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Research review paper

Towards enzymatic breakdown of complex plant xylan structures: State of the art

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ABSTRACT

Significant progress over the past few years has been achieved in the enzymology of microbial degradation and saccharification of plant xylan, after cellulose being the most abundant natural renewable polysaccharide. Several new types of xylan depolymerizing and debranching enzymes have been described in microorganisms. Despite the increasing variety of known glycoside hydrolases and carbohydrate esterases, some xylan structures still appear quite recalcitrant. This review focuses on the mode of action of different types of depolymerizing endoxylanases and their cooperation with β-xylosidase and accessory enzymes in breakdown of complex highly branched xylan structures. Emphasis is placed on the enzymatic hydrolysis of alkali-extracted deesterified polysaccharide as well as acetylated xylan isolated from plant cell walls under non-alkaline conditions. It is also shown how the combination of selected endoxylanases and debranching enzymes can determine the nature of prebiotic xylooligosaccharides or lead to complete hydrolysis of the polysaccharide. The article also highlights the possibility for discovery of novel xylanolytic enzymes, construction of multifunctional chimeric enzymes and xylanosomes in parallel with increasing knowledge on the fine structure of the polysaccharide.

Contents

Abbreviations: Xyl*p*, D-xylopyranosyl residue; Ara*f*, L-arabinofuranosyl residue; MeGlcA, 4-*O*-methyl-D-glucuronic acid; GH, glycoside hydrolase; CE, carbohydrate esterase; Xyl_n, β-1,4-xylooligosaccharide of n Xyl*p* residues; $MeGlcA^iXyl_n$, aldouronic acid containing one residue of MeGlcA and n Xyl*p* residues - the upper index i marks the number of Xyl*p* residue counted from the reducing end to which MeGlcA is linked (it would be equal to 2 if MeGlcA would be linked to the second Xyl*p* residue from the reducing end, and to 3 if MeGlcA would be linked e.g. to the non-reducing end Xyl*p* residue in Xyl_3); $Ac^3MeGlcA^3Xyl_3$, aldotetraouronic acid in which both MeGlcA and the acetyl group is linked to non-reducing end Xyl*p* residue; $Ac^3MeGlcA^3Xyl_4$, aldopentaouronic acid in which both MeGlcA and the acetyl group is linked to penultimate Xyl*p* residue from the non-reducing end; Ara^2Xyl_2, Xyl_2 with Ara*f* residue linked to the non-reducing end Xyl*p* residue; Ara^3Xyl_3, Xyl_3 with Ara*f* residue linked to the non-reducing end Xyl*p* residue; $Ara^{3,3}_2Xyl_3$, Xyl_3 with two Ara*f* residues linked to the non-reducing end Xyl*p* residue at position 2 and 3.

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1. Introduction

The increasing worldwide effort to develop economically feasible and environmentally friendly procedures for bioconversion of plant biomass involves also the fraction of hemicellulose polysaccharides that are separated from cellulose and lignin during various pretreatments. The major plant hemicellulose of interest is xylan, a branched plant polysaccharide, characteristic feature of which is the backbone built of β-1,4-linked D-xylopyranosyl (Xyl*p*) residues. Its fine structure changes from plant to plant, and even in different parts of the same plant (Ebringerová et al., 2005). Recent data indicates that differences in structure occur even within the same xylan molecule (Busse-Wicher et al., 2014). The variations are due to different side carbohydrate and acid substituents. With the exception of softwood xylan, in all other plants xylans are partially acetylated and in some cases also esterified with phenolic acids. Alkaline pretreatments afford deacetylated polysaccharide, e.g. glucuronoxylan from hardwood, arabinoxylan from cereal endosperm and arabinoglucuronoxylan from agricultural side products, such as straw and bran, and different maize residues. Milder alkaline pretreatments preserve ester linkages between Ara*f* side substituents and phenolic acids, mainly ferulic acid (Schendel et al., 2015). Non-alkaline pretreatments, such as steam explosion and autohydrolysis, yield partially acetylated hemicellulose (Alvira et al., 2010; Appeldoorn et al., 2013; Selig et al., 2008; Wyman et al., 2005).

Over the past two decades a great progress in enzymology of plant xylan degradation and saccharification has been achieved. New types of depolymerizing xylanases and debranching enzymes have been described in microorganisms and the structure-function relationship of accessory xylanolytic enzymes has become better understood. However, since the available data are scattered over the vast amount of published literature, and some xylan structures were recognized as recalcitrant to known enzymes only recently, we feel that it is of extreme importance to summarize current knowledge on microbial xylanolytic enzymes and outline possible strategies of enzymatic xylan degradation to oligosaccharides and also towards monosaccharide building blocks. The target sugar is naturally xylose which can serve as a source for production of a number of compounds including ethanol (Dodd and Cann, 2009; Gírio et al., 2010). As an added value to the previous reviews on this subject (Aachary and Prapulla, 2009; Beg et al., 2001; Deutschmann and Dekker, 2012; Keshwani and Cheng, 2009; Polizeli et al., 2005; Saha, 2003; Shallom and Shoham, 2003; Van den Brink and de Vries, 2011; Van Dyk and Pletschke, 2012), we distinguish here between xylans that were extracted from plant biomass in the presence of alkali which destroys almost all ester linkages, and xylans obtained under non-alkaline conditions which preserve considerable portion of ester linkages, and afford partially esterified polysaccharide that corresponds to its native form in plants. In all cases the enzymatic hydrolysis of xylan passes the stage of oligosaccharides which can also be considered as desired products. They find applications as prebiotics, dietary fibers and antioxidants (Broekaert et al., 2011).

2. Xylans and xylanolytic enzymes

2.1. Alkali extracted glucuronoxylan

Alkali extracted glucuronoxylan is the main hardwood hemicellulose which in average contains about one MeGlcA side residue per 10 main chain Xyl*p* residues (McGinnis and Shafizadeh, 1980; Takahashi and Koshijima, 1988). The reported yields of the polysaccharide extracted usually from delignified hardwood pulp reach 20% of the starting wood (Ebringerová et al., 1967). In native state, the polysaccharide is partially acetylated (Ebringerová et al., 2005; Timell, 1967; Wilkie, 1983), and also linked to lignin via ester linkages between lignin alcohols and carboxyl group of MeGlcA side residues (Jeffries, 1990; Takahashi and Koshijima, 1988). In situ reduction of MeGlcA esters to 4-*O*-methyl-D-glucose with $NaBH_4$ in beechwood sawdust suggested that about 30% of MeGlcA residues are esterified (Takahashi and Koshijima, 1988). However, any alkali treatment of hardwood leads to complete xylan deacetylation and obviously also to disruption of its ester linkages with lignin. The distribution of MeGlcA side residues shows some regularity, but several lines of evidence suggest that alkaline extraction offers a mixture of heterogeneous xylan molecules differing in the degree of substitution with MeGlcA. It is also highly possible that several or at least two types of xylans with varying content of MeGlcA are co-extracted. The ratio Xyl:MeGlcA 10:1 is most probably an average value, since DMSO-extracted acetylated glucuronoxylan from delignified hardwood pulp shows considerably lower MeGlcA content than the polysaccharide extracted by alkali [Naran et al., 2009; Vršanská and Biely, unpublished results]. Recent structural studies of xylans from softwood Japanese cedar and Hinoki cypress (Ishii et al., 2010) showed a strange distribution of MeGlcA residues, frequently also on two neighboring Xyl*p* residues. Hardwood xylan, e.g. from beechwood, appears to be quite heterogeneous since its more soluble fraction also possesses higher MeGlcA content (Biely et al., 2015). Thus, the MeGlcA content determines physico-chemical properties, mainly the polysaccharide solubility. Lower frequency of substitution leads to higher probability of association of xylan chains among themselves and with cellulose, via hydrogen bond interaction of unsubstituted regions of xylan molecules.

2.1.1. Glucuronoxylan depolymerization by xylanases

For simplification we shall discuss the enzymes needed to saccharify an average alkali extracted hardwood glucuronoxylan. Depolymerization can be achieved by three types of *endo*-β-1,4-xylanases that have been classified in glycoside hydrolase (GH) families 10, 11 and 30 (Biely et al., 1997; Collins et al., 2005; Pollet et al., 2010) (Fig. 1). The mode of glucuronoxylan cleavage by GH10, GH11 and GH30 enzymes differs mainly in the way of acceptance and recognition of the MeGlcA side residues in the process of formation of the productive enzyme-substrate complexes (Biely et al., 1997; Fujimoto et al., 2004; Pell et al., 2004; Pollet et al., 2010). With exception of the reducing end, GH10 xylanases require two consecutive unsubstituted Xyl*p* residues to attack the xylan main chain, and are capable of cleaving the glycosidic linkage to MeGlcA-substituted Xyl*p* residue. GH11 xylanases require three unsubstituted Xyl*p* residues in a row and attack xylan chain one linkage before the Xyl*p* substituted with MeGlcA (Fig. 1). The two glycosidic linkages following the MeGlcA branch are not attacked by both types of enzymes. For these reasons the GH10 xylanases generate, as a rule, products by one Xyl*p* residue shorter that GH11 xylanases (Fig. 1). While Xyl_2 and aldotetraouronic acid $MeGlcA^3Xyl_3$ predominate in the GH10 hydrolysate, Xyl_2, Xyl_3 and aldopentauronic acid $MeGlcA^3Xyl_4$ are the major products of GH11 xylanases. GH10 xylanases usually generate more Xyl that GH11 enzymes. Looking on Fig. 1, we immediately recognize what should be the other enzymes to complete the hydrolysis of generated oligosaccharides. Neutral, linear xylooligosaccharides, mainly Xyl_2 and Xyl_3, have to be hydrolyzed to the monomer by β-xylosidase, the second type of hydrolase attacking the β-1,4-xylosidic

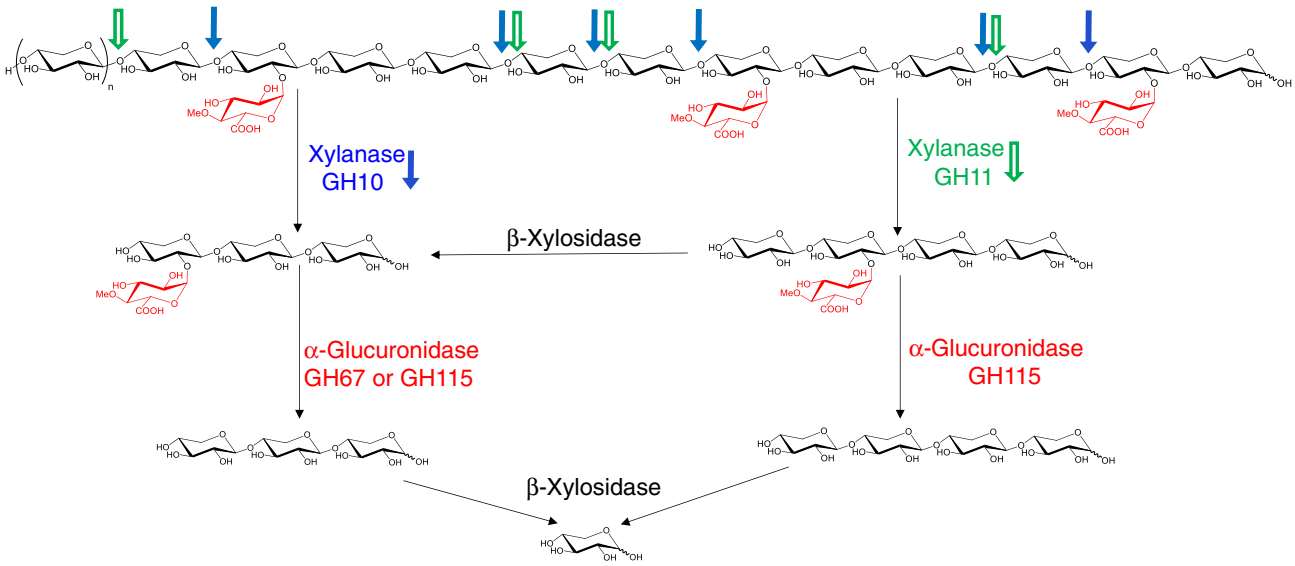

Fig. 1. Scheme of enzymatic breakdown of alkali extracted glucuronoxylan by xylanases of GH families 10 and 11. The enzymes generate neutral and acidic fragments. Only acidic oligosaccharides, aldouronic acids, and their hydrolysis to monosaccharides by β-xylosidase and GH67 and GH115 α-glucuronidases is shown.

linkages. These enzymes liberate Xyl from the non-reducing end of xylooligosaccharides and can shorten the aldopentaouronic acid MeGlcA^{3}Xyl$_4$ to aldotetraouronic acid MeGlcA^{3}Xyl$_3$ (Fig. 1). Since the structure-specificity relationship of β-xylosidases of different GH families (GH3, 39, 43 and 52) has not been sufficiently clarified (Lagaert et al., 2014), they may not behave in the same way on branched oligosaccharides. We shall dedicate more attention to the mode of action of β-xylosidases when discussing the saccharification of other types of xylans.

An important role in degradation of linear xylooligosaccharides is also played by exoxylanases releasing xylose from the reducing end. These enzymes occur in GH8 family and are active on both linear and branched oligosaccharides (Honda and Kitaoka, 2004; Hong et al., 2014; Juturu and Wu, 2014; Lagaert et al., 2007; Valenzuela et al., 2016).

Family GH8 harbours also endoxylanases. An enzyme isolated from a psychrophilic bacterium, exhibited lower specific activity on glucuronoxylan than on rhodymenan, a β-1,3-β-1,4-xylan, as a rhodymenanase (Collins et al., 2002). Since a detail mode of action of this enzyme is unknown it will not be discussed in this article.

Under the assumption that the Xyl:MeGlcA ratio in glucuronoxylan is around 10, xylanase and β-xylosidase treatment would theoretically leave about 30% of Xyl bound in the form of aldouronic acid. This underlines the importance of the accessory enzyme α-glucuronidase, to achieve complete saccharification.

2.1.2. α-Glucuronidase

The α-1,2-linkage of MeGlcA to the main chain Xyl*p* residue is one of the most acid stable glycosidic linkages in plant cell walls. Aldobiouronic acid MeGlcA-α-1,2-Xyl persists in the acid hydrolysate of glucuronoxylan. Fortunately the enzymes cleaving this linkage have been found in xylanolytic systems of microorganisms (Dekker, 1983; de Vries et al., 1998; Puls et al., 1987). Two types of microbial α-glucuronidases have been recognized up to now. Better known are α-glucuronidases classified in GH67 family. The members of this family can liberate MeGlcA only from oligosaccharides that carry the uronic acid on the non-reducing end Xyl*p* residue (Biely et al., 2000; Nagy et al., 2003; Nurizzo et al., 2002; Zaide et al., 2003). These enzymes are unable to debranch polysaccharide or oligosaccharides in which MeGlcA is linked to internal Xyl*p* residues. This means that aldopentaouronic acid MeGlcA^{3}Xyl$_4$ or longer aldouronic acids generated by GH11 xylanases will be debranched only after their non-substituted non-reducing Xyl*p* residues are removed by β-xylosidase (Fig. 1).

Another combination of the enzymes cleaving the β-1,4-glycosidic linkages of glucuronoxylan main chain would be with the second type of α-glucuronidase classified in GH115 (Fig. 1). In addition to the ability to catalyze the same reaction as GH67 enzymes, GH115 α-glucuronidases also liberate MeGlcA linked to internal Xyl*p* residues in oligo- and polysaccharides (Ryabova et al., 2009; Tenkanen and Siika-Aho, 2000). GH115 enzymes, in contrast to GH67 enzymes, possess a cleft that can accommodate Xyl*p* residues at both sides of the MeGlcA side substituent (Golan et al., 2003; Kolenová et al., 2010; Murciano Martínez et al., 2016; Rogowski et al., 2014; Wang et al., 2016).

Thus the combination of any of GH10 and GH11 xylanase with β-xylosidase and GH67 or GH115 α-glucuronidase should be in theory sufficient to achieve a complete saccharification of alkali extracted hardwood xylan. This usually does not happen due to several reasons: i) at high polysaccharide concentration the generated products may inhibit further action of hydrolases, so the hydrolysis yields are determined by a thermodynamic equilibrium; ii) part of the polysaccharide is resistant to the action of enzymes due to dense branching that blocks the formation of productive enzyme-substrate complexes; iii) retaining xylanases and xylosidases catalyze glycosyl transfer reactions at high oligosaccharide concentration. One of such reactions catalyzed by both GH10 and GH11 xylanases is for instance the conversion of Xyl$_3$ to Xyl$_2$ instead of its hydrolysis to Xyl and Xyl$_2$ which takes place at low substrate concentration (Biely et al., 1981; Vršanská et al., 1982). Thus the substrate concentration may regulate the monomer and dimer ratio. Moreover, in the presence of ethanol in the reaction mixtures, e.g. during simultaneous saccharification and fermentation, the transfer of xylosyl residues to ethanol instead of water leads to the formation of ethyl β-glycosides (Appeldoorn et al., 2013; Zhang et al., 2009), a reaction which is reversible.

An example of glucuronoxylan that contains structural features, complicating its enzymatic hydrolysis, is found in eucalyptus. It has been reported that approximately 30% of MeGlcA side residues of such polysaccharide is further 2-*O*-α-substituted by D-Gal*p* residues (Kabel et al., 2002b; Magaton et al., 2012; Shatalov et al., 1999; Teleman et al., 2000). Gal will obviously block the action of α-glucuronidases, so its removal with a proper α-galactosidase would be required. The galactosylation of MeGlcA side residue does not seem to prevent the GH10 xylanase attack of the glycosidic linkage to 2-*O*-α-substituted Xyl*p* residue since acidic fragments heavier by a hexose unit were found in the eucalyptus xylan GH10 hydrolysate (Kabel et al., 2002a). Recent evidence for GH10 and GH11 xylanase-generated glucuronoxylan

fragments containing also two MeGlcA residues on Xyl_5 and Xyl_6, suggests that the hydrolysis of the main chain does not take place when the substituted Xyl*p* residues are separated by one or two unsubstituted Xyl*p* residues (Biely et al., 2015). It remains to be established how close could be the substituted Xyl*p* residues to be accessible to GH115 α-glucuronidases.

2.1.3. GlcA/MeGlcA-dependent GH30 xylanases

The GH30 family harbours xylanases specialized for hydrolysis of glucuronoxylan. Originally the enzymes were classified in GH5 family. Their mode of cleavage of glucuronoxylan main chain is determined by MeGlcA or GlcA substituents. The first enzyme of this type was reported before the CAZy classification (Nishitani and Nevins, 1991). Majority of bacterial GH30 xylanases (St John et al., 2014) but also some fungal species, e.g. *Trichoderma reesei* XYN VI (Biely et al., 2014a), attack glucuronoxylan main chain at the second glycosidic linkage to the MeGlcA substituents towards the reducing end (Fig. 2) (Gallardo et al., 2010; St John et al., 2006; Vršanská et al., 2007). MeGlcA or GlcA thus determines the sites of polysaccharide cleavage. The enzymes show extremely low specific activities on unsubstituted xylan chain, linear xylooligosaccharides or xylan that does not contain uronic acid side residues. From hardwood glucuronoxylan they generate a mixture of aldouronic acids of general formula $MeGlcA^2Xyl_n$ where n is dependent on distribution of uronic acid side residues on the main chain (Fig. 2). All these aldouronic acids can be theoretically debranched by GH115 α-glucuronidase and then degraded to Xyl by other types of xylanases and β-xylosidase, or converted by the action of β-xylosidase to $MeGlcA^2Xyl_2$ which would serve as a substrate for GH67 α-glucuronidase (Fig. 2). More densely MeGlcA-substituted glucuronoxylan regions may be more recalcitrant to the action of GH30 enzymes.

There are reports that GH30 xylanases can also cleave neutral xylooligosaccharides (Gallardo et al., 2010) and acetylated xylan chain not substituted by MeGlcA (Busse-Wicher et al., 2014). We believe that this activity is just a side activity of GH30 xylanases observed at very high enzyme load. The enzymes undoubtedly recognize the carboxyl of the uronic acid residue. The elimination of the carboxyl group by esterification with methanol or conversion of MeGlcA to 4-*O*-methyl-D-glucose leads to enormous decrease of specific activity of GH30 xylanases. Interesting to mention, however, the cleavage of the modified glucuronoxylan takes place again at the second glycosidic linkage following the branch towards the reducing end (Biely et al., 2015).

2.2. Acetyl glucuronoxylan

All procedures for biorefining of plant biomass that do not involve alkaline pretreatments will generate partially acetylated hemicellulose. This material is structurally more similar to the native plant polysaccharide, but is usually partially depolymerized. It is also well soluble, since the acetyl groups prevent association of unsubstituted xylan segments and longer xylooligosaccharides via hydrogen bonding. Steam explosion of hardwood affords acetylated glucuronoxylan, in which about 60% Xyl*p* residues are acetylated. A major portion of Xyl*p* residues is monoacetylated at position 2 or 3, and minor portion is 2,3-di-*O*-acetylated. 3-*O*-Acetylation frequently accompanies the 2-substitution of Xyl*p* residues with MeGlcA (Evtuguin et al., 2003; Naran et al., 2009; Teleman et al., 2000, 2002) (Fig. 3). The acetylation makes the polysaccharide more xylanase resistant and some structures in which the MeGlcA substitution is combined with acetylation of the vicinal hydroxyl group, appear to be the most recalcitrant. On the other hand, high solubility acetylated xylan positively affects its hydrolysis, although xylanases attack the polysaccharide only at regions that are unsubstituted or where the acetyl group at position 2 or 3 is tolerated by xylanase. The latter aspect of the mode of action of xylanases on acetylated polysaccharide remains to be elucidated. GH10 xylanase can tolerate acetyl groups at subsite +1 and also at −2 subsite, but only at *O*-3 position (Fujimoto et al., 2004; Pell et al., 2004). Consequently, the spectrum of products generated by GH10 and GH11 xylanases from hardwood acetyl glucuronoxylan is more complex than that from alkali extracted polysaccharide. Linear xylooligosaccharides cannot be hydrolyzed to shorter fragments because the presence of the acetyl group on any of the three positions of the non-reducing end Xyl*p* residue blocks the action of β-xylosidase (Biely et al., 2004). A similar situation occurs also with acidic xylooligosaccharides. Both types of xylanases generate from acetylated glucuronoxylan series of longer acidic oligosaccharides with different number of acetyl groups reaching almost the number of Xyl*p* residues (Table 1). With exception of a small amount of xylobiose, the hydrolysates essentially do not contain non-acetylated fragments. Table 1 shows the dramatic reduction of the number of products of xylanase action on deacetylated polysaccharide and also indicates that GH10 xylanases generate shorter products than GH11 xylanases also from acetylglucuronoxylan.

Addition of GH67 or GH115 α-glucuronidase to a mixture of xylanase and β-xylosidase would bring about only small changes in acetylglucuronoxylan saccharification yield, because the xylan

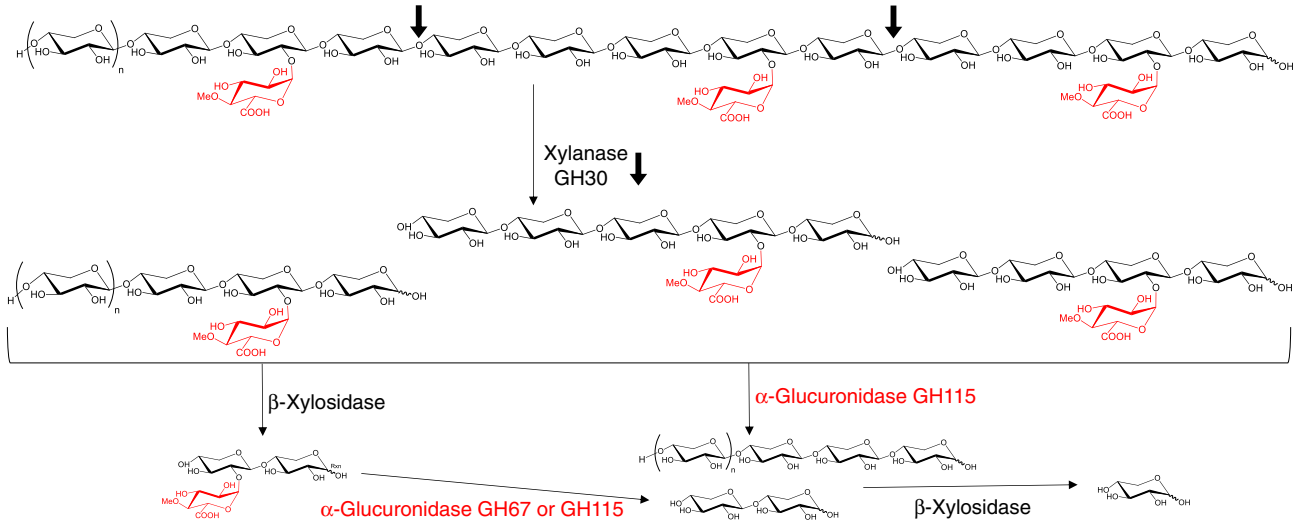

Fig. 2. Scheme of enzymatic breakdown of alkali extracted glucuronoxylan by xylanases of GH family 30. The enzymes generate acidic fragments, aldouronic acids, with one unsubstituted Xyl residue at the reducing end. The aldouronic acids are further hydrolyzed to monosaccharides by β-xylosidase and GH67 and GH115 α-glucuronidases. The enzymes appear to hydrolyze also linear parts of the polysaccharide, however at a rate by several orders of magnitude lower.

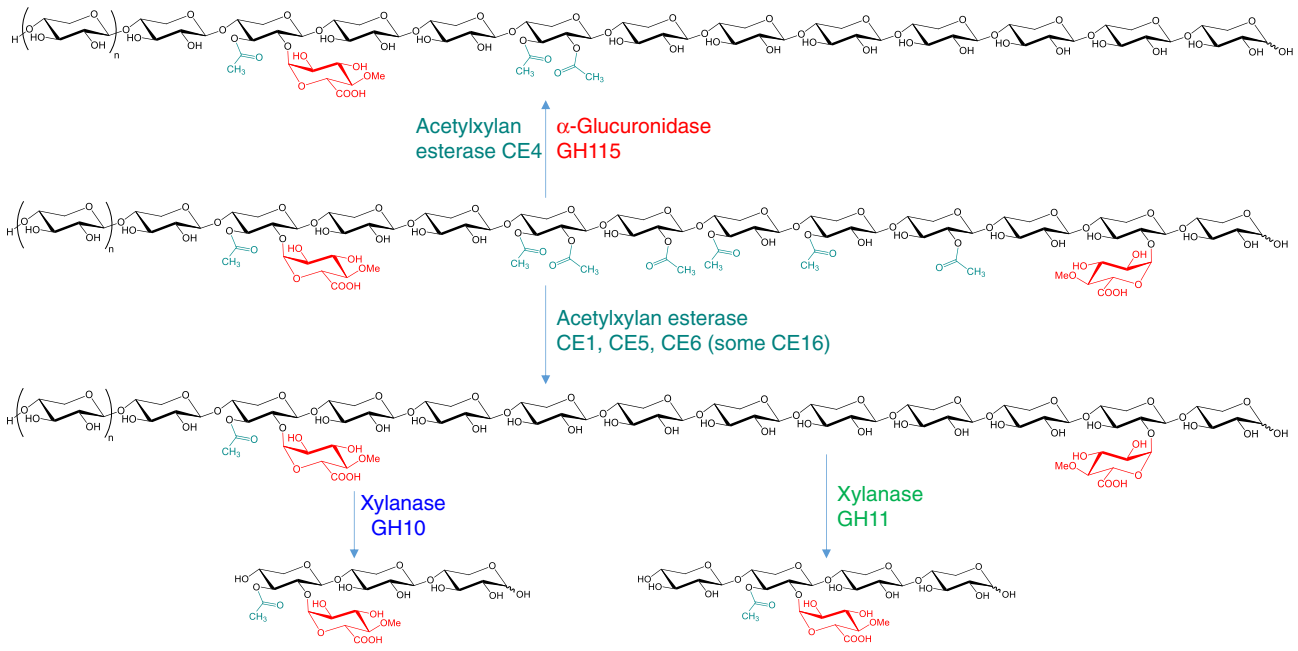

Fig. 3. Scheme of deacetylation of acetylglucuronoxylan (the second chain from the top) by acetylxylan esterases of CE families 1, 4, 5 and 6 and subsequent degradation by GH10 and GH11 xylanases. Acetylxylan esterases do not attack the 3-*O*-acetyl group on Xyl*p* residue substituted with MeGlcA. Doubly acetylated Xyl*p* residues do not serve as substrate for CE4 acetylxylan esterases. Xylanases of GH families 10 and 11 generate the same aldouronic acids as from deacetylated xylan (Fig. 1; MeGlcA^{3}Xyl$_3$ and MeGlcA^{3}Xyl$_4$, respectively), but also those which are 3-*O*-acetylated on Xyl*p* residue substituted with MeGlcA (formulas on the bottom).

Table 1
Sodium adducts of neutral and acidic xylooligosaccharides generated from aspen acetylglucuronoxylan by GH10 (*Clostridium thermocellum*) and GH11 (Optimase, Genencor) endoxylanases as revealed by MALDI ToF MS (reproduced with permission in a slightly modified form from Journal of Applied Glycoscience (Biely et al., 2014b). Symbols: ★, sodium adducts of major products and (★) minor products found in the enzymatic hydrolysate of chemically deacetylated glucuronoxylan. $^+$, sodium adducts of aldouronic acids with two MeGlcA residues.

Compound	Ions of neutral oligosaccharides by GH10	Ions of acidic oligosaccharides by GH11	Compound	Ions of neutral oligosaccharides by GH10	Ions of acidic oligosaccharides by GH11
Xyl	★ (★)	(★)	MeGlcAXyl$_2$	(★)	
Xyl$_2$	305 ★	★	MeGlcAXyl$_2$Ac	537	
Xyl$_2$Ac	347		MeGlcAXyl$_2$Ac$_2$		
Xyl$_2$ Ac$_2$	389		MeGlcAXyl$_3$	★	(★)
Xyl$_3$	(★)	★	MeGlcAXyl$_3$Ac	669	669
Xyl$_3$Ac	479	479	MeGlcAXyl$_3$Ac$_2$	711	
Xyl$_3$Ac$_2$	521		MeGlcAXyl$_3$Ac$_3$	753	
Xyl$_3$Ac$_3$	563		MeGlcAXyl$_4$	(★)	★
Xyl$_4$		(★)	MeGlcAXyl$_4$Ac	(801)	(801)
Xyl$_4$Ac			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$_5$		(★)
Xyl$_4$Ac$_4$	737		MeGlcAXyl$_5$Ac$_2$	(975)	975
Xyl$_5$Ac			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$_6$		1307	MeGlcAXyl$_9$Ac$_7$	1713	1713
Xyl$_8$Ac$_7$		1349	MeGlcAXyl$_9$Ac$_8$	1755	1755
Xyl$_8$Ac$_8$			MeGlcA$_2$Xyl$_7$Ac$_4$ $^+$		1513
Xyl$_9$Ac$_7$			MeGlcA$_2$Xyl$_7$Ac$_5$ $^+$		1555
Xyl$_9$Ac$_7$		1439	MeGlcA$_2$Xyl$_8$Ac$_4$ $^+$		1645
Xyl$_9$Ac$_8$		1481	MeGlcA$_2$Xyl$_8$Ac$_5$ $^+$		1687

fragments would remain still esterified with acetic acid. Therefore, on deglucuronylated oligosaccharides the action of β-xylosidase would stop at the first acetylated Xyl*p* residue from the non-reducing end. It becomes clear that enzymatic saccharification cannot proceed to completion without deacetylation of xylooligosaccharides by acetylxylan esterases. Due to the lack of information on the action of MeGlcA/GlcA-dependent GH30 xylanases on acetylglucuronoxylan, these xylanases will not be discussed in connection with saccharification of acetylated xylan.

2.2.1. Acetylxylan esterases

These are the enzymes which liberate acetic acid esterifying position 2 and 3 on mono- and di-*O*-acetylated Xyl*p* residues in polysaccharides and oligosaccharides and make the xylan main chain accessible to xylanases and β-xylosidase (Fig. 3). The enzymes which show this ability have been classified in carbohydrate esterase (CE) families 1, 4, 5 and 6 (Biely, 2012; Biely et al., 2013; Neumüller et al., 2015). A low specific activity on the polymeric substrate was recently observed with some deacetylases of family CE16 (Puchart et al., 2016). Acetylxylan esterases of CE4 family differ from the rest by requiring for the deacetylation of position 2 or 3 a free vicinal hydroxyl group (Biely et al., 2007), which means that CE4 acetylxylan esterases do not attack doubly acetylated Xyl*p* residues (Fig. 3). None of the enzymes in the four mentioned families can deacetylate position 3 on Xyl*p* residues substituted with MeGlcA (Neumüller et al., 2015; Uhliariková et al., 2013). The uronic acid represents a serious steric barrier for esterases. Thus the corresponding 3-*O*-acetylated aldouronic acids generated from acetylated glucuronoxylan by a mixture of xylanase, β-xylosidase, α-glucuronidase and acetylxylan esterase behave as the most recalcitrant structures in hardwood acetylglucuronoxylan (Fig. 3). The aldouronic acids with acetyl group and MeGlcA on the non-reducing end of Xyl_3, $Ac^3MeGlcA^3Xyl_3$, will persist in the GH10 hydrolysate as the main acidic product in the presence of the above mentioned xylanolytic enzymes (Puchart et al., 2016). The 3-*O*-acetyl group on the non-reducing end Xyl*p* residue substituted with MeGlcA blocks the action of GH67 α-glucuronidase, while the presence of MeGlcA prevents the deacetylation of position 3 by acetylxylan esterases. It remains to be established whether a similar enzyme cocktail with GH11 xylanase would end up at the level of $Ac^3MeGlcA^3Xyl_4$ or $Ac^3MeGlcA^3Xyl_3$, and whether $Ac^3MeGlcA^3Xyl_4$ would serve as a substrate for β-xylosidase to be shortened to $Ac^3MeGlcA^3Xyl_3$. At least GH3 β-xylosidases, such as those from *Trichoderma reesei* or *Aspergillus niger*, do not liberate a non-substituted non-reducing Xyl*p* residue linked to a 3-*O*-glycosylated Xyl*p* residue (Herrmann et al., 1997; Kormelink et al., 1993a; Smaali et al., 2006; Tenkanen et al., 1996). The xylose is cleaved off only when the penultimate Xyl*p* residue is either non-substituted or substituted by a sugar residue at position 2. It has not been established whether the enzyme will react similarly to the smaller 3-*O*-acetyl group, e.g., whether the presence of the acetyl group at position 3 of $Ac^3MeGlcA^3Xyl_4$ will also block the release of the non-reducing unsubstituted Xyl*p* residue (Fig. 4, question mark). Preliminary data suggest that the same acetyl group prevents the action of GH115 α-glucuronidase on $Ac^3MeGlcA^3Xyl_3$, $Ac^3MeGlcA^3Xyl_4$ and the polymeric substrate (Puchart V., unpublished data).

The only enzyme having the capability of deacetylating $Ac^3MeGlcA^3Xyl_3$ is the CE16 acetyl esterase from *Trichoderma reesei* (Puchart et al., 2016), an inducible constituent of the cellulolytic/hemicellulolytic system of the fungus (Bischof et al., 2013; Häkkinen et al., 2012; Li et al., 2008; Poutanen and Sundberg, 1988). In the above aldotetraouronic acid the *Tr*CE16 deacetylated both position 3 and position 4, where the acetyl group migrated (Fig. 4). Hence, it is obvious that the $Ac^3MeGlcA^3Xyl_3$ degradation requires in the first step deacetylation of position 3 or 4, which is then followed by action of α-glucuronidase and β-xylosidase. *A. niger* and *P. anserina* CE16 esterases show *exo*-mode of action similar to *T. reesei* CE16, however, they exhibit a slow deacetylation of positions 2 and 3 also on internal Xyl*p* residues not substituted with MeGlcA. In $Ac^3MeGlcA^3Xyl_3$ they deacetylated only position 4 (Koutaniemi et al., 2013; Neumüller et al., 2015; Puchart et al., 2015; Puchart et al., 2016) (Fig. 4). A unifying catalytic property of CE16 esterases is their capability to deacetylate also position 4 on non-reducing end Xyl*p* residues where the acetyl group migrates easily from position 3 (Puchart and Biely, 2015; Puchart et al., 2016). We cannot exclude the existence of esterases releasing the 3-*O*-acetyl group from internal Xyl*p* residues substituted with MeGlcA, however, such enzymes have not been discovered yet.

In conclusion we could define the enzyme mixture required for complete breakdown of acetylated glucuronoxylan. Depolymerization by an endoxylanase should be accompanied by the action of β-xylosidase, α-glucuronidase, acetylxylan esterase and an *exo*-acting acetyl esterase deacetylating oligosaccharides at the non-reducing ends (Fig. 4). α-Galactosidases could contribute to a higher degree of saccharification in the case of xylans similar to that found in the eucalyptus wood. The enzymes would act in a synergistic manner as demonstrated by several examples (Biely et al., 1986; Poutanen et al., 1990; Zheng et al., 2013).

2.3. Alkali extracted arabinoglucuronoxylan

There are two major types of plant arabinoxylans. The first type, called neutral, corresponds to highly viscous arabinoxylan of cereal seed endosperms. It is the same polysaccharide which appears after grain milling in flour (Cleemput et al., 1995; Courtin and Delcour,

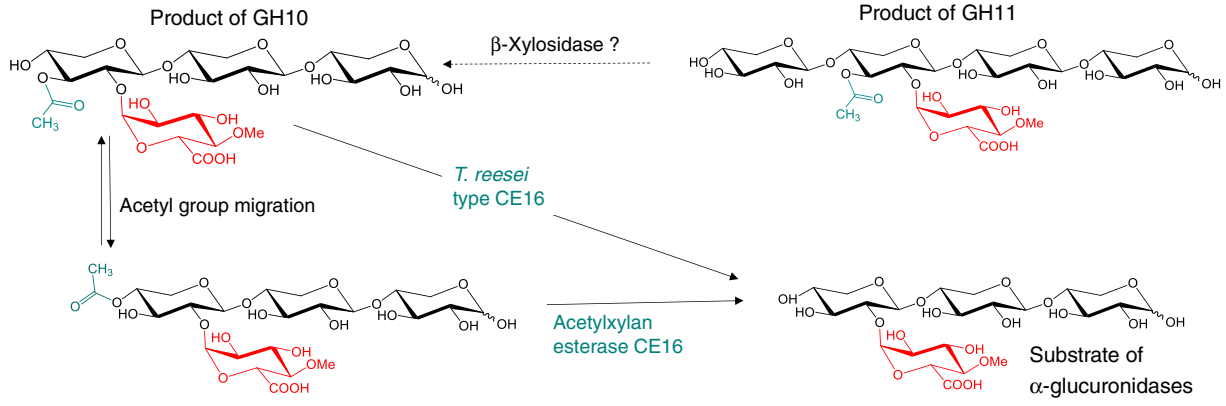

Fig. 4. Degradation pathway of acetylated aldouronic acids generated from acetylglucuronoxylan by GH10 and GH11 xylanases (Fig. 3). The 3-*O*-acetylated aldotetraouronic acid ($Ac^3MeGlcA^3Xyl_3$) can be deacetylated directly by some CE16 deacetylases, e.g. by CE16 from *Trichoderma reesei*. Alternatively, it can be deesterified by CE16 enzymes in the form of its isomer formed by acetyl group migration to position 4. It remains to be established whether the acetylated aldopentauronic acid formed by GH11 xylanases ($Ac^3MeGlcA^3Xyl_4$) does serve as a substrate for β-xylosidase (question mark).

2002; Ebringerová and Hromádková, 1999). This xylan would not serve as a substrate of glucuronic acid-dependent GH30 xylanases since it has been reported to be free of uronic acids side substituents. In native material Xyl*p* residues are also partially acetylated (Appeldoorn et al., 2013; Naran et al., 2009), and some Ara*f* residues are esterified at position 5 with ferulic acid (Faulds et al., 2006), however, alkaline extraction saponifies all ester linkages and eliminates thus all acid substituents. Since neutral arabinoxylans occur mainly in cereal material used as food and fodder, we shall discuss here only the enzymatic saccharification of arabinoglucuronoxylan, so called acidic arabinoxylan that represents the main hemicellulose of several lignocellulosic feedstocks such as wheat bran, wheat straw, various corn and sunflower residues, bagasse, grasses and other annual plants (Brillouet et al., 1982). However, we should mention that an important application of endoxylanases is in bakery and animal diets which leads to reduction of viscosity of the neutral polysaccharide in dough and fodders (Courtin and Delcour, 2002; Hew et al., 1998).

The main chain of acidic arabinoglucuronoxylans is substituted at position 3 or at both position 3 and 2 by α-linked L-Ara*f* residues (Fig. 5). Alkali extracted polysaccharide will be free of acetyl and feruloyl groups. A portion of ferulic acid esters survives mild alkaline pretreatment. The polysaccharide main chain contains also single side residues of 2-*O*-α-linked MeGlcA or GlcA, however, at much lower frequency than in hardwood glucuronoxylan. The degree of xylan main chain substitution by L-Ara*f* and MeGlcA is variable and depends strongly on the extraction conditions and on the plant part. In the group of arabinoglucuronoxylans we can clearly recognize two types differing in the accessibility to xylanase degradation. Arabinoglucuronoxylans of straw, stalks and cobs are branched to a lower degree that those from outer layers of seeds, e.g., of corn or cereal brans (Hespell et al., 1997). Consequently, xylans from the seed coat structures, pericarps, are more resistant to enzyme breakdown than xylans from other parts of plants. Maybe the most xylanase resistant is the corn bran (also called corn fiber) xylan with so densely substituted main chain that it is essentially non-hydrolysable by pure xylanases (Saha, 2001) and highly resistant even to commercial hemicellulose preparations (Saha and Bothast, 1999). We shall dedicate a special section to this hairy polysaccharide.

Interesting structural features of xylan that can occur in other plants including cereals was recently reported in *Arabidopsis thaliana* (Bromley et al., 2013). Xylan of this plant was suggested to contain two different domains. The major domain which is acetylated but less decorated by side chain carbohydrates, may play a role in attachment to cellulose fibrils by hydrogen bonding. The minor, more heavily branched domain, which has a different conformation and does not interact with cellulose, fills the space between cellulose fibrils and contributes to the three-dimensional structure of the cell wall (Busse-Wicher et al., 2014). Degradation of such xylans requires the presence of several debranching enzymes, however, their activity may also be hindered by high density of substituents on the same or neighboring sugar residues.

2.3.1. Deacetylated arabinoglucuronoxylan hydrolysis by xylanases

The polysaccharide serves as a substrate of xylanases classified in four families, GH10, GH11, GH30 and GH5. Each family generates different products (Fig. 5). As we mentioned earlier, GH10 xylanase can cleave the xylan main chain if there are two consecutive unsubstituted Xyl*p* residues. Linear xylooligosaccharides would be shortened mainly to Xyl_2 and all branched oligosaccharides would have the substituents on the non-reducing end. The reducing end should contain two unsubstituted Xyl*p* residues like in the case of glucuronoxylan hydrolysis. However, this mode of cleavage of the main chain is not the only one. In GH10 hydrolysate of arabinoglucuronoxylan one can find branched oligosaccharides with only single unsubstituted Xyl*p* residue on the reducing end, or with additional Xyl*p* on the non-reducing end (Kormelink et al., 1993a; Pastell et al., 2008; Rantanen et al., 2007; Vardakou et al., 2003). Some of such fragments could originate from the reducing ends of the polysaccharide, however, their abundance

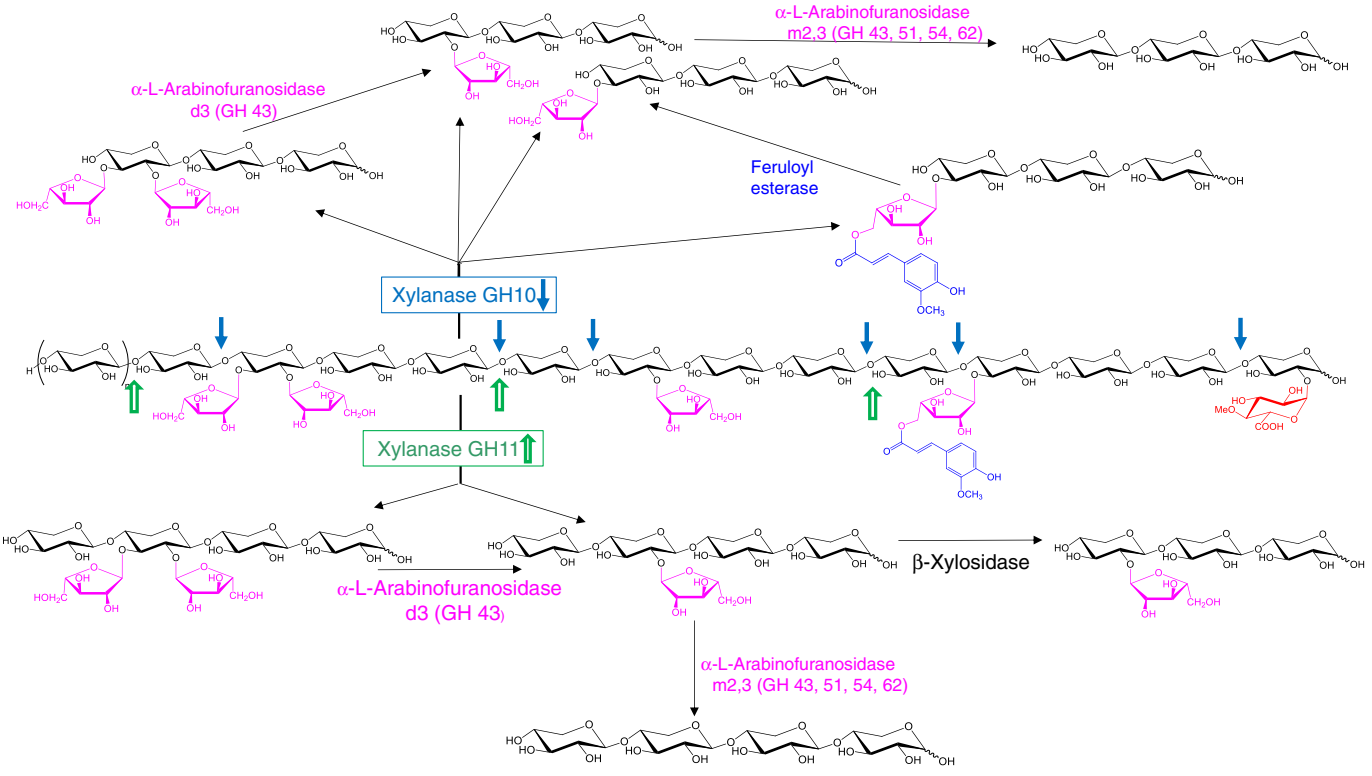

Fig. 5. Scheme of enzymatic breakdown of alkali extracted arabinoglucuronoxylan by xylanases of GH families 10 and 11. Only arabinosylated fragments are shown. Their hydrolysis to monosaccharides requires action of two types of α-L-arabinofuranosidases and β-xylosidase. The α-L-arabinofuranosidases are typically active either on 2- or 3-monoarabinosylated Xyl*p* residues (m2,3 type enzymes; GH43, GH51, GH54 and GH62 families) or on doubly arabinosylated Xyl*p* residues (d3 type enzymes; some GH43 members and one GH51 member) from which they liberate solely Ara*f* from position 3.

appears to be a consequence of so far not clarified aspects of the mode of their action.

According to Kormelink et al. (1993a, 1993b), the number of Xyl*p* residues following the branch in products generated by GH10 xylanases depends on the position substituted by Ara*f*. If Ara*f* is linked to position 3, the enzymes generate Ara^2Xyl_2, if to position 2, Ara^3Xyl_3 appears to be the shortest branched product. In case Xyl is doubly arabinosylated, the product is always $Ara_2^{3,3}Xyl_3$ (Fig. 5). The feruloyl substituents on 3-linked Ara*f* do not impede the action of xylanases, as the feruloyl arabinoxylobiose (FeA^2Xyl_2; FAXX) is the shortest feruloylated oligosaccharide liberated by GH10 xylanase. A similar rule applies to some β-xylosidases. They liberate the non-reducing Xyl*p* residue linked to a 2-*O*-substituted one, but do not attack the same residue linked to a 3-*O*-substituted Xyl*p* residue (Herrmann et al., 1997; Tenkanen et al., 1996).

As mentioned earlier, GH11 requires three consecutive unsubstituted Xyl*p* residues. Linear xylooligosaccharides are shortened to Xyl_2 and Xyl_3, depending on the enzyme, but all GH11 xylanases produce branched oligosaccharides with unsubstituted non-reducing end. This is either a Xyl*p* residue in the case when the enzyme attacks the main chain locus with three unsubstituted Xyl*p* residues, or a xylobiosyl residue when the enzyme attack took place in the middle of four consecutive unsubstituted Xyl*p* residues. As a rule, all branched oligosaccharides generated by GH11 xylanases contain two unsubstituted Xyl*p* residues at the reducing end (Kormelink et al., 1993b; Wan et al., 2014) (Fig. 5). The shortest products generated by GH11 xylanases correspond to Xyl_4 substituted by one or two Ara*f* residues at the penultimate Xyl*p* residue from the non-reducing end, i.e. Ara^3Xyl_4 or $Ara_2^{3,3}Xyl_4$ (Kormelink et al., 1993a, 1993b; Puchart and Biely, 2008). Though the composition of the hydrolysate, however, is more complex than indicated here, the oligosaccharides can be produced in industrial scale and used as prebiotics (e.g. Broekaert et al., 2016; Rivas et al., 2013).

The action of GH30 enzymes of arabinoglucuronoxylan has not been investigated. Based on what we know about the mode of action on glucuronoxylan we can expect cleavage of the main chain at the second glycosidic linkage following the GlcA or MeGlcA branch towards the reducing end of the polysaccharide chain. The generated oligosaccharides would be very large due to a low degree of substitution of the main chain with uronic acids. It remains to be established how the neighboring Ara*f* substituents will affect the recognition of the uronic acid residues or whether other branches could also be recognized by GH30 xylanases.

The GH5 xylanase specialized for hydrolysis of arabinoxylans was described only recently and was found to be an appendage-dependent xylanase, that recognizes α-1,3-linked Ara*f* side residues (Correia et al., 2011). The enzyme does not attack linear regions of xylan or linear xylooligosaccharides or glucuronoxylan. The enzyme cleaves xylan main chain at the glycosidic linkage of arabinosylated Xyl*p* residue which means that it generates fragments branched at the reducing end (Fig. 6A). Their nature and mainly their length will depend mainly on density of substitutions. Detail studies of the mode of action of this type of xylanase are not available. It remains unknown whether the enzyme would cleave also the glycosidic linkage if the side chain Ara*f* is feruloylated or the linkage of the doubly arabinosylated Xyl*p* residue (Fig. 6A, question marks). Important role in hydrolysis of arabinoglucuronoxylans is naturally played by α-glucuronidases as demonstrated on a softwood polysaccharide (McKee et al., 2016).

2.3.2. α-L-Arabinofuranosidases

These enzymes release Ara*f* side residues from arabinoglucuronoxylan or from arabinan, a minor plant hemicellulose (Lagaert et al., 2014). We shall discuss here only the α-L-arabinofuranosidases acting on the former polysaccharide. They are classified in GH families 43, 51, 54 and 62. According to positional specificity and requirement for a free vicinal hydroxyl group on Xyl*p* residue, α-L-arabinofuranosidases can be

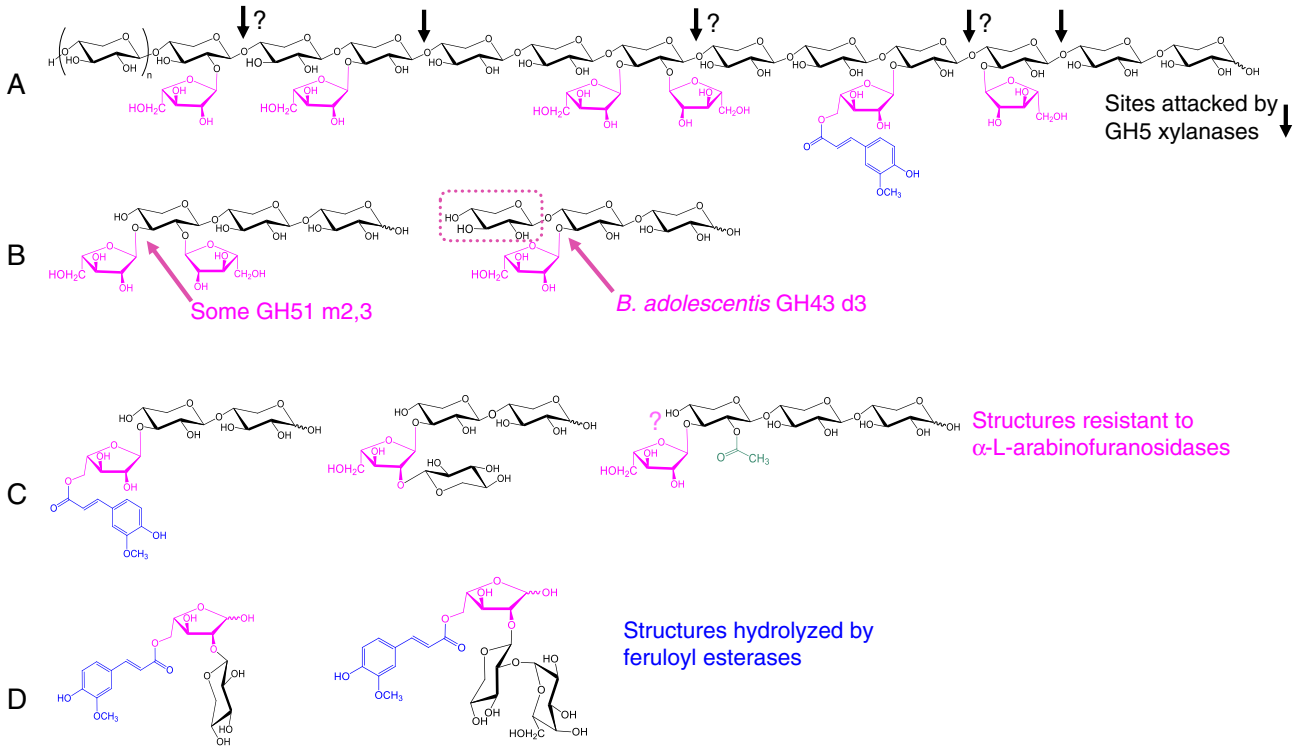

Fig. 6. (A) Scheme of the action of GH5 arabinoxylan-specific β-1,4-xylanase on (glucurono)arabinoxylan. The question marks point to linkages, cleavage of which has not been experimentally demonstrated. (B) The action of some GH51 m2,3 α-L-arabinofuranosidases on doubly arabinosylated non-reducing end Xyl*p* residue ($Ara_2^{3,3}Xyl_3$, the formula on the left), and the action of *Bifidobacterium adolescentis* d3 (GH43) on singly 3-*O*-arabinosylated Xyl_3 (Ara^2Xyl_3, the formula on the right). The Xyl*p*, interacting with the enzyme instead of 2-linked Ara*f*, is encircled. (C) Examples of arabinoxylooligosacchrides resistant to the action of α-L-arabinofuranosidases. Experimental evidence for the resistance of the compound with question mark has not been provided. (D) 5-*O*-Feruloylated arabinooligosaccharide xylan side chains serving as feruloyl esterase substrates.

divided into two groups. The major group consists of enzymes active on Xyl*p* residues monosubstituted by Ara*f* at either position 2 or 3 (Fig. 5). They are marked as m2,3 α-L-arabinofuranosidases (Van Laere et al., 1999) and are disseminated in all mentioned GH families (Wang et al., 2014). The minor group includes enzymes referred to as d3 α-L-arabinofuranosidases that are specific for doubly arabinosylated Xyl*p* residues from which they selectively liberate only the α-1,3-linked Ara*f*, leaving the α-1,2-linked Ara*f* on the main chain (Fig. 5). These enzymes are exemplified by GH43 family α-L-arabinofuranosidases from *Bifidobacterium adolescentis* (Van Laere et al., 1997) and *Humicola insolens* (Sørensen et al., 2006). An exception is the GH51 enzyme produced by *B. adolescentis* (Lagaert et al., 2010). On the basis of phylogenetic analysis the m2,3 and d3 enzymes are grouped to separate clusters within the GH43 and GH51 families (Lagaert et al., 2014; Mewis et al., 2016). However, the division of α-L-arabinofuranosidases into the two substrate and positional specificity groups is not sharp since there are other GH51 and GH54 enzymes that also show significant activity on diarabinosylated Xyl*p* residues (Koutaniemi and Tenkanen, 2016; Lee et al., 2001; Sakamoto et al., 2013). It should be noted that somewhat relaxed specificity of α-L-arabinofuranosidases is observed on oligosaccharides that are doubly arabinosylated at the non-reducing end (Fig. 6B). Such oligosaccharides are generated by GH10 xylanases (Pastell et al., 2008). They were found to be debranched by several GH51 enzymes that are otherwise specialized for internal monoarabinosylated Xyl*p* residues. The primary attack of such enzymes on doubly arabinosylated non-reducing end is the Ara*f* residue at position 3 (Ferré et al., 2000). This preference is explained by its better accessibility in comparison to the neighboring Ara*f* at position 2 (Borsenberger et al., 2014). It is also noteworthy that *B. adolescentis* GH43 d3 enzyme releases the single Ara*f* residue from tetrasaccharide Ara^2Xyl_3 (Biely P., unpublished) (Fig. 6B). Obviously the productive complexes of the enzymes with non-reducing ends or with shorter oligosaccharides must be formed in a different way than with arabinosylated Xyl*p* residues linked to the xylan main chain. In the enzyme-substrate complex formation with the non-reducing end, the role of one Xyl*p* residue is taken by Ara*f* residue, and vice versa. This idea is also supported by the observation that GH43 d3 enzyme from *H. insolens* and GH43 d3 and GH51 m2,3 enzymes from *B. adolescentis* liberate 3-linked Ara*f* not only from 2,3-doubly substituted Xyl*p* but also from 2,3-doubly substituted Ara*f* residues in arabinan (Cartmell et al., 2011; Lagaert et al., 2010).

The two different types of α-L-arabinofuranosidases exhibit synergism of mutual action and also with xylanases (Sørensen et al., 2007b; Valls et al., 2016). α-L-Arabinofuranosidases are unable to liberate Ara*f* residues that are esterified with phenolic acids or otherwise substituted, e.g. with Xyl as shown in Fig. 6C. A portion of side chain Ara*f* remains esterified with ferulic acid even after mild alkaline treatment (Fang et al., 2000; Ford, 1989; Pan et al., 1998). It has been demonstrated that the enzymes are inactive even on Ara*f* residues acetylated at any of the available positions for esterification, that is positions 2, 3 and 5 (Biely et al., 2011). α-L-Arabinofuranosidases that would liberate esterified or otherwise substituted Ara*f* side residues either do not exist or have not been discovered yet.

From the above said we can conclude that deacetylated, not heavily branched arabinoglucuronoxylan with a portion of Ara*f* esterified with ferulic acid, could be completely saccharified by xylanases of GH10 or GH11 family in the presence of two α-L-arabinofuranosidases, one m2,3 and one d3 enzyme, and, of course, in the presence of β-xylosidase (Lagaert et al., 2014; Sørensen et al., 2007a), α-glucuronidase and feruloyl esterase.

2.3.3. Feruloyl esterases

These enzymes have been extensively treated in several reviews (Crépin et al., 2004; Faulds and Williamson, 2003; Topakas et al., 2007; Wong, 2006). They are quite difficult to be properly classified (Benoit et al., 2008; Udatha et al., 2011), and that is the reason why CAZy currently ignores these esterases. Missing are also detail data on structure-function relationship. According to substrate specificities on a few natural and artificial substrates, mainly on alkyl ferulates, four groups of feruloyl esterases have been recognized (Crépin et al., 2004). All of the enzymes liberate *trans*-ferulic acid from 5-*O*-feruloylated Ara*f*. Some of them belong to CE1 family similarly as acetylxylan esterases, however, the substrate cross specificity of the CE1 enzymes has not been observed (Puchart et al., 2007). Some enzymes are capable of hydrolyzing also ester linkage to Ara*f* further substituted at position 2 with Xyl or disaccharide 2-*O*-α-L-galactopyranosyl-Xyl*p* (Faulds et al., 1995) (Fig. 6D).

2.4. Acetylated arabinoglucuronoxylan

Processes for extraction of hemicellulose from agricultural side products using steam explosion or hydrothermal reactor (Kabel et al., 2002a, 2002b, 2003; Makishima et al., 2009; Wyman et al., 2005) yield hemicellulose with a large portion of preserved ester linkages. They are represented by acetyl esters of Xyl*p* residues of the main chain and feruloyl esters of Ara*f* side residues. The amount of acetyl groups in arabinoglucuronoxylan is not as high as in hardwood acetylglucuronoxylan, but it may be still relatively high to hinder enzymatic depolymerization and saccharification (Naran et al., 2009). The acetyl groups occur at the same positions of the main chain Xyl*p* residues as in hardwood glucuronoxylan. The main chain has 2- and 3-*O*-monoacetylated and 2,3-di-*O*-acetylated residues and also 3-*O*-acetylated Xyl*p* residues which are 2-*O*-substituted with MeGlcA. A distinct acetylation site not occurring in hardwood polysaccharide is position 2 on Xyl*p* residue 3-*O*-substituted with Ara*f* which can be 5-*O*-esterified with ferulic acid (Appeldoorn et al., 2013). Detail studies of enzymatic degradation of acetylated arabinoglucuronoxylan have not been carried out, but from what we have learned on hardwood acetyl glucuronoxylan, acetylxylan esterases will also be indispensable to achieve complete saccharification here. The enzymes of CE families 1, 4, 5 and 6 would deacetylate the xylan main chain except of the 3-*O*-acetyl group on the MeGlcA-substituted Xyl*p* residues. As we have discussed earlier, this group becomes susceptible to deacetylation by some CE16 acetyl esterases after enzymatic cleavage of the main chain by GH10 xylanases or combination of GH11 xylanase and β-xylosidase (Fig. 4). CE16 esterases will be required to liberate the acetyl group from position 4 of the non-reducing end Xyl*p* residues where the acetyl group migrates (Puchart and Biely, 2015). The enzymatic degradation of highly substituted arabinoglucuronoxylan regions has not been elucidated, but it is obvious that sequential or simultaneous attack by GHs and CEs will be required. Due to steric hindrance by other substituents in the vicinity of the target linkage, some of the enzymes may exhibit extremely low specific activities on otherwise excellent substrates. In this context it would be important to find out whether the 2-*O*-acetyl group on Xyl*p* 3-*O*-substituted by Ara*f* (Fig. 6C) will influence the action of α-L-arabinofuranosidases. The blocking of the action of acetylxylan esterases with the neighboring Ara*f* residue can be anticipated similarly as that with MeGlcA in acetylated glucuronoxylan. A study of degradation of heavily branched acetylated arabinoglucuronoxylan using an enzyme mixture consisting of α-L-arabinofuranosidase, endoxylanase and β-xylosidase has clearly demonstrated importance of the action of acetylxylan esterase and feruloyl esterase for release of both Xyl and Ara (Agger et al., 2010).

2.4.1. Enzymatic breakdown of highly branched xylan

Corn fiber xylan is a typical example of xylan resistant to the action of pure endoxylanases. Structural analysis suggested that over 70% of the xylose backbone residues have one or more arabinose, 4-*O*-methylglucuronic acid, or other side chains (Nghiem et al., 2011; Saha, 2003; Saulnier et al., 1995a). In addition, some of the Xyl*p* residues are acetylated and Ara*f* side chains are not only esterified with ferulic acid, but further substituted at position 2 with β-linked Xyl*p* or disaccharide

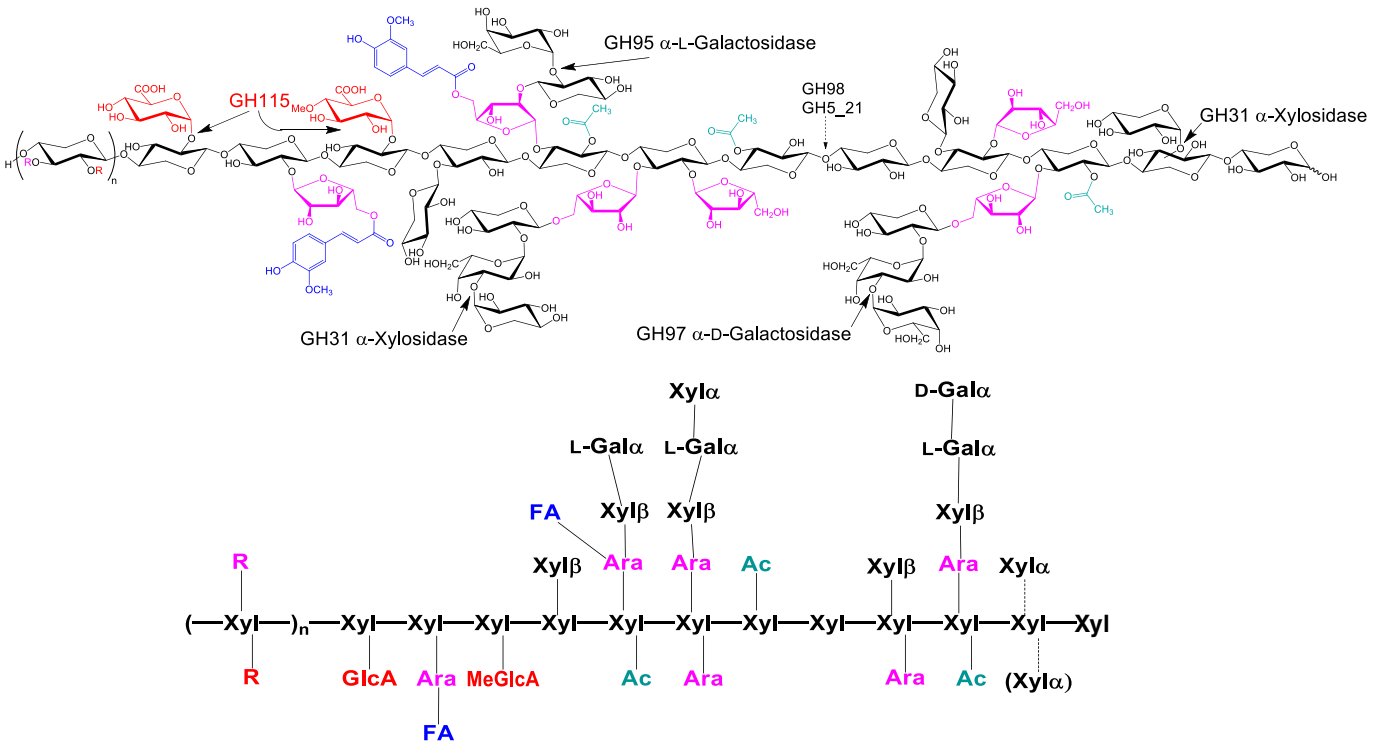

Fig. 7. Scheme of highly branched corn fiber glucuronoarabinoxylan resistant to GH10 and GH11 xylanases. The constituents of the polysaccharide are shown in chemical formulas and abbreviations. Sites of the cleavage by corn fiber xylan-specific endoxylanases (GH98 and GH5, Rogowski et al., 2015) have not been established. In the bacterial genomes the corresponding endoxylanase genes are clustered with the genes of other corn fiber degrading enzymes, e.g. the genes of α-L-galactosidase, α-D-galactosidase and α-xylosidase.

2-*O*-α-L-galactopyranosyl-Xyl*p* or trisaccharides α-D-xylopyranosyl-(1 → 3)-α-L-galactopyranosyl-(1 → 2)-β-D-xylopyranose and α-D-galactopyranosyl-(1 → 3)-α-L-galactopyranosyl-(1 → 2)-β-D-xylopyranose (β-D-xylopyranosyl-(1 → 2)-5-*O*-(*trans*-feruloyl)-L-arabinofuranose side chain) (Allerdings et al., 2006; Appeldoorn et al., 2010; Doner et al., 2001) (Fig. 7). The presence of α-glycosidically linked Xyl and the rare sugar L-galactose contributes to the recalcitrance. As a result, there may be no regions in corn fiber xylan where several contiguous xylose residues are unsubstituted and accessible to attack by xylanases. These structural impediments are the major factors regulating xylan degradation. These are the reasons why one of the best naturally occurring GH11 xylanase producer, *Thermomyces lanuginosus* (Singh et al., 2003), cannot grow on corn fiber xylan because of the lack of secretion of debranching xylanolytic enzymes (Puchart and Biely, 2008).

The pretreatment of highly branched corn fiber is crucial before any success in enzymatic hydrolysis (Saha et al., 1998; Saulnier et al., 1995b). Access of xylanases can be facilitated by mild acid hydrolysis. Examples of the corn fiber xylan structural arrangements that survived such a treatment have been recently reported by Appeldoorn et al. (2013).

Acetylated corn fiber xylan isolated by hot water extraction is even more branched because it is also acetylated (Agger et al., 2010). Its enzymatic hydrolysis by xylanase, β-xylosidase and α-L-arabinofuranosidase required consorted action with acetylxylan esterase and feruloyl esterase, however, the efficiency of hydrolysis was still not satisfactory.

Fortunately, there is a new development on this aspect. Recently, a novel *endo*-acting corn fiber xylan-specific xylanase was discovered in *Bacteroides ovatus* (Rogowski et al., 2015). The enzyme is classified in GH98 family, comprising exclusively endo-β-1,4-galactosidases. The inactivity of the enzyme on hardwood glucuronoxylan and cereal arabinoxylan suggests that some of the corn xylan decorations, such as 2,3-di-arabinosylated Xyl*p* residues or 3-*O*-β-xylosylated side chain Ara*f* residues are the enzyme specificity determinants (Rogowski et al., 2015). The same authors reported on another novel xylanase active on corn xylan, namely the *Bacteroides xylanisolvens* protein BXY29320, grouped to GH5 family. This enzyme seems to exhibit a slightly relaxed specificity since it depolymerizes both corn xylan as well as cereal arabinoxylan, but does not show activity on hardwood glucuronoxylan. In this respect the *B. xylanisolvens* enzyme resembles arabinoxylan-specific GH5 xylanase (Correia et al., 2011). Interesting information is that the genes of two mentioned corn fiber xylan degrading xylanases are clustered with genes encoding novel enzymes participating in breakdown of the superbranched corn fiber xylan, an α-L-galactosidase belonging to GH95 family harboring exclusively α-L-fucosidases, and a GH31 α-xylosidase role of which might be the removal of Xyl*p* residues α-linked to the xylan main chain. The GH31 family harbours, besides α-glucosidases, α-xylosidases involved in degradation of xyloglucan. Current knowledge on debranching xylanolytic enzymes is summarized in Table 2.

3. Conclusions and future prospects

Despite obvious progress in understanding the structure of plant xylans and microbial enzymes of their degradation, we can recognize several aspects in both lines of research that require further clarification and deeper understanding. Minimal attention has been paid to learning more about the nature of the residues of the polysaccharide which are resistant to further enzyme digestion. The recent work suggesting two different xylan conformations in one xylan molecule in *Arabidopsis* as a consequence of density of substituents (Busse-Wicher et al., 2014), leads to an idea that the more resistant portion of the polysaccharide could correspond to more densely substituted parts of the xylan main chain. Structural studies of these recalcitrant domains could certainly stimulate the search for novel enzymes. In this connection we should mention certain confusion in the literature concerning the presence of D-galactose vs L-galactose in highly branched xylans. Clarification of this point could define the requirement of α-L-galactosidases. Recent discovery of unique xylanases catalyzing efficient hydrolysis of

Table 2
Summary of xylanolytic accessory (debranching) enzymes and their substrates.

Accessory enzyme[a]	CAZy family	Substrate
α-Glucuronidase	GH67	Unsubstituted α-1,2-linked MeGlcA or GlcA residues bound to non-reducing end Xyl*p* residue that is not substituted at position 3 or 4.
	GH115	Unsubstituted α-1,2-linked MeGlcA and GlcA residues bound to terminal or internal Xyl*p* residue not substituted at position 3.
α-L-Arabinofuranosidase (m2,3)	GH43, GH51, GH54, GH62	Singly α-1,2- and α-1,3-arabinosylated internal or non-reducing end Xyl*p* residues.
α-L-Arabinofuranosidase (d3)	Some GH43, one GH51 member	2,3-Doubly arabinosylated internal and non-reducing end Xyl*p* residues (release Ara*f* from position 3 and leave singly 2-arabinosylated product).
Acetylxylan esterase	CE1, CE5, CE6	2- and 3-monoacetylated as well as 2,3-diacetylated Xyl*p* residues (inactive on 3-acetylated Xyl*p* residues carrying MeGlcA at position 2).
	CE4	2- and 3-monoacetylated (inactive on 2,3-diacetylated Xyl*p* residues (inactive on 3-acetylated Xyl*p* residues carrying MeGlcA at position 2).
	CE16	Acetyl group at position 2, 3 and 4 (migration) on the non-reducing end Xyl*p* residue. Low activity on internal acetylated Xyl*p* residues.
Non-reducing end exodeacetylase	CE16 of *Trichoderma reesei* type	Acetyl group at position 3 and 4 (migration) on the non-reducing end Xyl*p* residue. Low activity also on the non-reducing end 2-acetyl group.
Feruloyl esterase	Some in CE1 family	Ara*f* residues feruloylated at position 5 (in arabinoglucuronoxylan).

[a] Less known debranching enzymes such as α-D-xylosidase, α-D-galactosidase, α-L-galactosidase and β-xylosidase operating on xylan side oligosaccharides (Fig. 7) are not included.

superbranched corn fiber xylan (Rogowski et al., 2015) could open a new chapter in the field of enzymatic breakdown of corn fiber hemicellulose. We are anxious to see the impact of this new development.

An exciting challenge is to search for lytic xylan monooxygenases (Agger et al., 2014; Hemsworth et al., 2015; Horn et al., 2012), even though xylan represents soluble or well hydrated cell wall polysaccharide. The first example of such activity was recently demonstrated with a cellulose attacking protein of the fungus *Meliophtora thermophile C1* (Frommhagen et al., 2015). The work describes the oxidative cleavage of alkali-extracted xylan, but only in the presence of cellulose, suggesting that the xylan cleavage requires its close association with cellulose. This interesting observation suggested that there might be microbial enzyme systems designed to degrade efficiently cellulose-xylan complexes as they occur in plant cell walls.

Naturally, detailed knowledge of the plant xylan structures is the way towards new defined substrates that could be used for search of novel enzymes exploring genomic and metagenomics libraries. A significant contribution to the fine structure of xylan can be expected from growing number of studies of xylan and plant cell wall biosynthesis. There are also challenges on the side of protein engineers to construct enzyme mutants that would accommodate substituted xylan side chains. An example could be the engineering of α-L-arabinofuranosidases to be capable to accommodate in their substrate binding site and liberate substituted Ara*f* side residues, e.g. Ara*f* residues esterified with ferulic acid eventually substituted with other sugars. Phenolic acid containing xylooligosaccharides are attractive products as antioxidants.

Another challenge for protein engineers is to construct chimeric proteins that would be composed of several catalytic domains that act in synergy, for example xylanases and arabinofuranosidases or xylanases and acetylxylan esterases. An alternative way would be a combination of catalytic domain(s) with various carbohydrate binding module(s). We can be inspired by the Nature that has developed multienzyme complexes called cellulosomes (Moraïs et al., 2011; Shoham et al., 1999) and xylanosomes (McClendon et al., 2012; Srikrishnan et al., 2013), or multidomain enzymes with incredible combination of catalytic and carbohydrate binding domains (Yang et al., 2009). Due to the fact that saccharification of acetylglucuronoxylan and arabinoxylan does not require a large number of enzymes, construction of "glycanosomes" can be reduced to their mini versions (Mingardon et al., 2007; Xu et al., 2013). We also would like to emphasize the fact that the microbial enzymes have evolved to attack plant cell wall structure in its complexity rather than single cell wall components such as individual isolated polysaccharides. Therefore investigation of their action on native plant cell walls could deliver new valuable data important in view of efficient bioconversion of plant biomass.

Finally we should mention genetic manipulation of the structure of plant cell walls as alternative way to facilitate xylan extraction or saccharification. An improvement of enzymatic hydrolysis yields of xylan due to lack of its branching with uronic acids has been demonstrated in *Arabidopsis* mutants (Mortimer et al., 2010). However, the attempts to reduce the xylan degree of acetylation to make it more accessible to degradation by glycoside hydrolases, did not lead to expected results (Manabe et al., 2011; Xiong et al., 2013). Xylan deacetylation decreases its hydration and solubility, and increases its selfassociation and association with cellulose. Expression of fungal glucuronoyl esterases in *Arabidopsis* and *Populus* leading to reduced xylan-lignin ester cross-links improved xylan extractability (Tsai et al., 2012; Gandla et al., 2015). The complexity of plant cell walls remains an infinite source of new knowledge leading to improvement of plant biomass bioconversion.

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