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Review

Recent Progress in Understanding the Mode of Action of Acetylxylan Esterases

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Abstract: Acetylation is one of the main obstacles to the effective enzymatic conversion of hemicelluloses to fermentable sugars. In nature, the microbial degradation of hemicellulose involves the action of deacetylating esterases that act synergistically with glycoside hydrolases. In the industrial processing of lignocelluloses biomass, alkaline pretreatments remove acetyl groups by saponification, but other non-alkaline pretreatment methods generate acetylated hemicelluloses. Complete saccharification of plant hemicelluloses can't be achieved without the deacetylating enzymes. Recent years have witnessed considerale progress in our understanding of the mode of acetylation of hemicellulose and mode of action of microbial polysaccharide deacetylases. In this article we focus on the diversity and role of acetylxylan esterases in the breakdown of acetylxylan, the most abundant hemicellulose in nature.

Key words: plant biomass conversion, xylan, acetylation, acetylxylan esterases, mode of action

INTRODUCTION

The major obstacle of efficient and economically feasible bioconversion of renewable plant biomass is its recalcitrance as a result of complexity of the plant cell wall structure. The structural complexity is, in part, the result of plant evolution under strong pressure to enhance the resistance to microbial invaders. With the exception of insoluble cellulose, almost all other cell wall polysaccharide components, mainly hemicelluloses and pectin, are to some degree branched and partially esterified with acetic acid.[1,2)] Esterification of the side chain sugar residues of polysaccharides with acetyl groups is apparently critical in determining the physical properties of the cell wall, although other polymer modifications are also important; for example, esterification with phenolic acids plays a role in cross-linking.[3,4)] Here we review recent progress in understanding of enzymatic degradation of native acetylated hemicellulose using NMR and MS approaches. Particular attention is paid to the breakdown of partially acetylated hardwood glucuronoxylan, as studies of the action of deacetylating carbohydrate esterases have principally focused on this polysaccharide.

ROLE OF ACETYLATION OF PLANT CELL WALL POLYSACCHARIDES

Acetylation has been clearly shown to hinder the action of microbial glycanases.[5)] However, acetylation also dramatically changes the physico-chemical properties of polysaccharides. While full acetylation makes polysaccharides water-insoluble, partial acetylation, as observed in plant xylans, increases their solubility in water. Partial acetylation increases the bulk volume of the polysaccharide due to a higher degree of hydration. This is likely to have important consequences for transport of water and nutrients through the plant cell walls and also increase their susceptibility to enzymatic hydrolysis. This idea finds support in recent reports that the knock-out of acetyl transferase genes in *Arabidopsis* resulted in a lower degree of hemicellulose acetylation, enhanced the recalcitrance of the cell wall and the plant resistance to infection by the necrotrophic fungal pathogen *Botrytis cinerea*.[6–8)] Acetyl groups in the main xylan chain obviously prevent closer association of xylan with cellulose, thus increasing the plant resistance to pathogen attack.

It is not known at which stage of plant evolution the acetylation of polysaccharides was introduced, however, it is likely that deacetylating enzymes evolved in parallel with microorganisms invading plants or proliferating on plant residues. The substrate specificity of some carbohydrate esterases for elements of the polysaccharide main chain suggests that some of these enzymes evolved from glycoside hydrolase ancestors.[5,9–11)]

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Abbreviations: AcXE, acetylxylan esterase; GH, glycoside hydrolase; CE, carbohydrate esterase; DMSO, dimethyl sulfoxide; Xyl*p*, D-xylopyranose or D-xylopyranosyl; MeGlcA, 4-*O*-methyl-D-glucuronic acid; MeGlcA^{3}Xyl$_3$, 4-*O*-methyl-D-glucuronosyl-α-1,2-D-xylopyranosyl-β-1,4-D-xylopyranosyl-β-1,4-D-xylopyranose (the upper index indicates the number of the xylosyl residue from the reducing end substituted with MeGlcA); Xyl$_x$Ac$_y$, acetylated β-1,4-xylooligosaccharide containing x xylose residues and y acetyl groups; MeGlcAXyl$_x$Ac$_y$, acetylated aldouronic acid containing one MeGlcA, x xylose residues and y acetyl groups; MeGlcA$_2$Xyl$_x$Ac$_y$, acetylated aldouronic acids containing two MeGlcA residues.

PRETREATMENTS AFFORDING ACETYLATED HEMICELLULOSES

The bioconversion of insoluble plant biomass to soluble fermentable sugars is an essential step in the production of liquid fuels and a variety of other chemicals and useful products including food additives. Typically, the first and crucial step of this bioconversion process is the mechanical and physico-chemical pretreatment to render the lignocellulosic biomass susceptible to enzymatic saccharification. Alkaline pretreatment of biomass results in saponification of ester linkages, therefore, here we will consider only the acetylated hemicellulose fractions obtained under non-alkaline conditions, such as steam explosion, hot water treatment and autohydrolysis at increased pressure and temperature.[12–17] Hemicellulose also remains acetylated after ionic liquid, organosolvent or super-critical CO_2 pretreatments.[15–17]

PATTERNS OF XYLAN ACETYLATION IN PLANTS

Acetylation is the most common esterification of plant xylans. In hardwood glucuronoxylans more than half of the main chain xylopyranosyl (Xyl*p*) residues are acetylated.[18–19] The majority of Xyl*p* residues are singly acetylated at positions 2 or 3, but a smaller fraction is also doubly 2,3-acetylated. There are also reports of 3-*O*-acetylation of Xyl*p* residues substituted with 4-*O*-methyl-D-glucuronic acid residues (MeGlcA).[18–21] Similar acetylation patterns have been observed for Xyl*p* residues substituted by GlcA.[8] Several lines of evidence suggest that there could be several types of glucuronoxylan, which differ in their content of MeGlcA and acetyl groups.[8,22] We have shown that a DMSO-extracted beechwood acetylglucuronoxylan contains a much lower content of MeGlcA residues than alkali extracted polymer (M. Vršanská and P. Biely, unpublished results), suggesting that the reported values of the degree of substitution with MeGlcA and the degree of acetylation are average values. Acetylation of Xyl*p* residues has been confirmed in cereal arabinoglucuronoxylan[20] and in xylan from *Arabidopsis thaliana*.[23] Although only monoacetylation of Xyl*p* residues was reported in cornstalk arabinoxylan,[20] it has clearly been demonstrated that the acetylation features of the hardwood polysaccharide are also seen in dimethylsulfoxide extracted acetylglucuronoxylan from *Arabidopsis*.[24] As in the hardwood, Xyl*p* residues in *Arabidopsis* xylan are mono- and diacetylated at positions 2 and 3, and also 3-*O*-acetylated on Xyl*p* residues 2-*O*-substituted with uronic acid.[24] The distinct feature of the *Arabidopsis* xylan is that in addition to MeGlcA it also contains GlcA side residues.[8] A detailed structural analysis of one of the most recalcitrant plant xylans occurring in corn fiber[25] revealed novel sites of polysaccharide acetylation.[26] Acetyl groups were found at position 2 in approximately half of Xyl*p* residues. Some of these Xyl*p* residues carry at position 3 Ara*f* residues esterified at position 5 with ferulic acid or substituted at position 2 with additional Xyl*p* residue or with the disaccharide 2-*O*-α-D-galactopyranosyl-Xyl*p*.[26] The known patterns of acetylation of hardwood and cereal xylans, and the xylan of annual plants, are depicted in Fig. 1.

EFFECT OF XYLAN ACETYLATION ON XYLAN HYDROLYSIS BY ENDOXYLANASES

The inhibition of endoxylanase-catalyzed hydrolysis of xylan by its acetylation is illustrated in Table 1. As shown by a MALDI ToF MS analysis of oligosaccharides released from acetylated xylan and its deacetylated counterpart, the number of products generated from acetylated polysaccharides by family 10 or family 11 xylanases is very large in comparison with the few products generated by the same enzymes acting on chemically deacetylated polysaccharide.[27] With exception of traces of non-acetylated Xyl and Xyl_2, the acetylxylan hydrolysates do not contain other

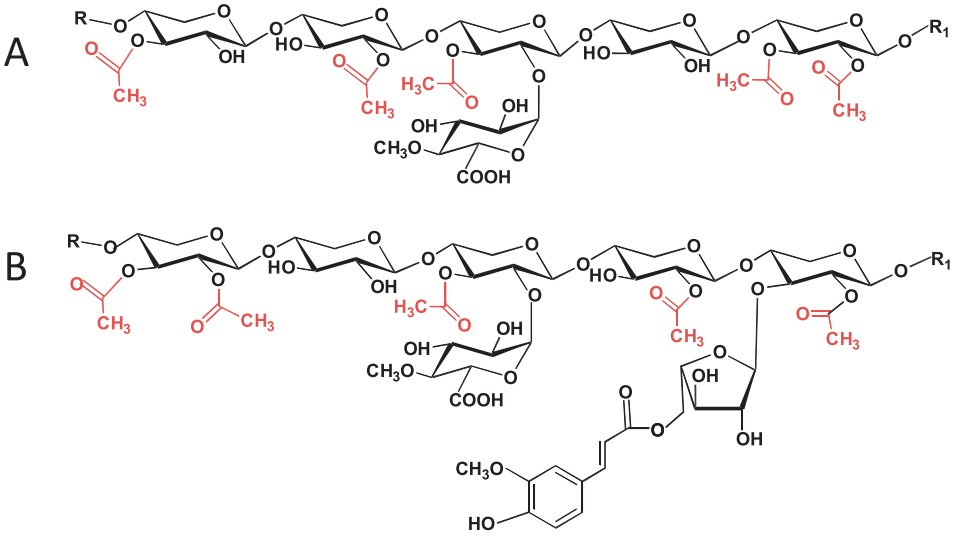

Fig. 1. Acetylation of xylosyl residues in hardwood acetylglucuronoxylan (A) and cereal or annual plant arabinoglucuronoxylan (B).

MeGlcA in xylan B can be replaced by GlcA. The acetylation of position 2 in Xyl*p* residues 3-*O*-substituted with L-arabinose and esterified at position 5 with ferulic acid has been reported only recently in highly substituted corn fiber xylan.[26] Another structural feature of corn fiber xylan is substitution of L-arabinose at position 2 by xylose residue or 2-*O*-α-galactosylated xylosyl residue.[26]

non-acetylated xylan fragments. These results highlight the important role of acetylxylan esterases (AcXEs) in microbial degradation of native acetylated polysaccharides. As expected, the GH10 xylanase produced shorter products than GH11 xylanase from both acetylated and deacetylated polysaccharide. Aldotetraouronic acid MeGlc^{3}Xyl$_3$ is the major acidic product of GH10 xylanase and aldopentaouronic acid MeGlcA^{3}Xyl$_4$ the major acidic product of GH11 xylanase acting on the deacetylated polysaccharide. Another difference showing the lower tolerance of the GH11 xylanases towards substitution by MeGlcA is the appearance of aldouronic acids with two residues of MeGlcA among the products of the GH11 xylanase hydrolysis (Biely and Westereng, unpublished data; see the bottom of Table 1). The crystal structure of GH10 xylanases with aldouronic ligands indicate that these require two consecutive unsubstituted Xyl*p* residues for the cleavage of the xylanase main chain.[28,29] Several lines of evidence, particularly the analysis of the structure of products generated by GH11 xylanases, suggest that these enzymes require three consecutive unsubstituted Xyl*p* residues.[27] It remains to be elucidated which of these Xyl*p* residues could be acetylated. Exactly how acetyl groups affect the mode of cleavage of the xylan main chain remains unknown and little information is available on the tolerance of acetyl groups on the xylan main chain by appendage-dependent GH30 and GH5 xylanases (glucuronoxylan hydrolases and arabinoxylan hydrolases).[30–33]

DIVERSITY OF ACETYLXYLAN ESTERASES

The variability of acetylation of Xyl*p* residues (Fig. 1) poses various questions on the effective hydrolysis of the acetylated polysaccharide. For example, how many different AcXEs are needed to remove acetyl groups from all positions? Can complete deacetylation be achieved by a single enzyme in the absence of endoxylanases? Is the diversity of carbohydrate esterases linked also to variety of acetyl groups in acetylated xylans? According to the CAZy classification,[34] among the 16 carbohydrate esterase families we can identify characterized AcXEs in families 1, 3, 4, 5 and 6. Here we define AcXEs as only those enzymes that can deacetylate the polymeric substrate so efficiently as to cause it to precipitate from solution.[5]

AcXEs acting on acetylated polysaccharides which are too large to be transported into the cells, are likely to be extracellular, and maybe bound in bacterial cellulosomes[35,36] (the polymer is too large to be transported into the cell). The enzymes may also be intracellular, where they would act on soluble acetylated xylooligosaccharides imported into the cells.[37–40] A transport system for linear and branched arabinoxylooligosaccharides also occurs in the probiotic strain of *Bifidobacter animalis* subsp. *lactis* Bl-04.[41] A xylobiose permease was earlier demonstrated as a part of the xylanolytic system of the yeast *Cryptococcus albidus*[42] and a *Streptomyces* strain.[43] None of the above reports deals with acetylated xylooligosaccharides, but they are most probably transported by the same carriers as non-acetylated

Table 1. Neutral and acidic xylooligosaccharides generated from aspen acetylglucuronoxylan by GH10 (*Clostridium thermocellum*) and GH11 (Optimase, Genencor) endoxylanases as revealed by MALDI ToF MS (masses correspond to sodium adducts; for experimental details see Ref. 55).

Compound	Ions of neutral oligosaccharides by GH10	Ions of neutral oligosaccharides by GH11	Compound	Ions of acidic oligosaccharides by GH10	Ions of acidic oligosaccharides by GH11
Xyl$_2$	(305)		MeGlcAXyl$_2$Ac	537	
Xyl$_2$Ac	347		MeGlcAXyl$_3$Ac	669	669
Xyl$_2$Ac$_2$	389		MeGlcAXyl$_3$Ac$_2$	711	
Xyl$_3$Ac	479	479	MeGlcAXyl$_3$Ac$_3$	753	
Xyl$_3$Ac$_2$	521		MeGlcAXyl$_4$Ac	(801)	(801)
Xyl$_3$Ac$_3$	563		MeGlcAXyl$_4$Ac$_2$	843	843
Xyl$_4$Ac$_2$	653	653	MeGlcAXyl$_4$Ac$_3$	885	885
Xyl$_4$Ac$_3$	695	695	MeGlcAXyl$_5$Ac$_2$	(975)	975
Xyl$_4$Ac$_4$	737		MeGlcAXyl$_5$Ac$_3$	1017	1017
Xyl$_5$Ac$_2$	785	785	MeGlcAXyl$_5$Ac$_4$	1059	1059
Xyl$_5$Ac$_3$	827	827	MeGlcAXyl$_6$Ac$_3$	1149	1149
Xyl$_5$Ac$_4$	869	869	MeGlcAXyl$_6$Ac$_4$	1191	1191
Xyl$_5$Ac$_5$	911		MeGlcAXyl$_6$Ac$_5$	1233	1233
Xyl$_6$Ac$_3$	959	959	MeGlcAXyl$_7$Ac$_4$	(1323)	1323
Xyl$_6$Ac$_4$	1001	1001	MeGlcAXyl$_7$Ac$_5$	1365	1365
Xyl$_6$Ac$_5$	1043	1043	MeGlcAXyl$_7$Ac$_6$	1407	1407
Xyl$_6$Ac$_6$	1085	1085	MeGlcAXyl$_8$Ac$_4$	1455	1455
Xyl$_7$Ac$_4$	1133	1133	MeGlcAXyl$_8$Ac$_5$	1497	1497
Xyl$_7$Ac$_5$	1175	1175	MeGlcAXyl$_8$Ac$_6$	1539	1539
Xyl$_7$Ac$_6$	1217		MeGlcAXyl$_8$Ac$_7$	1581	1581
Xyl$_7$Ac$_7$	1259		MeGlcAXyl$_9$Ac$_6$	1671	1671
Xyl$_8$Ac$_5$		1307	MeGlcAXyl$_9$Ac$_7$	1713	1713
Xyl$_8$Ac$_6$		1349	MeGlcAXyl$_9$Ac$_8$	1755	1755
Xyl$_8$Ac$_7$			MeGlcA$_2$Xyl$_7$Ac$_4$*		1513
Xyl$_8$Ac$_8$			MeGlcA$_2$Xyl$_7$Ac$_5$*		1555
Xyl$_9$Ac$_5$		1439	MeGlcA$_2$Xyl$_8$Ac$_4$*		1645
Xyl$_9$Ac$_6$		1481	MeGlcA$_2$Xyl$_8$Ac$_5$*		1687

Acidic oligosaccharides containing two MeGlcA residues are marked by asterisks. Xyl and Xyl$_2$ are the only deacetylated products observed in small quantities in the enzymatic hydrolysates of acetylglucuronoxylan.

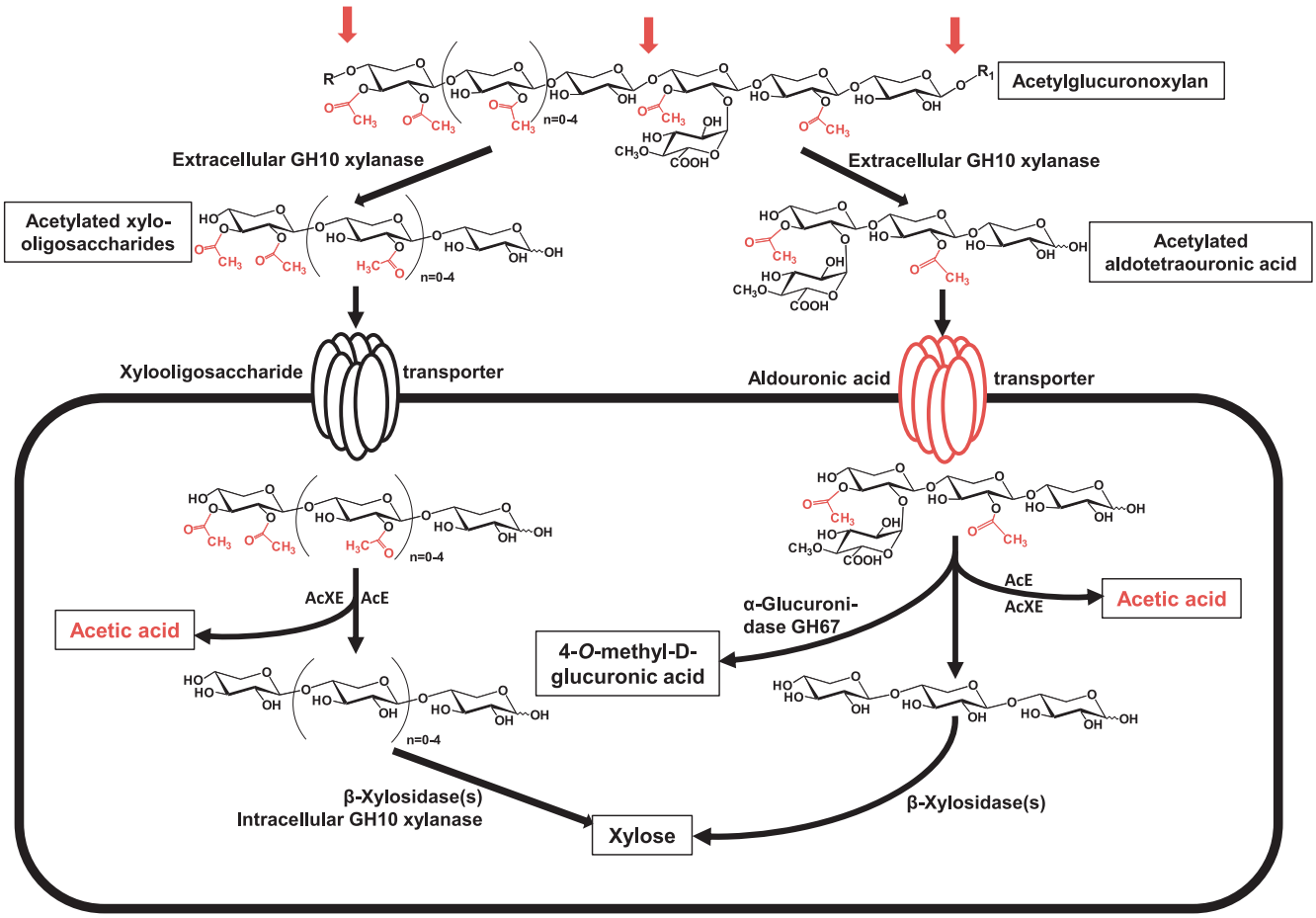

Fig. 2. Proposed model for intracellular breakdown of xylooligosaccharides in some bacteria.

The acetylglucuronoxylan is cleaved extracellularly into acetylated xylooligosaccharides and aldotetrauronic acid, which are internalized by specific oligosaccharide transport systems. Subsequently these oligosaccharides are deacetylated and hydrolyzed to monosaccharides by intracellular esterases and glycoside hydrolases. Based on Refs. 37−40) and 44).

compounds. Once in the cell, acetylated oligosaccharides would be substrates for intracellular esterases.[40,44] Candidate for this process would be esterases of CE family 7. This family includes intracellular acetylesterases which do not recognize large polymeric substrates but deacetylate glycosides (alkyl and aryl β-xylopyranosides) and oligosaccharides.[45–47] A model for intracellular breakdown of extracellularly generated acetylated xylooligosaccharides that involves the interplay of intracellular deacetylases and glycosidases can be deduced from the genomic and biochemical evidence from a number of bacterial species (Fig. 2).[37–40,44]

MODE OF ACTION OF AcXEs

The positional specificity of AcXEs was earlier studied using artificial substrates; mono- or di-*O*-acetylated methyl β-xylopyranoside, their deoxy- and deoxy-fluoroderivatives, 4-nitrophenyl β-xylopyranoside and glycosides of hexoses (reviewed in Ref. 5). However, the use of these substrates did not provide unequivocal guidelines for specificity. Several AcXEs deacetylated methyl β-xylopyranoside at position 2 and 3, in contrast to their strong preference for position 2 in monoacetyl derivatives of 4-nitrophenyl β-xylopyranoside.[48] A study on the mode of action of several AcXEs on partially acetylated cellulose showed that some AcXEs exhibited regioselectivity, while others did not.[49] The data obtained with deoxy and deoxy-fluoro-analogues of 2- or 3-monoacetylated methyl β-xylopyranoside showed major differences in specificities between serine-type esterases belonging to carbohydrate esterase families 1 and 5, and AcXEs of family CE4, the aspartate metalloenzymes.[5] The CE4 AcXEs required the vicinal OH-group free for deacetylation of position 2 or 3 in Xyl*p* residues, such that 2,3-di-*O*-acetyl or fully acetylated methyl β-D-xylopyranoside did not serve as substrates.[50–52]

The evaluation of the total amount of released acetyl groups from a mixture of acetylated xylooligosaccharides by two AcXEs (CE1 and CE5 family) from *Chrysosporium lucknowense* C1 (renamed as *Myceliophtora thermophila* C1[53]) suggested that the enzymes deacetylate both positions 2 and 3, which was in accord once with the mode of action of enzymes on acetylated methyl xylopyranosides but in contradiction with the data observed on monoacetyl derivatives of 4-nitrophenyl β-D-xylopyranoside.[48] These observations stimulated a more detailed analysis of the mode of action of AcXEs on native, partially acetylated glucuronoxylan extracted from hardwood using ^{1}H-NMR spectroscopy. The complete assignment of the proton resonances of all acetyl groups in the polysaccharide, both of resonances of esterified Xyl*p* residues and those of the methyl protons of the acetyl groups was achieved.[54] This approach was used to follow the positional specificity and mode of action of AcXEs in deuterized buffer by observing the decrease in the intensity of the signals of particular acetyl groups. Members

of the following CE families were examined: *Schizophyllum commune* CE1 AcXE, *Streptomyces lividans* and *Clostridium thermocellum* CE4 AcXEs and *Orpinomyces* CE6 AcXE.[54,55] All enzymes tested were found to de-esterify Xyl*p* residues monoacetylated at both positions 2 and 3. The major difference found between the modes of action of serine type AcXEs belonging to CE families 1, 5 and 6 and aspartate metallo-enzymes of CE4 family, was the inability of the latter to deesterify doubly acetylated Xyl*p* residues. This is apparently the consequence of the requirement for a free vicinal OH-group which participates in the formation of the enzyme-substrate complex by coordinating with the catalytic metal ion. This interaction appears to be indispensable for deacetylation of the vicinal position, regardless whether it is position 2 or 3.[56] None of the enzymes were capable of attacking the 3-*O*-acetyl group on the Xyl*p* residue substituted with MeGlcA residue. However, for serine-type esterases attacking diacetylated Xyl*p* residues, which do not require a vicinal hydroxyl group, the MeGlcA residue in position 2 is a much larger substituent than the acetyl group, and is likely to represent a serious steric hindrance to hydrolyze the neighbouring 3-*O*-acetyl group.

The ^{1}H-NMR studies of the mode of action of AcXEs confirmed the capability of members of four different CE families to catalyze deacetylation of internal Xyl*p* residues in the polymeric substrate.[21,55] It is probable that some of the enzymes recognize several Xyl*p* residues of the main chain as is typical for the glycoside hydrolases. Examples are found in chitin deacetylases, where the 3D structures show substrate binding sites comprising several subsites binding *N*-acetyl-D-glucosaminyl residues.[10] The deacetylation of hexopyranosides[57] and cellulose acetate by AcXEs[49] suggests that the enzymes tolerate the replacement of Xyl*p* residues by hexopyranosyl residues. This is consistent with the observation that CE4 AcXEs catalyze *N*-deacetylation of chitin, an example of their action on acetylated hexopyranosyl residues.[56,58]

One of the most striking observations from studies of the positional specificity of AcXEs is the finding that both serine- and aspartate-type AcXEs (the CE4 family enzymes) deacetylate monoacetylated Xyl*p* residues at positions 2 and 3, earlier observed with methyl β-D-xylopyranosides.[5] It was originally proposed that the deacetylation of the two positions involved acetyl group migration in the active site of the enzyme.[5] However this concept lost favour after the 3D structure of *T. reesei* AcXE was elucidated.[59] The authors suggested that the 2- or 3-acetylated methyl β-D-xylopyranoside could form two different 180° oriented enzyme-substrate complexes[59] with identical stereochemical arrangements of the hydroxyl groups.[5] While this model is clearly feasible for small substrates, it is not readily compatible for polymeric substrates. A strong argument against the formation of two alternative 180° flipped enzyme-substrate complexes is the fact that deacetylation of positions 2 and 3 is also catalyzed by CE4 AcXEs which comprise the same protein fold topology as chitin deacetylases, but whose active site structures are not homologous to that of *T. reesei* AcXE.

The hypothesis that deacetylation of both positions 2 and 3 is facilitated by acetyl group migration between the hydroxyls OH-2 and OH-3 has also been weakened by recent structural studies of GH62 α-L-arabinofuranosidases, which are capable of removing both α-1,2- and α-1,3-linked L-arabinofuranosyl side residues from the xylan main chain.[60,61] The studies demonstrate that these enzymes form two oppositely oriented productive complexes with the polymeric substrate,[60,61] as was once proposed for AcXEs.[59]

DEACETYLATION OF XYLOOLIGOSACCHARIDES BY AcXEs

It has been clearly demonstrated that AcXEs and endoxylanases act on acetyl xylan synergistically; *i.e.* xylanases stimulate the rate of deacetylation and AcXE stimulate the rate of hydrolysis of glycosidic linkages.[62–65] The AcXEs create new sites on the xylan main chain for productive binding with endoxylanases. Conversely, cleavage of the main chain exposes new sites for deacetylation. The depolymerization of the polysaccharide may also have additional positive effects on the rate of substrate deacetylation. Decreasing the size of the substrate increases its diffusion rate and thus the rate of deacetylation.

The effect of AcXEs belonging to CE families 1, 4, 5 and 6 on aspen acetylglucuronoxylan hydrolysis products generated by the action of GH10 from *Clostridium thermocellum* (*Ct*GH10), was elucidated by MALDI ToF MS.[55] This method allowed a detailed comparison of the oligosaccharides before and after AcXE treatment.

The deacetylation of oligosaccharides by all AcXEs tested was consistent with their performance on polysaccharides. For both neutral and acidic oligosaccharides, CE4 AcXEs hydrolyzed one or two acetyl group less than the serine-type AcXEs. While serine-type esterases deacetylated neutral oligosaccharides either completely or to monoacetates, the CE4 AcXEs left a considerable portion of the products also in the form of diacetates. Similarly, while the serine type esterases converted acidic oligosaccharides either to the deacetylated or the mono- and di-*O*-acetate forms, the products of CE4 AcXE activity included derivatives with three acetyl groups.[55] This difference is obviously the consequence of the inability of the CE4 AcXEs to attack doubly acetylated Xyl*p* residues. Incomplete deacetylation by AcXEs of CE families 1 and 5 was also observed with xylooligosaccharides derived from the eucalyptus wood,[66] where a considerable fraction of both neutral and acidic xylooligosaccharides remained monoacetylated. The exact mode of action of AcXEs on polymeric substrate[54,55] has yet to be clarified, however, based on the mode of action of AcXEs on artificial substrates like monoacetates of 4-nitrophenyl β-xylopyranosides,[48] it can hypothesized that the monoacetates of neutral xylooligosaccharides carry single acetyl group at position 4, as a result of acetyl group migration. In the monoacetates of the acidic xylooligosaccharides generated by GH10 endoxylanase, *i.e.* aldouronic acids with α-1,2-linked MeGlcA residues, the nonreducing Xyl*p* residue may have the single acetyl group either at the original position 3 or at position 4 as a result of acetyl group migration from position 3. In the case of CE4 AcXEs, di- and tri-*O*-acetates are also observed to be resistant to enzymatic hydrolysis. The proposed structures of xylooligosaccharide mono- di- and triacetates resistant to serine-type and aspartate-type AcXEs are depicted in Fig. 3.

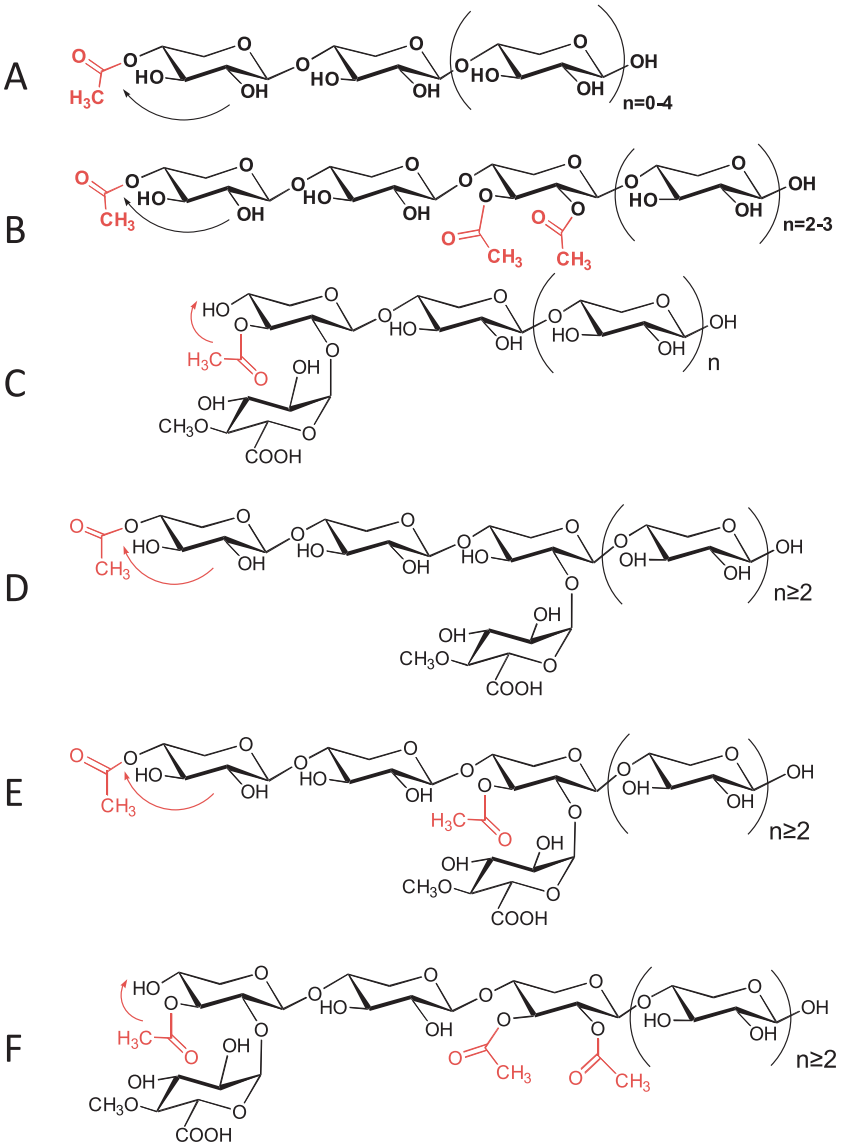

Fig. 3. Suggested structural features of acetylated neutral (A, B) and acidic oligosaccharides (C-D) in the hydrolysate of acetylglucuronoxylan with GH10 xylanase resistant to AcXEs (GH10 xylanase was denatured before the AcXE treatment).

With exception of the structures C and F, all xylooligosaccharides are shown acetylated in position 4 of the non-reducing Xyl*p* residue, where the acetyl group migrated (thin red arrows). The 4-acetate is in the quilibrium with the 3-and 2-*O*-acetyl derivatives in linear xylooligosaccharides (A and B) and with 3-*O*-acetylated derivative in aldouronic acids carrying MeGlcA residue on the non-reducing end (C and F). The internal 2,3-di-*O*-acetylated Xyl*p* residues (B and F) are resistant only to CE4 AcXEs. Based on Refs. 55, 66) and 71).

ACETYL GROUP MIGRATION TO POSITION 4

The proposal for the resistance of the 4-acetyl group to the action of AcXEs is based, in part, on kinetic measurements of the migration of the acetyl group in monoacetates and diacetates of 4-nitrophenyl β-D-xylopyranosides.[67] At equilibrium, the 4-*O*-acetyl derivative predominates in monoacetates and the 3,4-derivative in diacetates. The 2-*O*-acetyl derivatives are the most kinetically labile. Acetyl group migration was assessed at 40°C,[67] a temperature much lower than used in many non-alkaline hydrothermal pretreatments of lignocellulose biomass. Examination of the rate of acetyl group migration on monoacetates of 4-nitrophenyl β-xylopyranoside at 100°C confirmed that the positional isomer equilibrium is established very rapidly (within a few minutes, even at pH < 7; M. Cziszárová and P. Biely, unpublished data). Acetyl migration has also been observed for monoacetyl derivatives of methyl β-D-xylotrioside prepared by *T. reesei* CE16 acetylesterase transacetylation.[68] The enzyme transfers the acetyl group exclusively to the OH-3 of the non-reducing Xyl*p* residue, although a single reaction product cannot be isolated due to migration of the acetyl group from position 3.

Other data supporting the resistance of the 4-acetyl group at the non-reducing terminal to the action of AcXEs include the relative rates of deacetylation of the monoacetates of 4-nitrophenyl β-xylopyranoside by AcXEs[48] and the regioselectivity of deacetylation of per-*O*-acetylated glycosides.[57] AcXEs show negligible or extremely low activity on the acetyl group at position 4 of a variety of acetylated glycosides, but deacetylate rapidly positions 2 and 3.[57] Treatment of the equilibrium mixture of three monoacetyl

derivatives of methyl β-D-xylotrioside by AcXEs belonging to CE families 1, 4 and 6 clearly confirmed that the 4-*O*-acetyl derivative is the most resistant, although a slow but significant hydrolysis was observed with the CE1 AcXE (V. Puchart and P. Biely, unpublished data).

On the basis of these studies we conclude that any heat treatment of xylooligosaccharides acetylated at the non-reducing end, such as those extracted under high temperatures or generated from endoxylanase digestion of acetylated polysaccharide, will rapidly generate 4-*O*-acetylated derivatives which are not susceptible to deacetylation by AcXEs. However, since the 4-acetyl derivative are in equilibrium with 2-*O*- and 3-*O*-acetyl compounds, which are viable AcXE substrates, over long incubation period, the 4-acetyl derivatives will slowly be removed.

ENZYMES DEACETYLATING NON-REDUCING ENDS

Enzymes deacetylating the non-reducing end of oligosaccharides are found in CE family16. The first enzyme identified with this specificity was the acetylesterase of *T. reesei* classified as the first member of the CE family 16.[69,70] The enzyme did not deacetylate polymeric substrates but acted on acetylated xylobiose.[69] On monoacetyl derivatives of 4-nitrophenyl β-D-xylopyranoside the enzyme showed preference for deacetylation of position 3 and 4,[48] with specific activities for the 2-acetate approximately 20 times lower. The CE16 esterase also efficiently catalyzed transacetylation to position 3 of the non-reducing ends of cello-, manno- and xylooligosaccharides[68] with removal of the acetyl in the absence of an acetyl donor.[68] Based on these catalytic properties the enzyme was assigned as an exo-deacetylase,[5] and exo-activity was subsequently confirmed on acetylated xylooligosaccharides using MALDI ToF MS.[66,71] The application of this enzyme with AcXEs leads to almost complete deacetylation of neutral and acidic xylooligosaccharides. When applied as the sole enzyme, the CE16 esterase typically removes only one acetyl group. A similar specificity is observed with MeGlcA-substituted non-reducing ends of aldouronic acids, although evidence whether the deacetylation takes place at position 3 or 4, or both is currently lacking. The migration of the acetyl group to position 4 would move the acetyl group away from the MeGlcA, thereby potentially reducing steric inhibition. Support for this suggestion was obtained by testing the enzyme on di-*O*-acetates and tri-*O*-acetate of methyl β-D-xylopyranoside at 10 mM substrate concentration.[71] The enzyme showed a preference for deacetylation of position 3 in 2,3-diacetate. The 2,4-diacetate was deacetylated about 5-fold slower at position 4 and the 3,4-diacetate about 2 to 3 orders of magnitude slower. The fully acetylated glycoside was resistant to the enzyme.[71] These observations suggest that for the deacetylation of position 3 substituents at position 2 show the lowest steric influence.

A higher degree of xylooligosaccharide deacetylation was reported with the CE16 acetyl esterase from *M. thermophila* C1,[66] only the second CE16 esterase investigated in this regard. In the presence of a GH family 10 endoxylanase and a β-xylosidase, the enzyme released 79% of acetic acid from a mixture of neutral and acidic acetylated xylooligosaccharides. Combination with *T. reesei* AcXE (CE5) led to 88% deacetylation. Addition of GH115 α-glucuronidase to the enzyme mixture not only resulted in almost complete release of MeGlcA but also in near quantitative release of acetic acid.[66]

Naturally, the migration of the acetyl group to position 4 may not be of such significance in nature where the acetylated xylan is attacked simultaneously with a mixture of endoxylanases, esterases and accessory xylanolytic enzymes. The acetyl group at the non-reducing end of oligosaccharides generated by endoxylanases and eventually β-xylosidase would be primarily located at position 2 or 3 where it could immediately be attacked by AcXEs and CE16 acetylesterase. Debranching of the main xylan chain or oligosaccharides by α-glucuronidase and α-arabinofuranosidase would proceed synergistically with simultaneous deacetylation.

CONCLUDING REMARKS AND OUTLOOK

The importance of the participation of deacetylating carbohydrate esterases in the breakdown of native partially acetylated plant hemicelluloses by microorganisms is clear, where the esterases appear to work synergistically with glycoside hydrolases. The high chemical and structural complexity of plant hemicelluloses appears to be balanced in nature by a very high diversity of degradative enzymes differing in the modes of action and substrate specificities. Here we have reviewed the recent progress in our knowledge of a specific group of esterases which participate in the degradation of partially acetylated plant xylans. The AcXEs are unique enzymes harbouring two structurally diverse groups; the aspartate metalloenzymes and serine-type enzymes with differing capabilities to deacetylate doubly acetylated Xyl*p* residues. One of the most interesting questions posed from recent studies is the mechanistic basis of AcXE deacetylation at positions 2 and 3 in the polysaccharide. A deeper insight into the active site structure of the esterases will be needed to clarify this "positional" non-specificity.

Microorganisms also produce specialized esterases capable of removing acetyl groups not readily accessible to AcXEs. An important recent contribution to this area is a partial recognition of the role of CE16 acetylesterases, which effectively deacetylate positions which do not occur in native polysaccharide.[5,66,71] We refer in particular to the acetyl group at position 4 of non-reducing Xyl*p* residues, where this group migrates rapidly during heat treatment of the acetylated oligosaccharides. This is an important issue for the development of processes for the complete saccharification of acetylated xylan. In nature, where the polymer is attacked simultaneously by a consortium of glycoside hydrolases and esterases the 4-acetylation at the non-reducing end may not be of such significance.

None of the members of the CE16 acetylesterases has been crystallized to date. Acquisition of a high resolution 3D structure will be particularly important in understanding the interaction of the enzyme with MeGlcA substituted and 3-*O*-acetylated non-reducing Xyl*p* residues as well as resolving the mechanism of the activation of the CE16 enzyme with carbohydrates when acting on non-carbohydrate substrates.[70]

We should emphasize that all new data on the mode of action of AcXEs and of CE16 esterases on xylooligoaccharides comes from the experiments with hydrolysis products generated by family 10 endoxylanase, which cleaves the acetylglucuronoxylan main chain at the glycosidic linkage adjacent to 3-*O*-acetylated and by MeGlcA- or GlcA-substituted Xyl*p* residues.[27–29] Similar studies should be carried out with acetylated xylooligosaccharides generated by other types of endoxylanases, including the recently discovered appendage-dependent xylanases of family GH30 and GH5. GH family 11 xylanase would most probably generate aldouronic acids with MeGlcA-substituted and 3-*O*-acetylated Xyl*p* residues on the penultimate residue from the non-reducing end. The 3-*O*-acetyl group could become the substrate of CE16 acetyesterases only after removal of the terminal xylose by β-xylosidase.

Whether polymer-attacking enzymes deacetylating position 3 in internal Xyl*p* residues substituted with MeGlcA or acetyl group at position 2 in Xyl*p* residues substituted by L-Ara occurring in cereal arabinoxylans[26] exist in nature, remains to be investigated.

There are numerous esterases reported in the literature which have not been examined for their abilities to deacetylate hemicellulose-derived carbohydrates. Many microorganisms utilize xylan as a carbon sourse, but do not degrade xylan fragments extracellularly. These oligosaccharides are transported into the cells by inducible transport systems and fully degraded intracellularly. Evidence has been presented that there are transport systems for both neutral and acidic oligosaccharides,[37–40] but it is not known whether these systems also translocate acetylated xylooligosaccharides. Some intracellular acetylesterases, like those of the CE7 family[44–47] appear to have very wide substrate specificity, potentially offering a variety of useful applications.

Recent attempts to reduce the degree of acetylation of plant cell wall polysaccharides by genetic modifications, with the aim of making them more accessible to degradation by glycoside hydrolases and to reduce the acetate-mediated inhibition of fermentations, have clearly demonstrated that acetylation of hemicelluloses is inevitable for plants and that the reduction of the acetyl groups content in plant polysaccharides may not lead to expected results.[6–8] Suggestions to genetically modify plant hemicellulose structure by replacing the acetyl group side residues by fermentable sugars[7] is an exciting prospect for future research.

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