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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 acetylxylan 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 *Tr*CE16 AcE. The most prominent target is the 3-*O*-acetyl group at the non-reducing terminal Xyl*p* 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 *Tr*CE16 esterase.
Conclusion: Concerted action of *Ct*GH10 xylanase, an AcXE and *Tr*CE16 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 *Tr*CE16 AcE is 3-*O*-acetylated Xyl*p* residue followed by 4-*O*-acetylated Xyl*p* 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.

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 acetylxylan esterases (AcXEs), whose role is to deacetylate xylopyranosyl (Xyl*p*) 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 Xyl*p* residues in the main chain: singly 2- or 3-*O*-acetylated Xyl*p* residues, doubly 2,3-di-*O*-acetylated Xyl*p* residues and 3-*O*-acetylated Xyl*p* 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. ^{1}H NMR was used to establish the positional specificity of the enzymes on

Abbreviations: AcE, acetyl esterase; AcXE, acetylxylan esterase; CE, carbohydrate esterase; Xyl*p*, D-xylopyranose or D-xylopyranosyl; MeXyl*p*, methyl β-D-xylopyranoside; Xyl_2–Xyl_7, β-1,4-xylobiose–β-1,4-xyloheptaose; MeGlcA, 4-*O*-methyl-D-glucuronic acid or 4-*O*-methyl-D-glucuronosyl; Xyl_xAc_y, acetylated β-1,4-xylooligosaccharide containing x xylose residues and y acetyl groups; $MeGlcAXyl_xAc_y$, acetylated aldouronic acid containing one MeGlcA, x xylose residues and y acetyl groups; $HexXyl_xAc_y$, 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 (*Ct*GH10) [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 Xyl*p* residues. Aspartate metalloenzymes, which are members of the CE4 family, deacetylated effectively only singly 2- or 3-*O*-acetylated Xyl*p* 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 Xyl*p* 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 Xyl*p* 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 Xyl*p* residues of xylooligosaccharides, both for non-substituted Xyl*p* or Xyl*p* 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 (*Tr*CE16) 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 *Tr*CE16 enzyme appears to be a good candidate for deacetylation of non-reducing end Xyl*p* 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 *Tr*CE16 on natural substrates and the interplay of this enzyme with various AcXEs. The exo-type action of *Tr*CE16 on the non-reducing end sugar residues was demonstrated on xylooligosaccharides generated from aspen acetyl glucuronoxylan by the *Ct*GH10 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 *Tr*CE16 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-Xyl*p* 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 Quimica Organica General, CSIC, Madrid, Spain). 2,3,4-Tri-*O*-acetate of MeXyl*p* was prepared by a standard acetylation procedure [20].

Endo-β-1,4-xylanase of GH10 family from *C. thermocellum* (*Ct*GH10) (xylanase catalytic module of Xyn Z) was a recombinant enzyme prepared as described previously [6]. A CE1 family AcXE from *Schizophyllum commune* (*Sc*CE1) was purified as described [20]. A CE4 family AcXE from *Streptomyces lividans* (*St*CE4, Ref. [21]) was supplied by Drs. Claude Dupont and Dieter Kluepfel (Institute of Armand Frappier, Laval, Canada). A similar enzyme from *C. thermocellum* (*Ct*CE4) [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* (*Tr*CE5) [14] was kindly provided by Prof. Maija Tenkanen (University of Helsinki, Finland). A family CE6 AcXE from *Orpinomyces* sp. (*O*CE6) was from Megazyme Int. (Ireland). The recombinant CE16 AcE from *T. reesei* (*Tr*CE16) was produced and purified as reported by Li et al. [16].

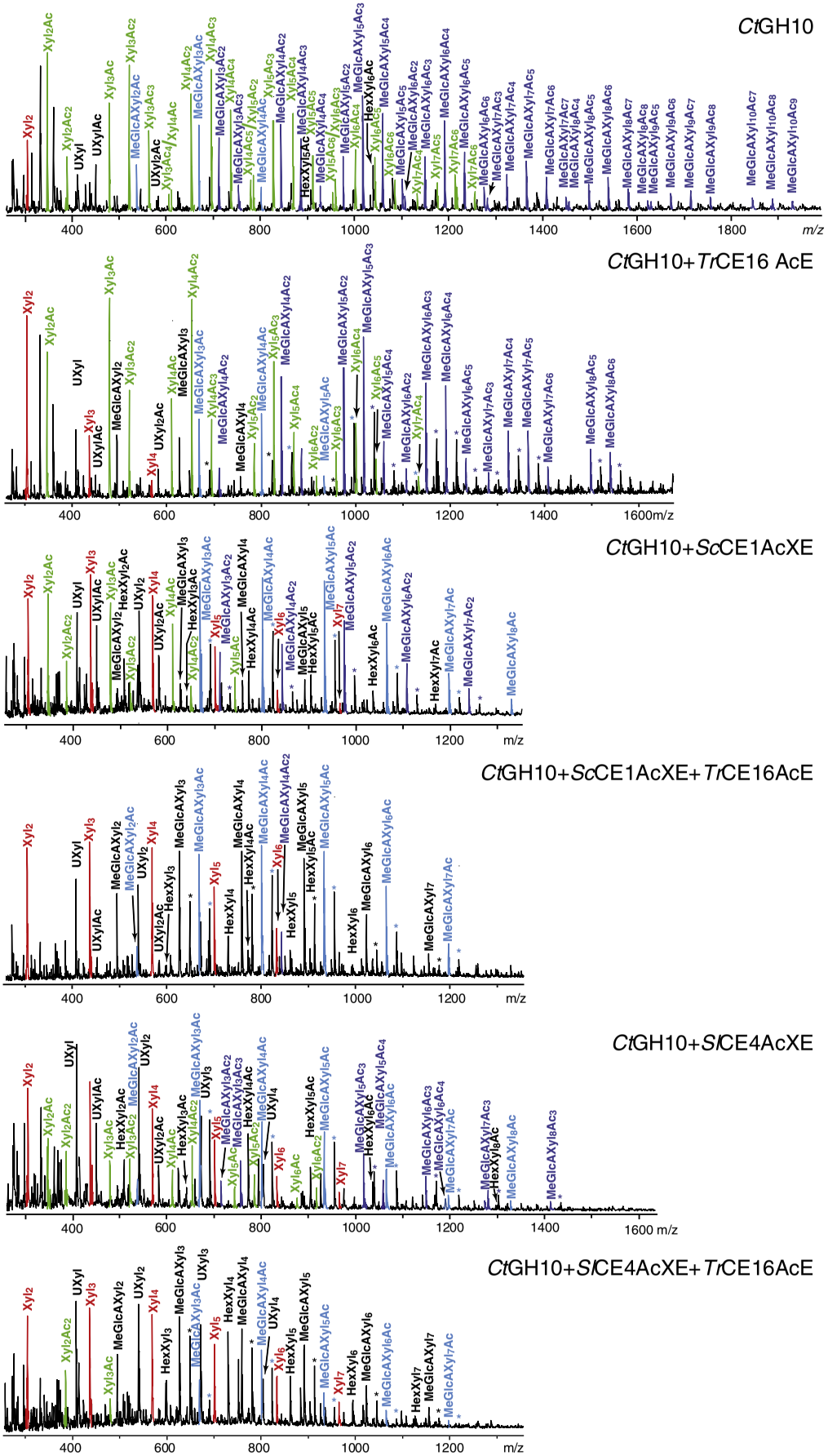
2.2. Hydrolysis of aspen acetyl glucuronoxylan with enzymes

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 (*Ct*GH10; 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 μl aliquots of the mixture with heat-inactivated xylanase were mixed with small volumes of solutions of AcXEs alone or in combination with *Tr*CE16 to give the final enzyme concentrations: *Sc*CE1 (0.13 mg/ml), *Sl*CE4 (0.045 mg/ml), *Ct*CE4 (0.4 mg/ml), *Tr*CE5 (0.13 mg/ml), *Tr*CE16 (0.022 mg/ml). All incubation mixtures containing a combination of an AcXE and *Tr*CE16 acetylesterase were run in parallel with the mixtures containing the *Ct*GH10 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 *Tr*CE16. The action of *Tr*CE16 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 *Ct*CE10 (without the denaturing step) in combination with *Sc*CE1 or *Sl*CE4 AcXE in the presence or absence of *Tr*CE16 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-Xyl*p* at 10 mM concentration in 0.05 M sodium phosphate buffer (pH 6.0) by *Tr*CE16 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].


CtGH10
CtGH10+TrCE16 AcE
CtGH10+ScCE1AcXE
CtGH10+ScCE1AcXE+TrCE16AcE
CtGH10+SlCE4AcXE
CtGH10+SlCE4AcXE+TrCE16AcE
m/z

3. Results

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

*Tr*CE16 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 *Ct*GH10 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 *Tr*CE16 [6]. The analysis of the product mixtures showed that the effect of *Tr*CE16 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 *Tr*CE16 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, *Tr*CE16 AcE appears to liberate only one acetyl group from most compounds (Table 1). The data for the reaction with the *Tr*CE16 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 Xyl_3 is deacetylated to the di-*O*-acetate, the di-*O*-acetate to the monoacetate and the monoacetate to free Xyl_3. Xyl_3 was not detected among the products released by the treatment with *Ct*GH10 endoxylanase alone.

A similar reduction in the number of acetyl groups upon treatment with *Tr*CE16 AcE also occurred for the aldouronic acids. Comparison of the mass spectra in Fig. 1 clearly shows that the treatment with *Tr*CE16 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 aldotetraouronic acid, monoacetates of aldohexaouronic acids, diacetate of aldoheptaouronic acids, etc.

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

The effect of *Tr*CE16 esterase was also tested on mixture of acetylated xylooligosaccharides in combination with four different AcXEs, *Sc*CE1, *Tr*CE5, *Sl*CE4 and *Ct*CE4 (see [6]), *Tr*CE16 contributed to a higher degree of deacetylation in all cases, but the effect was notably not the same with all AcXEs.

*Sc*CE1 in the absence of *Tr*CE16 enzyme deacetylated neutral xylooligosaccharides mainly to free and monoacetylated oligosaccharides, and acidic xylooligosaccharides to mono and di-*O*-acetates. The combination of *Sc*CE1 and *Tr*CE16 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, *Tr*CE16 removes all remaining acetyl groups from *Sc*CE1 treated xylooligosaccharides, except for one acetyl group on MeGlcA substituted oligomers.

The combination of *Tr*CE16 with *Tr*CE5 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 *Tr*CE16 (not shown). The contamination of *Tr*CE5 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 Xyl*p*) the treatment with CE4 AcXEs alone left more acetyl groups on the products than treatment with CE1 or CE5 AcXEs. Remarkably, co-incubation of *Sl*CE4 with *Tr*CE16 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 *Sl*CE4 alone were converted to a mixture of deacetylated and mono-*O*-acetylated derivatives by the combined actions of *Sl*CE4 and *Tr*CE16.

The effect of *Tr*CE16 was also examined in combination with another enzyme of the CE4 family, *Ct*CE4. The acetylated neutral oligosaccharides, persisting in the reaction mixture with *Ct*CE4 alone, were converted to free oligosaccharides and their di-*O*-acetates by *Tr*CE16 (Table 2). The aldouronic acids resistant to further deacetylation with *Ct*CE4 (mostly mono-, di- and tri-*O*-acetates), were converted to a mixture of free, mono- and di-*O*-acetylated derivatives in the presence of *Tr*CE16. These results correspond again to a removal of one acetyl group by *Tr*CE16. The persistence of doubly acetylated oligosaccharides corresponds to the notion that neither *Ct*CE4 nor *Tr*CE16 act on internal doubly acetylated Xyl*p* residues.

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

The aspen acetyl glucuronoxylan hydrolysate by *Ct*GH10 xylanase treated with *Sc*CE1, *Tr*CE5, *Sl*CE4 and *Ct*CE4 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 *Tr*CE16 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 *Ct*GH10 endoxylanase and two different AcXEs (*Sc*CE1 and *Sl*CE4) either in the presence or absence of *Tr*CE16 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 *Sc*CE1 AcXE did not yield any acetylated neutral products even in the absence of *Tr*CE16. The only neutral acetylated product persisting in the reaction mixture with *Sl*CE4 AcXE (not attacking doubly acetylated Xyl*p* residues) was Xyl_3Ac_2. 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 *Ct*GH10 xylanase only. The other panels show product mixtures obtained after denaturation of the GH10 xylanase and subsequent treatment with: *Tr*CE16 AcE, *Sc*CE1 AcXE, *Sc*CE1 AcXE and *Tr*CE16 AcE, *Sl*CE4 AcXE and *Sl*CE4 AcXE and *Tr*CE16 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 *Ct*GH10 alone or after treatment with *Ct*GH10 followed by denaturation of the endoxylanase ("den") and subsequent treatment with family CE1 or CE5 esterases and/or *Tr*CE16. Note that the *Tr*CE5 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 *Sc*CE1 are shown in Fig. 1 and the data in columns GH10, GH10den + *Sc*CE1, GH10den + *Tr*CE5 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 + *Tr*CE16	GH10den + *Sc*CE1	GH10den + *Sc*CE1 + *Tr*CE16	GH10den + *Tr*CE5	GH10den + *Tr*CE5 + *Tr*CE16	Acidic products	GH10	GH10den + *Tr*CE16	GH10den + *Sc*CE1	GH10den + *Sc*CE1 + *Tr*CE16	GH10den + *Tr*CE5	GH10den + *Tr*CE5 + *Tr*CE16
Xyl_2	305	305	305	305	305	305	**$MeGlcAXyl_2$**		495	495	495	495	495
Xyl_2Ac	347	347	347		347		$MeGlcAXyl_2Ac$	537				537	
Xyl_2Ac_2	389		389				$MeGlcAXyl_2Ac_2$						
Xyl_3		437	437	437	437	437	**$MeGlcAXyl_3$**		627	627	627	627	627
Xyl_3Ac	479	479	479		479		$MeGlcAXyl_3Ac$	669	669	669	669	669	669
Xyl_3Ac_2	521	521	521				$MeGlcAXyl_3Ac_2$	711	711	711			
Xyl_3Ac_3	563						$MeGlcAXyl_3Ac_3$	753					
Xyl_4			569	569	569	569	**$MeGlcAXyl_4$**			759	759	759	759
Xyl_4Ac		611	611		611		$MeGlcAXyl_4Ac$	801	801	801	801	801	801
Xyl_4Ac_2	653	653	653				$MeGlcAXyl_4Ac_2$	843	843	843	843		
Xyl_4Ac_3	695	695					$MeGlcAXyl_4Ac_3$	885	885				
Xyl_4Ac_4	737						$MeGlcAXyl_4Ac_4$	927					
Xyl_5			701	701	701		**$MeGlcAXyl_5$**			891	891	891	891
Xyl_5Ac		743	743				$MeGlcAXyl_5Ac$		933	933	933	933	933
Xyl_5Ac_2	785	785					$MeGlcAXyl_5Ac_2$	975	975	975			
Xyl_5Ac_3	827	827					$MeGlcAXyl_5Ac_3$	1017	1017				
Xyl_5Ac_4	869	869					$MeGlcAXyl_5Ac_4$	1059	1059				
Xyl_5Ac_5	911						$MeGlcAXyl_5Ac_5$	1101	1101				
Xyl_6			833	833			**$MeGlcAXyl_6$**				1023		
Xyl_6Ac							$MeGlcAXyl_6Ac$			1065	1065	1065	
Xyl_6Ac_2		917					$MeGlcAXyl_6Ac_2$		1107	1107			
Xyl_6Ac_3	959	959					$MeGlcAXyl_6Ac_3$	1149	1149				
Xyl_6Ac_4	1001	1001					$MeGlcAXyl_6Ac_4$	1191	1191				
Xyl_6Ac_5	1043	1043					$MeGlcAXyl_6Ac_5$	1233	1233				
Xyl_6Ac_6	1085						$MeGlcAXyl_6Ac_6$	1275					
Xyl_7			965	965			**$MeGlcAXyl_7$**				1155		
Xyl_7Ac_3	1091						$MeGlcAXyl_7Ac$			1197	1197		
Xyl_7Ac_4	1133						$MeGlcAXyl_7Ac_2$			1239			
Xyl_7Ac_5	1175	1133					$MeGlcAXyl_7Ac_3$		1281				
Xyl_7Ac_6	1217	1175					$MeGlcAXyl_7Ac_4$	1323	1323				
Xyl_7Ac_7	1259						$MeGlcAXyl_7Ac_5$	1365	1365				
							$MeGlcAXyl_7Ac_6$	1407	1407				
							$MeGlcAXyl_8$						
							$MeGlcAXyl_8Ac$			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 *Ct*GH10 alone or after treatment with *Ct*GH10 followed by denaturation of the endoxylanase ("den") and subsequent treatment with family CE4 esterases and/or *Tr*CE16. 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 *Sl*CE4 are shown in Fig. 1 and the data in columns GH10, GH10den + *Sl*CE4, GH10den + *Ct*CE4 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 + *Tr*CE16	GH10den + *Sl*CE4	GH10den + *Sl*CE4 + *Tr*CE16	GH10den + *Ct*CE4	GH10den + *Ct*CE4 + *Tr*CE16	Acidic products	GH10	GH10den + *Tr*CE16	GH10den + *Sl*CE4	GH10den + *Sl*CE4 + *Tr*CE16	GH10den + *Ct*CE4	GH10den + *Ct*CE4 + *Tr*CE16
Xyl_2	305	305	305	305	305	305	**$MeGlcAXyl_2$**		495		495		495
Xyl_2Ac	347	347	347		347	347	$MeGlcAXyl_2Ac$	537					
Xyl_2Ac_2	389						$MeGlcAXyl_2Ac_2$						
Xyl_3		437	437	437	437	437	**$MeGlcAXyl_3$**		627		627	627	627
Xyl_3Ac	479	479	479		479		$MeGlcAXyl_3Ac$	669	669	669		669	669
Xyl_3Ac_2	521	521	521		521	521	$MeGlcAXyl_3Ac_2$	711	711			711	
Xyl_3Ac_3	563						$MeGlcAXyl_3Ac_3$	753		753		753	
Xyl_4			569	569	**569**	569	**$MeGlcAXyl_4$**				759	759	759
Xyl_4Ac		611	611		611		$MeGlcAXyl_4Ac$	801	801	801	801	801	801
Xyl_4Ac_2	653	653	653		653	653	$MeGlcAXyl_4Ac_2$	843	843			843	843
Xyl_4Ac_3	695	695					$MeGlcAXyl_4Ac_3$	885	885	885		885	
Xyl_4Ac_4	737						$MeGlcAXyl_4Ac_4$	927					
Xyl_5			701	701	701	701	**$MeGlcAXyl_5$**				891		891
Xyl_5Ac		743	743		743		$MeGlcAXyl_5Ac$		933	933	933	933	933
Xyl_5Ac_2	785	785	785		785	785	$MeGlcAXyl_5Ac_2$	975	975			975	975
Xyl_5Ac_3	827	827			827		$MeGlcAXyl_5Ac_3$	1017	1017	1017		1017	
Xyl_5Ac_4	869	869					$MeGlcAXyl_5Ac_4$	1059	1059				
Xyl_5Ac_5	911						$MeGlcAXyl_5Ac_5$	1101	1101				
Xyl_6			833	833	833	833	**$MeGlcAXyl_6$**				1023		1023
Xyl_6Ac			875		875		$MeGlcAXyl_6Ac$			1065	1065	1065	1065
Xyl_6Ac_2		917	917		917	917	$MeGlcAXyl_6Ac_2$		1107			1107	1107
Xyl_6Ac_3	959	959			959		$MeGlcAXyl_6Ac_3$	1149	1149	1149		1149	
Xyl_6Ac_4	1001	1001			1001		$MeGlcAXyl_6Ac_4$	1191	1191				
Xyl_6Ac_5	1043	1043					$MeGlcAXyl_6Ac_5$	1233	1233				
Xyl_6Ac_6	1085						$MeGlcAXyl_6Ac_6$	1275					
Xyl_7			(965)	965	965	965	**$MeGlcAXyl_7$**				1155		1155
Xyl_7Ac_2						1049	$MeGlcAXyl_7Ac$			1197		1197	1197
Xyl_7Ac_3	1091						$MeGlcAXyl_7Ac_2$					1239	1239
Xyl_7Ac_4	1133	1133					$MeGlcAXyl_7Ac_3$		1281	1281		1281	
Xyl_7Ac_5	1175	1175					$MeGlcAXyl_7Ac_4$	1323	1323				
Xyl_7Ac_6	1217						$MeGlcAXyl_7Ac_5$	1365	1365				
							$MeGlcAXyl_7Ac_6$	1407	1407				
Xyl_8				1097			**$MeGlcAXyl_8$**						
							$MeGlcAXyl_8Ac$			1329		1329	

Table 3
Sodium adducts of molecular ions of monoacetylated hexose-containing xylooligosaccharides appearing in the hydrolysate of aspen acetyl glucuronoxylan by *Ct*GH10 after denaturation of the endoxylanase ("den") and subsequent treatment with three AcXEs, *Sc*CE1, *Sl*CE4 and *Tr*CE5, in the presence or absence of *Tr*CE16 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 + *Sc*CE1	GH10den + *Sc*CE1 + *Tr*CE16	GH10den + *Tr*CE5	GH10den + *Tr*CE5 + *Tr*CE16	GH10den + *Sl*CE4	GH10den + *Sl*CE4 + *Tr*CE16
$HexXyl_2$						466
$HexXyl_2Ac$	508				508	
$HexXyl_3$		599		599		599
$HexXyl_3Ac$	641		641		641	
$HexXyl_4$		730		730		730
$HexXyl_4Ac$	772	772	772		772	
$HexXyl_5$		862		862		862
$HexXyl_5Ac$	904	904	904		904	
$HexXyl_6$		994				994
$HexXyl_6Ac$	1036		1036		1036	
$HexXyl_7$						1126
$HexXyl_7Ac$					1168[a]	
$HexXyl_8$						
$HexXyl_8Ac$					1300	

[a] Not marked in Fig. 1 panel *Ct*GH10 + *Sl*CE4AcXE due to space limitations, but is immediately to the left of the $MeGlcAXyl_6Ac_4$ peak.

containing the *Tr*CE16 enzyme in addition. Aldouronic acids mainly appeared as mono-, di- and tri-*O*-acetylated derivatives in both reactions with AcXEs. In the presence of *Tr*CE16, 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 *Tr*CE16 on artificial substrates

The recombinant *Tr*CE16 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 *Tr*CE16 on diacetates and triacetate of MeXyl*p* 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 *Tr*CE16, 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 MeXyl*p* triacetate was almost resistant to deacetylation. Both 3,4-di-*O*- and 2,3,4-tri-*O*-acetyl MeXyl*p* 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 MeXyl*p* 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 MeXyl*p* and in 2,3- and 2,4-di-*O*-acetylated MeXyl*p*. However, position 3 is deacetylated much faster than position 4.

The resistance of position 2 towards deacetylation by *Tr*CE16 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 *O*CE6 from 2,3-di-*O*-acetate [4], was treated with *Tr*CE16 esterase. Both monoacetates served as outstanding substrates of the esterases and were rapidly converted to MeXyl*p*. These results provide another strong evidence that *Tr*CE16 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 *Ct*GH10 endoxylanase in combination with *Sc*CE1 or *Sl*CE4 AcXE in the presence or absence of *Tr*CE16 AcE. *Ct*GH10 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.) + *Sc*CE1	GH10 (nonden.) + *Sc*CE1 + *Tr*CE16	GH10 (nonden.) + *Sl*CE4	GH10 (nonden.) + *Sl*CE4 + *Tr*CE16
Xyl_2	**305**	**305**	**305**	**305**
Xyl_3	**437**	**437**	**437**	**437**
Xyl_3Ac_2			521	
Xyl_4	**569**	**569**	**569**	**569**
Xyl_5			**701**	**701**
$MeGlcAXyl_2$		**495**		**495**
$MeGlcAXyl_3$	**627 (1)**	**627 (1)**	**627 (1)**	**627 (1)**
$MeGlcAXyl_3Ac$	669 (12.5)	669 (0.4)	669 (2.6)	669 (0.09)
$MeGlcAXyl_3Ac_2$	711 (1)			
$MeGlcAXyl_4$	**759 (1)**	**759 (1)**	**759 (1)**	**759 (1)**
$MeGlcAXyl_4Ac$	801(4.6)	801 (0.25)	801 (2)	801 (0.25)
$MeGlcAXyl_4Ac_2$	843 (0.3)	843 (0.2)		
$MeGlcAXyl_5$		**891 (1)**		
$MeGlcAXyl_5Ac$	933	933 (1.5)		
$MeGlcAXyl_5Ac_2$	975		975	
$MeGlcAXyl_5Ac_3$			1017	
$MeGlcAXyl_6Ac_3$			1149	

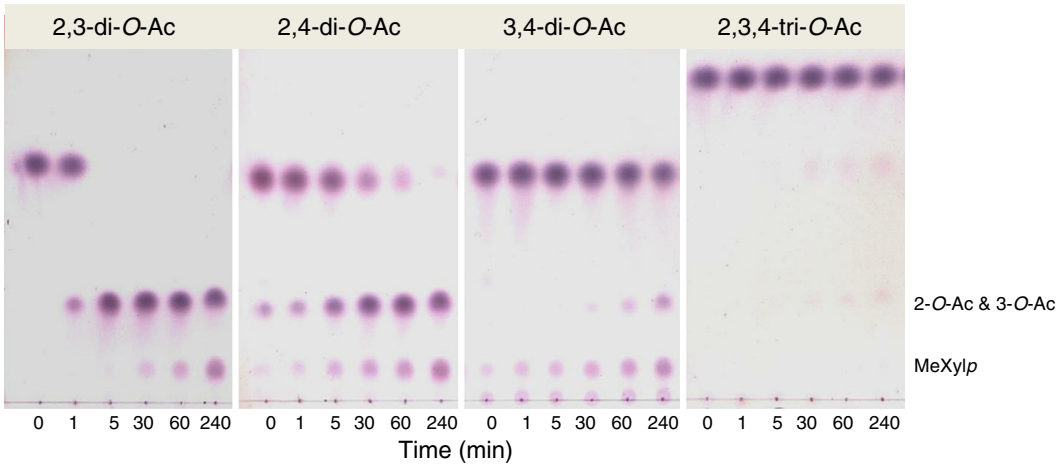


Fig. 2. TLC analyses of the products of deacetylation of MeXyl*p* diacetates and MeXyl*p* triacetate with *Trichoderma reesei* AcE (*Tr*CE16). The time of incubation is indicated.

compare the varying activities of *Tr*CE16 and *O*CE6 in deacetylating mono- and 2,3-di-*O*-acetylated non-reducing end Xyl*p* 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 *Tr*CE16 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 Xyl*p* 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 *Tr*CE16 AcE on synthetic diacetates and enzyme generated monoacetates of MeXyl*p*. The best substrate of the multiply acetylated substrates appeared to be the 3-*O*-acetyl group of the 2,3-di-*O*-acetylated Xyl*p*

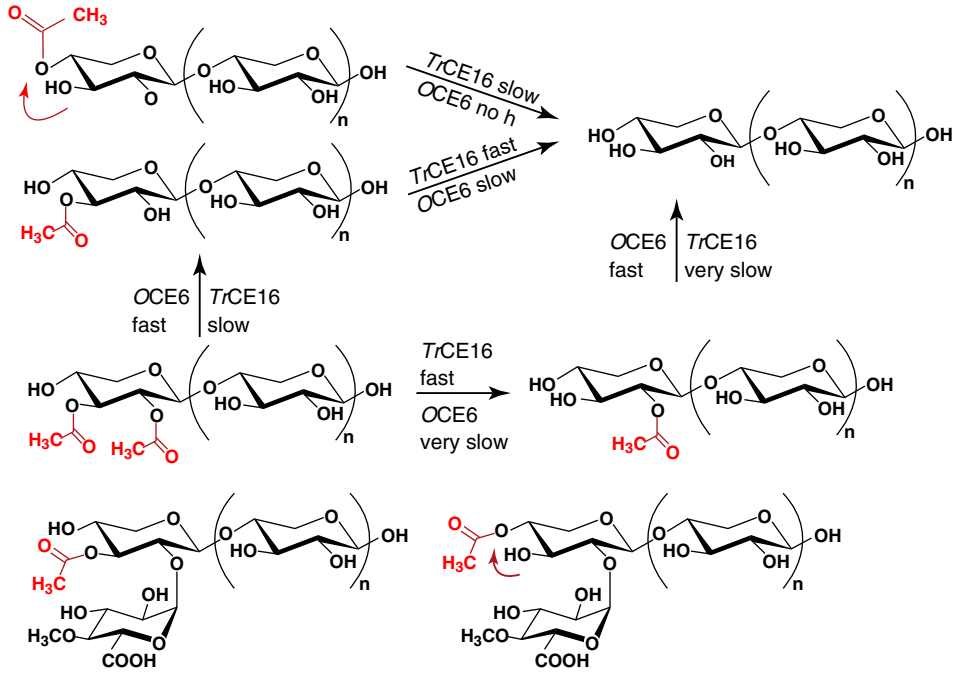


Fig. 3. Deacetylations of non-reducing end Xyl*p* residues in neutral oligosaccharides catalyzed by *Tr*CE16 and *O*CE6 suggested on the basis of the mode of action of both enzymes on acetylated derivatives of MeXyl*p*, acetylated xylooligosaccharides and acetyl glucuronoxylan. The abbreviations "fast", "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 *Tr*CE16 acetyl esterase. The aldouronic acids are deacetylated by *Tr*CE16, 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 *Tr*CE16 AcE after migration of the 2-*O*-acetyl group to a different position. *Tr*CE16 deacetylates the 4-*O*-acetyl group in 2,4-di-*O*-MeXyl*p* 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-MeXyl*p* or 2,3,4-tri-*O*-Ac-MeXyl*p*. 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 *Tr*CE16 AcE are 3-*O*-acetylated and doubly 2,3-di-*O*-acetylated Xyl*p* 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 Xyl*p* 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 Xyl*p* residues as substrates. The other targets of *Tr*CE16 AcE will of course be 3-*O*- and 4-*O*-monoacetylated Xyl*p* 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 *Tr*CE16 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 *Tr*CE16 removes an acetate from such sugars thus suggests that the enzyme can act on MeGlcA substituted Xyl*p*. 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 *Tr*CE16 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 Xyl*p* residues.

An important information regarding the physiological function of *Tr*CE16 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 *Tr*CE16 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*Tr*CE16 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 *Tr*CE16 AcE to catalyze acetyl transfer to position 3 of various hexosides [18] and to the non-reducing end of hexooligosaccharides, such as cello- and mannooligosaccharides [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 Xyl*p* residues are probably removed by esterases before migrating to position 4.

5. Conclusions

*Tr*CE16 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 *Tr*CE16 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 *Tr*CE16 on diacetates and monoacetates of MeXyl*p* facilitated interpretation of the results obtained with xylooligosaccharides, certain aspects of *Tr*CE16 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 Xyl*p* residues substituted by MeGlcA. Available data show that an acetyl group at position 2 of the non-reducing end Xyl*p* 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 Xyl*p* residues carrying MeGlcA side substituent has been revealed. In the case that the AcXE that deesterifies these Xyl*p* residues in the polymeric substrate exists awaits discovery.

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