed with small volumes of solutions of AcXEs alone or in combination with TrCE16 to give the final enzyme concentrations: ScCE1 (0.13 mg/ml), SICE4 (0.045 mg/ml), CtCE4 (0.4 mg/ml), TrCE5 (0.13 mg/ml), TrCE16 (0.022 mg/ml). All incubation mixtures containing a combination of an AcXE and TrCE16 acetyl esterase were run in parallel with the mixtures containing the CtGH10 generated xylooligosaccharides (with denatured or active GH10) treated with only AcXEs. Therefore, the products identified by MALDI ToF MS in the mixtures with only AcXEs, reported in our previous paper [6], are included in this work to aid a direct comparison of their action in the presence of TrCE16. The action of TrCE16 on xylooligosaccharides in the absence of AcXEs was investigated under identical conditions. The mixtures with esterases were then incubated further for 24 h at 40 °C. After denaturation of the esterases (5 min, 100 °C), the samples were cooled, and prior to MALDI ToF MS, they were examined by TLC on silica gel (Merck, Germany) and vacuum dried. TLC was done in ethyl acetate–acetic acid–2-propanol–formic acid–water (25:10:5:1:15, v/v) and the sugars were detected using the *N*-(1-naphthyl)ethylenediamine dihydrochloride reagent [22]. The conditions used in the enzyme treatments afforded samples suitable for MALDI ToF MS analysis, as described in detail in a recent study [6].

In a separate experiment, using the conditions described above, acetyl glucuronoxylan was incubated for 48 h with CtCE10 (without the denaturing step) in combination with ScCE1 or SICE4 AcXE in the presence or absence of TrCE16 AcE. This implies that the esterases acted on the polysaccharide in the presence of an active GH10 xylanase. The reaction mixtures were heated for 5 min at 100 °C and cooled prior to MALDI ToF MS analysis [6].

2.3. Action of TrCE16 AcE on acetylated derivatives of methyl β -D-xylopyranoside

The deacetylation of the triacetate and diacetates of Me- β -Xylp at 10 mM concentration in 0.05 M sodium phosphate buffer (pH 6.0) by TrCE16 AcE (0.4 mg/ml) at 40 °C was monitored by TLC on silica gel (Merck, Germany) in ethyl acetate–benzene–isopropanol (2:1:0.1, v/v). Sugars were detected with the *N*-(1-naphthyl)ethylenediamine dihydrochloride reagent [22].



3. Results

3.1. Effects of TrCE16 on acetylated xylooligosaccharides liberated by CtGH10 xylanase

TrCE16 is known to be inactive on polymeric substrates such as hardwood acetyl glucuronoxylan [13]. Therefore, we examined its action on a mixture of oligosaccharides generated from aspen acetyl glucuronoxylan by CtGH10 endoxylanase. The mixture was identical with that analyzed by MALDI ToF MS in our previous paper, so the data are reproduced to show the starting substrates of TrCE16 [6]. The analysis of the product mixtures showed that the effect of TrCE16 on the number of acetyl groups in neutral and acidic xylooligosaccharides differed strongly from the effect of AcXEs reported earlier [6]. These data are also reproduced here since the action of TrCE16 on acetylated xylooligosaccharides was also examined in combination with AcXEs in parallel incubation mixtures (Fig. 1, Tables 1 and 2). While the AcXEs removed a considerable number of acetyl groups, thus reducing the number of acetyl groups in the oligosaccharide products to one, two or three, TrCE16 AcE appears to liberate only one acetyl group from most compounds (Table 1). The data for the reaction with the TrCE16 esterase show a shift for each series of multiple acetylated derivatives of a single xylooligosaccharide: the derivative with the highest number of acetyl groups disappears, whereas a new derivative with one acetyl group less than the oligosaccharide acetylated to the lowest degree in the starting material appears. For example, the data strongly suggest that the tri-*O*-acetate of Xyl₃ is deacetylated to the di-*O*-acetate, the di-*O*-acetate to the monoacetate and the monoacetate to free Xyl₃. Xyl₃ was not detected among the products released by the treatment with CtGH10 endoxylanase alone.

A similar reduction in the number of acetyl groups upon treatment with TrCE16 AcE also occurred for the aldouronic acids. Comparison of the mass spectra in Fig. 1 clearly shows that the treatment with TrCE16 AcE reduced the abundance of ions corresponding to oligosaccharides containing an equal number of acetyl groups and xylose residues. In addition, new derivatives were formed with a number of acetyl groups lower than those observed in the starting material. The newly observed ions correspond, e.g. to free aldopentaouronic acid, monoacetates of aldohexaouronic acids, diacetate of aldohexaouronic acids, etc.

3.2. Effect on TrCE16 AcE on acetylated xylooligosaccharides treated with AcXEs

The effect of TrCE16 esterase was also tested on mixture of acetylated xylooligosaccharides in combination with four different AcXEs, ScCE1, TrCE5, SICE4 and CtCE4 (see [6]), TrCE16 contributed to a higher degree of deacetylation in all cases, but the effect was notably not the same with all AcXEs.

ScCE1 in the absence of TrCE16 enzyme deacetylated neutral xylooligosaccharides mainly to free and monoacetylated oligosaccharides, and acidic xylooligosaccharides to mono and di-*O*-acetates. The combination of ScCE1 and TrCE16 resulted in complete deacetylation of neutral oligosaccharides and conversion of all acetylated aldouronic acids to a mixture of free and mono-*O*-acetates (Fig. 1 and Table 1). The ion of a di-*O*-acetate was observed only in the case of aldopentaouronic acid. Thus, TrCE16 removes all remaining acetyl groups from ScCE1 treated xylooligosaccharides, except for one acetyl group on MeGlcA substituted oligomers.

The combination of TrCE16 with TrCE5 had similar effects. While neutral xylooligosaccharides were completely deacetylated (Table 1), the aldouronic acids were not (Table 1), but the ratio of fully deacetylated to monoacetylated aldouronic acids was considerably increased by the presence of TrCE16 (not shown). The contamination of TrCE5 AcXE preparation with an endoxylanase as observed previously [6], led to production of shorter oligosaccharides. This observation underlines the synergism between the action of endoxylanases and AcXEs [19].

As expected on the basis of slightly more limited deacetylating abilities of CE4 AcXEs (i.e. no deacetylation of doubly acetylated Xylp) the treatment with CE4 AcXEs alone left more acetyl groups on the products than treatment with CE1 or CE5 AcXEs. Remarkably, co-incubation of SICE4 with TrCE16 led to the removal of all acetyl groups, except for one on the aldouronic acids (Table 2). The acidic oligosaccharides persisting almost equally in the form of mono- and tri-*O*-acetyl derivatives in the mixture obtained upon incubation with SICE4 alone were converted to a mixture of deacetylated and mono-*O*-acetylated derivatives by the combined actions of SICE4 and TrCE16.

The effect of TrCE16 was also examined in combination with another enzyme of the CE4 family, CtCE4. The acetylated neutral oligosaccharides, persisting in the reaction mixture with CtCE4 alone, were converted to free oligosaccharides and their di-*O*-acetates by TrCE16 (Table 2). The aldouronic acids resistant to further deacetylation with CtCE4 (mostly mono-, di- and tri-*O*-acetates), were converted to a mixture of free, mono- and di-*O*-acetylated derivatives in the presence of TrCE16. These results correspond again to a removal of one acetyl group by TrCE16. The persistence of doubly acetylated oligosaccharides corresponds to the notion that neither CtCE4 nor TrCE16 act on internal doubly acetylated Xylp residues.

3.3. Effect on TrCE16 on acetylated hexose-containing xylooligosaccharides treated with AcXEs

The aspen acetyl glucuronoxylan hydrolysate by CtGH10 xylanase treated with ScCE1, TrCE5, SICE4 and CtCE4 contained ions of sodium adducts of a series of monoacetylated xylooligosaccharides containing one hexose residue (Fig. 1, Table 3). Interestingly, the corresponding ions of the same oligosaccharides acetylated to a higher degree, i.e. potential precursors of the products generated by the AcXEs, were not observed among the products generated by the GH10 xylanase (Fig. 1A). Thus, we do not know how these oligosaccharides were formed. The presence of TrCE16 led to complete deacetylation of the hexose-containing oligosaccharides (Table 3).

3.4. Effects of TrCE16 on deacetylation of xylooligosaccharides in the presence of active CtGH10 xylanase

The products generated after 48 h treatment of acetyl glucuronoxylan with a combination of CtGH10 endoxylanase and two different AcXEs (ScCE1 and SICE4) either in the presence or absence of TrCE16 AcE are shown in Table 4. Under these conditions, the number of products is considerably reduced since deacetylated xylooligosaccharides serve as substrate for the active endoxylanase. The reaction with ScCE1 AcXE did not yield any acetylated neutral products even in the absence of TrCE16. The only neutral acetylated product persisting in the reaction mixture with SICE4 AcXE (not attacking doubly acetylated Xylp residues) was Xyl₃Ac₂. This compound was not present in the reaction mixture

Fig. 1. MALDI ToF MS analysis of oligosaccharides generated from aspen acetyl glucuronoxylan by various enzyme treatments. The upper panel shows the result of treatment with CtGH10 xylanase only. The other panels show product mixtures obtained after denaturation of the GH10 xylanase and subsequent treatment with: TrCE16 AcE, ScCE1 AcXE, ScCE1 AcXE and TrCE16 AcE, SICE4 AcXE and SICE4 AcXE and TrCE16 AcE. Different ions (sodium adducts) are marked by different colors. The disodium ions of aldouronic acids are marked by asterisks with the same color as the peak of their corresponding sodium adduct. UXyl_xAc_y, oligosaccharides containing an unknown component U, x xylose residues and y acetyl groups. Note that the removal of acetyl groups reduces solubility, which explains why longer oligomeric products are not observed in the analysis of samples with a high degree of deacetylation. Observed products are listed in Tables 1–3.

Table 1
MALDI ToF MS analysis of oligosaccharides generated from aspen acetyl glucuronoxylan by various enzyme treatments. The table shows sodium adducts of molecular ions of oligosaccharides in the product mixtures obtained after treatment with CrGH10 alone or after treatment with CrGH10 followed by denaturation of the endoxylanase (“den”) and subsequent treatment with family CE1 or CE5 esterases and/or TrCE16. Note that the TrCE5 AcXE preparation was contaminated by an endoxylanase which hydrolyzed deacetylated xylooligosaccharides to shorter products. Also note that the removal of acetyl groups reduces solubility, which explains why longer oligomeric products are not observed in the analysis of samples with a high degree of deacetylation. The mass spectra for the experiments with the GH10 alone and for the reactions with ScCE1 are shown in Fig. 1 and the data in columns GH10, GH10den + ScCE1, GH10den + TrCE5 were also presented in our previous paper [6]. Xylooligosaccharides containing one hexose are presented in a separate table, Table 3. Completely deacetylated products are shown in bold letters.

Neutral products	GH10	GH10den + TrCE16	GH10den + ScCE1	GH10den + TrCE16	GH10den + TrCE5	GH10den + TrCE5 + TrCE16	Acidic products	GH10	GH10den + TrCE16	GH10den + ScCE1	GH10den + TrCE16	GH10den + TrCE5	GH10den + TrCE5 + TrCE16
Xyl₂	305	305	305	305	305	305	MeGlcAXyl₂		495	495	495	495	495
Xyl ₂ Ac	347	347	347		347		MeGlcAXyl ₂ Ac	537				537	
Xyl ₂ Ac ₂	389		389				MeGlcAXyl ₂ Ac ₂						
Xyl₃		437		437	437	437	MeGlcAXyl₃		627	627	627	627	627
Xyl ₃ Ac	479	479	479		479		MeGlcAXyl ₃ Ac	669	669	669	669	669	669
Xyl ₃ Ac ₂	521	521	521				MeGlcAXyl ₃ Ac ₂	711	711	711			
Xyl ₃ Ac ₃	563						MeGlcAXyl ₃ Ac ₃	753					
Xyl₄			569	569	569	569	MeGlcAXyl₄			759	759	759	759
Xyl ₄ Ac		611	611		611		MeGlcAXyl ₄ Ac	801	801	801	801	801	801
Xyl ₄ Ac ₂	653	653	653				MeGlcAXyl ₄ Ac ₂	843	843	843	843		
Xyl ₄ Ac ₃	695	695					MeGlcAXyl ₄ Ac ₃	885	885				
Xyl ₄ Ac ₄	737						MeGlcAXyl ₄ Ac ₄	927					
Xyl₅			701	701	701		MeGlcAXyl₅			891	891	891	891
Xyl ₅ Ac		743	743				MeGlcAXyl ₅ Ac		933	933	933	933	933
Xyl ₅ Ac ₂	785	785					MeGlcAXyl ₅ Ac ₂	975	975	975			
Xyl ₅ Ac ₃	827	827					MeGlcAXyl ₅ Ac ₃	1017	1017				
Xyl ₅ Ac ₄	869	869					MeGlcAXyl ₅ Ac ₄	1059	1059				
Xyl ₅ Ac ₅	911						MeGlcAXyl ₅ Ac ₅	1101	1101				
Xyl₆			833	833			MeGlcAXyl₆				1023		
Xyl ₆ Ac							MeGlcAXyl ₆ Ac			1065	1065	1065	
Xyl ₆ Ac ₂		917					MeGlcAXyl ₆ Ac ₂	1107	1107				
Xyl ₆ Ac ₃	959	959					MeGlcAXyl ₆ Ac ₃	1149	1149				
Xyl ₆ Ac ₄	1001	1001					MeGlcAXyl ₆ Ac ₄	1191	1191				
Xyl ₆ Ac ₅	1043	1043					MeGlcAXyl ₆ Ac ₅	1233	1233				
Xyl ₆ Ac ₆	1085						MeGlcAXyl ₆ Ac ₆	1275					
Xyl₇			965	965			MeGlcAXyl₇			1155			
Xyl ₇ Ac ₃	1091						MeGlcAXyl ₇ Ac			1197	1197		
Xyl ₇ Ac ₄	1133						MeGlcAXyl ₇ Ac ₂			1239			
Xyl ₇ Ac ₅	1175	1133					MeGlcAXyl ₇ Ac ₃		1281				
Xyl ₇ Ac ₆	1217	1175					MeGlcAXyl ₇ Ac ₄	1323	1323				
Xyl ₇ Ac ₇	1259						MeGlcAXyl ₇ Ac ₅	1365	1365				
							MeGlcAXyl ₇ Ac ₆	1407	1407				
							MeGlcAXyl₈						
							MeGlcAXyl ₈ Ac			1329			

Table 2
MALDI ToF MS analysis of oligosaccharides generated from aspen acetyl glucuronoxylan by various enzyme treatments. The table shows sodium adducts of molecular ions of oligosaccharides in the product mixtures obtained after treatment with C₁GH10 alone or after treatment with C₁GH10 followed by denaturation of the endoxylanase (“den”) and subsequent treatment with family CE4 esterases and/or TrCE16. Note that the removal of acetyl groups reduces solubility, which explains why longer oligosaccharide products are not observed in the samples with a high degree of deacetylation. The mass spectra for the experiments with the GH10 alone and for the reactions with SICE4 are shown in Fig. 1 and the data in columns GH10, GH10den + SICE4, GH10den + C₁CE4 were also presented in our previous paper [6]. Xylooligosaccharides containing one hexose are presented in a separate table, Table 3. Completely deacetylated products are shown in bold letters.

Neutral products	GH10	GH10den + TrCE16	GH10den + SICE4	GH10den + SICE4 + TrCE16	GH10den + C ₁ CE4	GH10den + C ₁ CE4 + TrCE16	Acidic products	GH10	GH10den + TrCE16	GH10den + SICE4	GH10den + SICE4 + TrCE16	GH10den + C ₁ CE4	GH10den + C ₁ CE4 + TrCE16
Xyl₂	305	305	305	305	305	305	MeGlcAXyl₂		495		495		495
Xyl ₂ Ac	347	347	347		347	347	MeGlcAXyl ₂ Ac	537					
Xyl ₂ Ac ₂	389						MeGlcAXyl ₂ Ac ₂						
Xyl₃		437	437	437	437	437	MeGlcAXyl₃		627		627	627	627
Xyl ₃ Ac	479	479	479		479	479	MeGlcAXyl ₃ Ac	669	669	669		669	669
Xyl ₃ Ac ₂	521	521	521		521	521	MeGlcAXyl ₃ Ac ₂	711	711			711	
Xyl ₃ Ac ₃	563						MeGlcAXyl ₃ Ac ₃	753		753		753	
Xyl₄			569	569	569	569	MeGlcAXyl₄				759	759	759
Xyl ₄ Ac		611	611		611	611	MeGlcAXyl ₄ Ac	801	801	801	801	801	801
Xyl ₄ Ac ₂	653	653	653		653	653	MeGlcAXyl ₄ Ac ₂	843	843			843	843
Xyl ₄ Ac ₃	695	695					MeGlcAXyl ₄ Ac ₃	885	885	885		885	
Xyl ₄ Ac ₄	737						MeGlcAXyl ₄ Ac ₄	927					
Xyl₅			701	701	701	701	MeGlcAXyl₅				891		891
Xyl ₅ Ac		743	743		743	743	MeGlcAXyl ₅ Ac		933	933	933	933	933
Xyl ₅ Ac ₂	785	785	785		785	785	MeGlcAXyl ₅ Ac ₂	975	975			975	975
Xyl ₅ Ac ₃	827	827			827		MeGlcAXyl ₅ Ac ₃	1017	1017	1017		1017	
Xyl ₅ Ac ₄	869	869					MeGlcAXyl ₅ Ac ₄	1059	1059				
Xyl ₅ Ac ₅	911						MeGlcAXyl ₅ Ac ₅	1101	1101				
Xyl₆			833	833	833	833	MeGlcAXyl₆				1023		1023
Xyl ₆ Ac			875		875		MeGlcAXyl ₆ Ac			1065	1065	1065	1065
Xyl ₆ Ac ₂		917	917		917	917	MeGlcAXyl ₆ Ac ₂		1107			1107	1107
Xyl ₆ Ac ₃	959	959			959		MeGlcAXyl ₆ Ac ₃	1149	1149	1149		1149	
Xyl ₆ Ac ₄	1001	1001			1001		MeGlcAXyl ₆ Ac ₄	1191	1191				
Xyl ₆ Ac ₅	1043	1043					MeGlcAXyl ₆ Ac ₅	1233	1233				
Xyl ₆ Ac ₆	1085						MeGlcAXyl ₆ Ac ₆	1275					
Xyl₇			(965)	965	965	965	MeGlcAXyl₇				1155		1155
Xyl ₇ Ac ₂						1049	MeGlcAXyl ₇ Ac			1197		1197	1197
Xyl ₇ Ac ₃	1091						MeGlcAXyl ₇ Ac ₂					1239	1239
Xyl ₇ Ac ₄	1133	1133					MeGlcAXyl ₇ Ac ₃		1281	1281		1281	
Xyl ₇ Ac ₅	1175	1175					MeGlcAXyl ₇ Ac ₄	1323	1323				
Xyl ₇ Ac ₆	1217						MeGlcAXyl ₇ Ac ₅	1365	1365				
							MeGlcAXyl ₇ Ac ₆	1407	1407				
Xyl₈				1097			MeGlcAXyl₈						
							MeGlcAXyl ₈ Ac			1329		1329	

Table 3

Sodium adducts of molecular ions of monoacetylated hexose-containing xylooligosaccharides appearing in the hydrolysate of aspen acetyl glucuronoxylan by CrGH10 after denaturation of the endoxylanase ("den") and subsequent treatment with three AcXEs, ScCE1, SICE4 and TrCE5, in the presence or absence of TrCE16 AcE. Note that that the GH10 hydrolysate (Fig. 1, upper panel) did not show any higher deacetylated forms of these hexose containing products; see text.

Neutral products	GH10den + ScCE1	GH10den + ScCE1 + TrCE16	GH10den + TrCE5	GH10den + TrCE5 + TrCE16	GH10den + SICE4	GH10den + SICE4 + TrCE16
HexXyl ₂						466
HexXyl ₂ Ac	508				508	
HexXyl ₃		599		599		599
HexXyl ₃ Ac	641		641		641	
HexXyl ₄		730		730		730
HexXyl ₄ Ac	772	772	772		772	
HexXyl ₅		862		862		862
HexXyl ₅ Ac	904	904	904		904	
HexXyl ₆		994				994
HexXyl ₆ Ac	1036		1036		1036	
HexXyl ₇					1168 ^a	1126
HexXyl ₇ Ac						
HexXyl ₈						
HexXyl ₈ Ac					1300	

^a Not marked in Fig. 1 panel CrGH10 + SICE4AcXE due to space limitations, but is immediately to the left of the MeGlcAXyl₆Ac₄ peak.

containing the TrCE16 enzyme in addition. Aldouronic acids mainly appeared as mono-, di- and tri-*O*-acetylated derivatives in both reactions with AcXEs. In the presence of TrCE16, aldouronic acids appeared mainly in deacetylated form, while their mono-, di- and tri-*O*-acetylated derivatives either disappeared or their proportion was considerably reduced in favor of the deacetylated products (Table 4).

3.5. Action of TrCE16 on artificial substrates

The recombinant TrCE16 used in this work is known to deacetylate monoacetylated derivatives of 4-nitrophenyl β-D-xylopyranoside [17]. The enzyme showed the highest activity towards the 3- and 4-*O*-acetyl derivatives, whereas the activity towards the 2-*O*-acetyl derivative was lower by almost two orders of magnitude. Here, we have analyzed the activity of TrCE16 on diacetates and triacetate of MeXylp at 10 mM substrate concentration. The reaction was followed by TLC and the rate of deacetylation was roughly estimated by visual inspection of the plates (Fig. 2). The results show that the 2,3-diacetate is clearly the best substrate. The 2,4-diacetate was also deacetylated, but at a much slower rate, estimated to be less than 10% of the rate seen for the 2,3-diacetate. In both cases, large amounts of mono-acetylated intermediates accumulate, which, considering the known positional

specificity of TrCE16, must be the 2-*O*-acetate. The 3,4-diacetate was deacetylated extremely slowly, and directly converted to deacetylated glycoside without significant accumulation of an intermediate monoacetate. The MeXylp triacetate was almost resistant to deacetylation. Both 3,4-di-*O*- and 2,3,4-tri-*O*-acetyl MeXylp persisted in the reaction mixtures even after 20 h (not shown). 2,3- and 2,4-di-*O*-acetate and the product of their first deacetylation, the 2-*O*-monoacetate, was converted completely to free MeXylp after 20 h (not shown). Taking the previous and present data together, the results suggest that the enzyme deacetylates positions 3 and 4 both in monoacetylated MeXylp and in 2,3- and 2,4-di-*O*-acetylated MeXylp. However, position 3 is deacetylated much faster than position 4.

The resistance of position 2 towards deacetylation by TrCE16 AcE was further confirmed by an experiment in which the monoacetate formed from 2,3-di-*O*-acetate (2-*O*-acetate), a poor substrate of the enzyme, was subjected to treatment with the *Orpinomyces* CE6 AcXE that favors deacetylation at position 2 [4]. And vice versa, the 3-*O*-acetate formed transitionally by OCE6 from 2,3-di-*O*-acetate [4], was treated with TrCE16 esterase. Both monoacetates served as outstanding substrates of the esterases and were rapidly converted to MeXylp. These results provide another strong evidence that TrCE16 deacetylates position 3 in 2,3-di-*O*-acetate and position 4 in 2,4-di-*O*-acetate and allow to

Table 4

Sodium adducts of molecular ions of oligosaccharides generated from aspen acetyl glucuronoxylan by CrGH10 endoxylanase in combination with ScCE1 or SICE4 AcXE in the presence or absence of TrCE16 AcE. CrGH10 xylanase was not denatured (nonden). Completely deacetylated products are shown in bold letters. The numbers in parentheses indicate relative intensities of signals in each series of aldouronic acids, where the signal for the fully deacetylated species has been set to 1.

Oligosaccharide	GH10 (nonden.) + ScCE1	GH10 (nonden.) + ScCE1 + TrCE16	GH10 (nonden.) + SICE4	GH10 (nonden.) + SICE4 + TrCE16
Xyl₂	305	305	305	305
Xyl₃	437	437	437	437
Xyl ₃ Ac ₂			521	
Xyl₄	569	569	569	569
Xyl₅			701	701
MeGlcAXyl₂		495		495
MeGlcAXyl₃	627 (1)	627 (1)	627 (1)	627 (1)
MeGlcAXyl ₃ Ac	669 (12.5)	669 (0.4)	669 (2.6)	669 (0.09)
MeGlcAXyl ₃ Ac ₂	711 (1)			
MeGlcAXyl₄	759 (1)	759 (1)	759 (1)	759 (1)
MeGlcAXyl ₄ Ac	801 (4.6)	801 (0.25)	801 (2)	801 (0.25)
MeGlcAXyl ₄ Ac ₂	843 (0.3)	843 (0.2)		
MeGlcAXyl₅		891 (1)		
MeGlcAXyl ₅ Ac	933	933 (1.5)		
MeGlcAXyl ₅ Ac ₂	975		975	
MeGlcAXyl ₅ Ac ₃			1017	
MeGlcAXyl ₆ Ac ₃			1149	

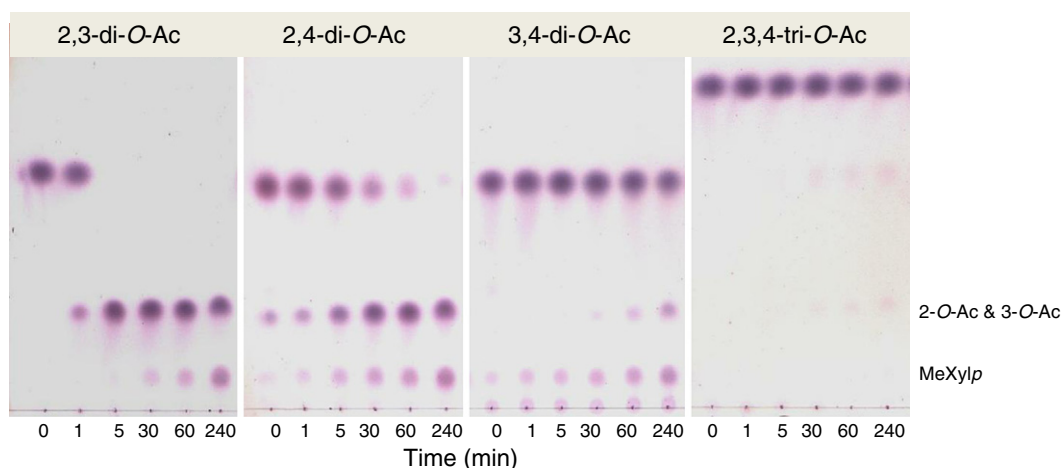


Fig. 2. TLC analyses of the products of deacetylation of MeXylp diacetates and MeXylp triacetate with *Trichoderma reesei* AcE (TrCE16). The time of incubation is indicated.

compare the varying activities of TrCE16 and OCE6 in deacetylating mono- and 2,3-di-O-acetylated non-reducing end Xylp residues (Fig. 3).

4. Discussion

Using a variety of natural substrates and enzymatic set-ups, we have confirmed the proposed exo-deacetylating ability of the TrCE16 AcE [19]. We show that the application of this enzyme in combination with other deacetylases leads to a higher degree of overall deacetylation of xylooligosaccharides. With a few exceptions, all data indicate that the enzyme removes only one acetyl group from both neutral and acidic xylooligosaccharides which is compatible with deacetylation of Xylp residues at the non-reducing ends. Removal of two acetyl groups might be a result of double acetylation of the non-reducing end sugar

residues. Deacetylation of branched oligosaccharides acetylated at two different non-reducing sugar residues also cannot be excluded.

We should emphasize that the xylanase denaturation step prior to addition of esterases accelerates acetyl group migration to such a degree that acetylated non-reducing end sugars will be close to or already in a thermodynamic equilibrium with all possible positional isomers (unpublished observations), that is 2-O-, 3-O- and 4-O-monoacetyl derivatives, and 2,3-, 2,4- and 3,4-diacetates. 4-Acetate predominates among monoacetates and 3,4-diacetate in the mixtures of diacetates [10]. This has to be taken into consideration whenever discussing the mode of action of ACXEs and AcE. The interpretation of the results obtained on oligosaccharides is facilitated by the activity of TrCE16 AcE on synthetic diacetates and enzyme generated monoacetates of MeXylp. The best substrate of the multiply acetylated substrates appeared to be the 3-O-acetyl group of the 2,3-di-O-acetylated Xylp

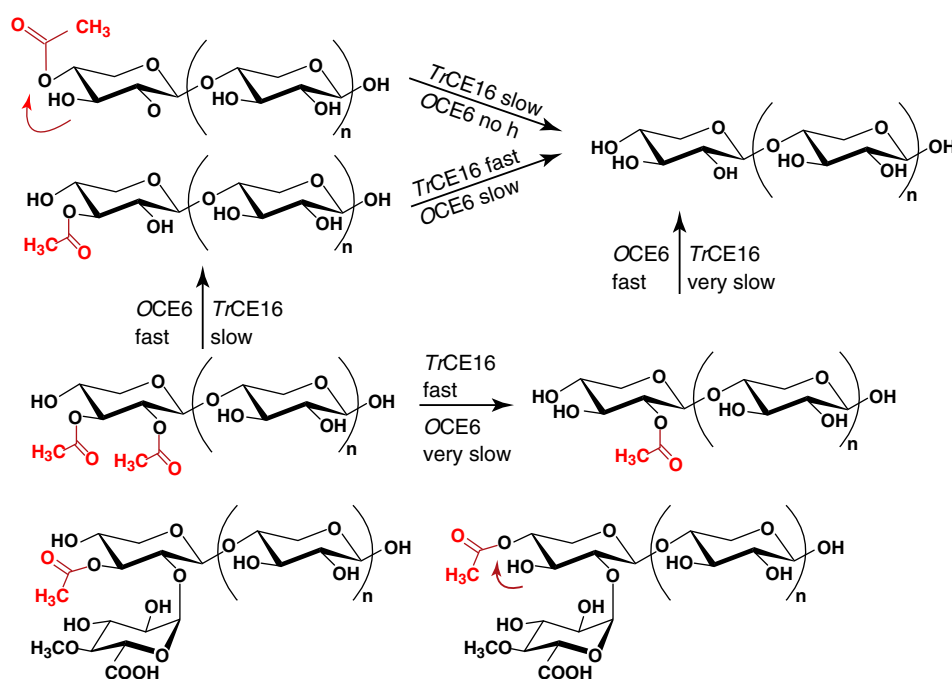


Fig. 3. Deacetylations of non-reducing end Xylp residues in neutral oligosaccharides catalyzed by TrCE16 and OCE6 suggested on the basis of the mode of action of both enzymes on acetylated derivatives of MeXylp, acetylated xylooligosaccharides and acetyl glucuronoxylan. The abbreviations “fast”, “slow”, “very slow” mark the relative rates of substrate hydrolysis. The abbreviation “no h” means “no hydrolysis”. The formulas on the bottom show two acetylated aldouronic acids as additional substrates of TrCE16 acetyl esterase. The aldouronic acids are deacetylated by TrCE16, however, we do not know whether from both positions (see text). Arrows indicate acetyl group migration to position 4.

residue. Thus, the presence of the 2-*O*-acetyl group does not seem to hinder deacetylation of the 3 position. However, the 2-*O*-acetate resulting from the 3-*O*-deacetylation of 2,3-diacetate serves as a poor substrate which is in accordance with the positional specificity of the enzyme established by studying activity on monoacetates of 4-nitrophenyl xylopyranoside [17]. In the absence of AcXEs, any native terminally 2-*O*-acetylated substrate would be more rapidly deacetylated by TrCE16 AcE after migration of the 2-*O*-acetyl group to a different position. TrCE16 deacetylates the 4-*O*-acetyl group in 2,4-di-*O*-MeXylp much slower than the 3-*O*-acetyl group in the 2,3-diacetate, but much faster than any of the acetyl groups in 3,4-di-*O*-Ac-MeXylp or 2,3,4-tri-*O*-Ac-MeXylp. Hence, deacetylation of position 3 or 4 is much faster when the hydroxyl group at position 4 or position 3, respectively, is not esterified. Hence, one can conclude that a favored substrates of TrCE16 AcE are 3-*O*-acetylated and doubly 2,3-di-*O*-acetylated Xylp residue at the non-reducing end of xylooligosaccharides from which the 3-*O*-acetyl group is easily removed. Such xylooligosaccharides will be formed upon the action of GH10 xylanase on acetyl glucuronoxylan [6,23,24], and they will survive to different extents in the reaction mixtures incubated sequentially with GH10 xylanase and AcXEs. The doubly acetylated Xylp residue at non-reducing ends should be rapidly deacetylated by the serine-type esterases (families CE1, CE5) [6] and may become a target of CE4 AcXEs as soon as the 3-*O*-acetyl group moves to position 4. The CE4 AcXEs do not recognize the internal 2,3-di-*O*-acetylated Xylp residues as substrates. The other targets of TrCE16 AcE will of course be 3-*O*- and 4-*O*-monoacetylated Xylp residues at the non-reducing end, where the 4-acetate is the result of acetyl group migration.

The above considerations do not allow us to draw conclusions on the behavior of TrCE16 AcE on 3-*O*-acetylated aldouronic acids, since the large MeGlcA group could interfere with AcXE and AcE activity in a more pronounced and different manner than 2-*O*-acetylation. The xylanase is likely to mainly produce aldouronic acids with the MeGlcA substitution of the non-reducing sugar [6,23,24] and the observation that TrCE16 removes an acetate from such sugars thus suggests that the enzyme can act on MeGlcA substituted Xylp. Notably, this activity may involve preceding migration of the 3-*O*-acetyl group to the 4 position. Fig. 3 provides a graphical summary of the activities of TrCE16 AcE that can be derived from combining previous and present results. Further insight into this matter awaits the preparation of model compounds that would be used not only as substrates for CE16 enzymes but also as model compounds to examine acetyl group migration to position 4 in MeGlcA-substituted non-reducing end Xylp residues.

An important information regarding the physiological function of TrCE16 AcE is the finding that the enzyme deacetylated all monoacetylated hexose-containing oligosaccharides. The pentose in these oligosaccharides is considered to be xylose since the monosaccharide analysis of the extracted acetyl glucuronoxylan used as a substrate only showed traces of arabinose (results not shown). Nevertheless, the chemical nature of these oligosaccharides remains to be confirmed. Deacetylation of these oligosaccharides by TrCE16 AcE serves as evidence that they are esterified at the non-reducing end, and most probably at positions 3 or 4. At this stage of knowledge it remains unknown whether the non-reducing end sugar is hexose or xylose. Deacetylation of a hexosyl residue at the non-reducing end by TrCE16 should not be a problem because the enzyme has been shown to deacetylate glucose penta-*O*-acetate [13] and also the non-reducing end hexopyranosyl residues in oligosaccharides [11]. The strongest argument to believe that hexose is 3- or 4-*O*-acetylated on the non-reducing end is the capability of the TrCE16 AcE to catalyze acetyl transfer to position 3 of various hexosides [18] and to the non-reducing end of hexooligosaccharides, such as cello- and manooligosaccharides [11]. However, it was also shown that the reducing end glucose residue of cellooligosaccharides served as an

acetyl group acceptor [11]. However, the reducing end acetylation was very slow and could be completely eliminated by converting the oligosaccharides to methyl glycosides [11]. As mentioned earlier [6], the structural requirements of GH10 xylanase substrate binding sites should not allow cleavage of the main acetylxylan chain to generate fragments acetylated at the reducing end [23,24]. Finally, we should emphasize that in nature, when all glycoside hydrolases and esterases are available for a simultaneous action on acetylated hemicelluloses, the migration of the acetyl group to position 4 may not be so significant. The acetyl groups at positions 2 or 3 on non-reducing Xylp residues are probably removed by esterases before migrating to position 4.

5. Conclusions

TrCE16 AcE is secreted by a biotechnologically highly important fungus relevant to plant biomass degradation. At the same time, the enzyme remains somewhat enigmatic, partly because there is no structural information for this CE family [5]. Using both natural and artificial substrates, we have shown that TrCE16 has an exo-deacetylating ability and that it collaborates with AcXEs to obtain complete or almost complete deacetylation of neutral and MeGlcA-substituted xylooligosaccharides. Although the examination of the activity of TrCE16 on diacetates and monoacetates of MeXylp facilitated interpretation of the results obtained with xylooligosaccharides, certain aspects of TrCE16 substrate specificity remain to be elucidated. This concerns the mode of action on the non-reducing ends of aldouronic acids, specifically the 3-*O*-deacetylation of Xylp residues substituted by MeGlcA. Available data show that an acetyl group at position 2 of the non-reducing end Xylp residue does not interfere with deacetylation of position 3, however, it remains to be established whether substitution of the 2 position with MeGlcA is equally well tolerated. This study shows that CE16 acetyl esterases similarly as α -glucuronidases are crucial enzymes facilitating deacetylation and consequently saccharification of acetylated xylans which is an important task in current biotechnology. An important aspect of this work is that the esterase deacetylating the non-reducing end Xylp residues carrying MeGlcA side substituent has been revealed. In the case that the AcXE that deesterifies these Xylp residues in the polymeric substrate exists awaits discovery.

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