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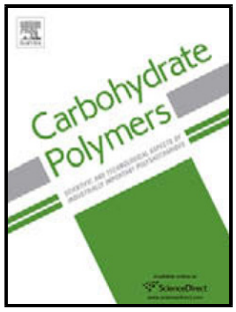

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Positional specificity of *Flavobacterium johnsoniae* acetylxylan esterase and acetyl group migration on xylan main chain

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Highlights

- This is the second study of a novel unclassified acetylxylan 3-*O*-deacetylase
- The acetylxylan esterase is a product of a new *Flavobacterium johnsoniae* isolate
- The xylan 3-*O*-deacetylation proceeds most rapidly on 2-*O*-substituted Xyl*p* residues
- The enzyme converts acetylglucuronoxylan to 2-*O*-acetylated polysaccharide
- The latter served for the first time to study acetyl group migration in the polymer

Abstract

A new *Flavovacterium johnsoniae* isolate encodes an enzyme that is essentially identical with a recently discovered novel acetylxylan esterase, capable of liberating 3-*O*-acetyl group from 4-*O*-methyl-D-glucuronic acid-substituted xylopyranosyl residues (Xyl*p*) (Razeq et al., 2018). In addition to deesterification of the 2-*O*-MeGlcA-substituted Xyl*p* residues in acetylglucuronoxylan, the enzyme acts equally well on doubly acetylated Xyl*p* residues from which it liberates only the 3-*O*-acetyl groups, leaving the 2-*O*-acetyl groups untouched. 3-*O*-Monoacetylated Xyl*p* residues are attacked with a significantly reduced affinity. The resulting 2-*O*-acetylated xylan was used to investigate for the first time the migration of the 2-*O*-acetyl group to position 3 within the polysaccharide. In contrast to easy acetyl group migration along the monomeric xylopyranosides or non-reducing-end terminal Xyl*p* residues of xylooligosaccharides, such a migration in the polymer required much longer heating at 100 °C. The specificity of the xylan 3-*O*-deacetylase was, however, no so strict on acetylated methyl xylopyranosides.

Keywords: hardwood acetylglucuronoxylan; novel acetylxylan esterase; novel regiospecificity; 3-*O*-deacetylase; 2-*O*-acetylglucuronoxylan; acetyl group migration

Abbreviations

CE, carbohydrate esterase; MeGlcA; 4-*O*-methyl-α-D-glucopyranosyluronic acid; Xyl*p*, β-D-xylopyranosyl;

1. INTRODUCTION

Major hemicellulose in hardwoods is acetylglucuronoxylan. It is a polysaccharide with main chain of β-1,4-linked xylopyranosyl (Xyl*p*) residues, 10 % of which carry 4-*O*-methyl-α-D-glucopyranuronosyl side residues attached to position 2. About 60 % of Xyl*p* residues are acetylated. Major portion is monoacetylated at position 2 and 3 and minor portion is 2,3-di-*O*-acetylated. Recent observations suggested that in various hardwood xylans 3-*O*-acetyl group occurs on almost all MeGlcA-substituted Xyl*p* residues (Evtuguin, Tomás, Silva, & Neto, 2003; Naran, Black, Decker, & Azadi, 2009; Puchart, Mørkeberg Krogh, & Biely, 2019; Teleman, Lundqvist, Tjerneld, Stålbrand, & Dahlman, 2000). Thus, there are four types of acetylated Xyl*p* residues within the xylan chain: 2-*O*-monoacetylated, 3-*O*-monoacetylated, 2,3-di-*O*-acetylated and 3-*O*-acetylated-2-MeGlcA-glycosylated (Fig. 1).

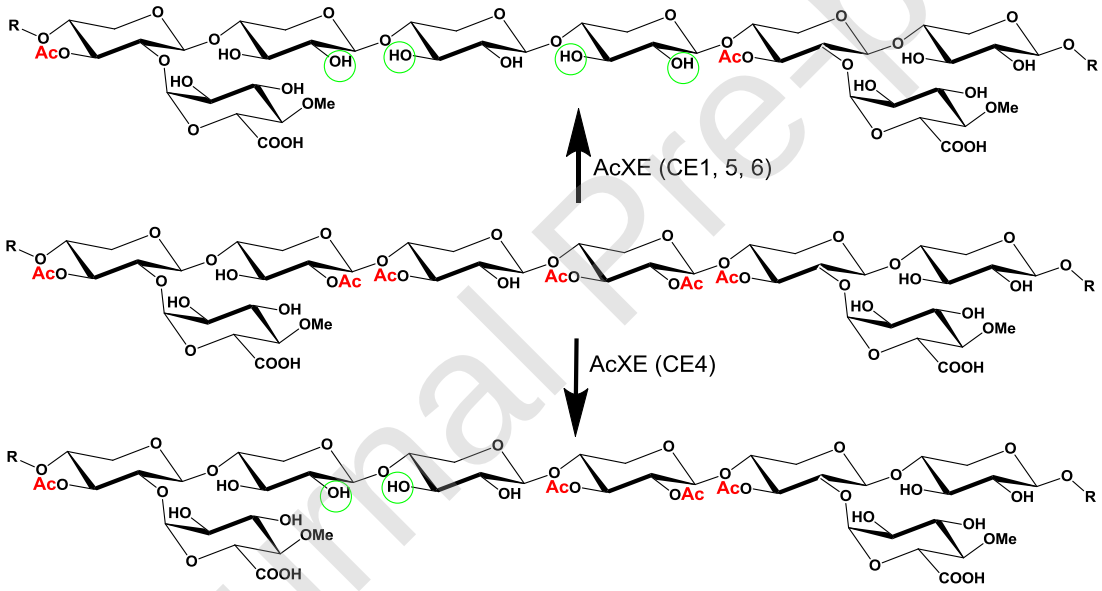


Fig. 1 Hypothetical hardwood acetylglucuronoxylan containing four types of acetylated Xyl*p* residues (the middle formula) and their deacetylation by AcXEs belonging to CE1. CE4, CE5 and CE6 families. AcXEs of all four CE families are unable to release the 3-*O*-acetyl on MeGlcA-subtituted Xyl*p* residues, and AcXEs of family CE4 attack neither doubly acetylated Xyl*p* residues. The upper polysaccharide is acetylated exclusively at the 3-position of MeGlcA-substituted Xyl*p* residues.

Microbial decomposition of such a complex polysaccharide by glycoside hydrolases, namely endo-β-1,4-xylanases (EC 3.2.1.8, 3.2.1.136), β-xylosidases (EC 3.2.1.37) and α-glucuronidases (EC 3.2.1.131, 3.2.1.139), requires also polysaccharide deesterification catalyzed by acetylxylan esterases. They are a quite diverse group of enzymes differing in primary and tertiary structures as well as in regiospecificity. In Carbohydrate Active enZymes (CAZy) database they are classified in carbohydrate esterase (CE) families 1, 4, 5, 6 and 16 (Lombard, Golaconda Ramulu, Drula, Coutinho, & Henrissat, 2014). The members of these families differ slightly in the mode of action on singly and doubly acetylated Xyl*p* residues, however, enzymes of neither mentioned families were found capable of liberating the acetyl group from position 3 on Xyl*p* residue MeGlcA-glycosylated at position 2 (Fig. 1) (Biely et al., 2012). These heterogeneously doubly substituted Xyl*p* residues represent the most recalcitrant structural arrangement in the acetylated polysaccharide. MeGlcA is a steric barrier for AcXEs and the 3-*O*-acetyl interferes with the action of α-glucuronidase. In view of the yields of hardwood xylan saccharification such a new deacetylase could play an important role, since its absence in enzyme cocktails may leave almost one third of the polysaccharide in the form of enzyme resistant tetrasaccharide (Biely, Singh, & Puchart, 2016).

Recently a new AcXE was identified in a xylan utilization locus of *Flavobacterium johnsoniae* UW101 which showed capability of attacking the 3-*O*-acetyl group vicinal to the MeGlcA side residue (Razeq et al., 2018). A clear experimental evidence for this action was obtained by ^{1}H-NMR spectroscopy. Attracted by this new development in the area of glucuronoxylan deacetylation, but also due to an incomplete interpretation of the changes in the acetyl group signals in the NMR spectra caused by the enzyme (Razeq et al., 2018), we have prepared highly similar recombinant enzyme (98.6% identity) from a different *F. johnsoniae* strain. The enzyme was applied to glucuronoxylan acetylated solely at MeGlcA-substituted Xyl*p* residues. Moreover, the regiospecificity of the enzyme was reexamined on aspen acetylglucuronoxylan extracted by steam explosion. The results obtained were similar as those reported by Razeq et al. (2018) on a mixture of acetylated aldouronic acids, however, our NMR signal assignments also expand current knowledge on the mode of action of these novel type AcXEs on singly and doubly acetylated Xyl*p* residues. The action of the enzyme resulted in a 2-*O*-acetylated polysaccharide which was exploited to study for the first time the acetyl group migration between position 2 and 3 on internal Xyl*p* residues. The positional specificity of the enzyme on the polymeric substrate was also

compared with its action on synthetic substrates, partially and fully acetylated β-xylopyranosides.

2. METHODS

2.1. Substrates

All acetates derived from methyl β-D-xylopyranoside (Puchart et al., 2016, and references therein) and its deoxy- and deoxy-fluoro- analogues (Mastihubová & Biely, 2004b) as well as monoacetates of 4-nitrophenyl β-D-xylopyranoside (Mastihubová & Biely, 2004a) have been synthesized previously. Isolation of steam explosion extracted aspen acetylglucuronoxylan has also been reported (Biely et al., 2013; Horn, Nguyen, Westereng, Nilsen, & Eijsink, 2011). This hemicellulose was used as a substrate for *Orpinomyces* sp. CE6 AcXE (Megazyme, Bray, Ireland)-catalyzed deacetylation according to published procedure (Puchart, Mørkeberg Krogh, & Biely, 2019) that resulted in a polysaccharide selectively 3-*O*-acetylated at MeGlcA-substituted Xyl*p* residues. Monosaccharide composition as well as acetic acid and MeGlcA content of the polysaccharide have been published (Puchart, Mørkeberg Krogh, & Biely, 2019). The degree of polymerization was estimated to be ca. 35 based on the reducing sugar determination.

2.2. Cloning and expression of an acetylxylan esterase from *Flavobacterium johnsoniae* 1WA

The gene encoding the acetylxylan esterase (GenBank accession number: MN306533) from the bacterium *Flavobacterium johnsoniae* 1WA which was originally isolated from a soil sample collected in the USA, was codon optimized for *Bacillus subtilis* codon usage via a publicly available codon optimizer tool (Raab, Graf, Notka, Schödl, & Wagner, 2010). The resulting optimized gene was further modified by exchanging the coding region for the natural signal peptide with the coding region from the *Bacillus clausii* AprE signal peptide and by addition of a C-terminal 6×His affinity purification tag. The gene was then ordered as a synthetic gene from the commercial provider GeneArt, Regensburg, Germany.

The synthetic gene was fused via overlap extension PCR to a strong triple promoter consisting of the $P_{amyL4199}$, P_{amyQsc} and P_{cry3A} promoters in series (Jørgensen & Pedersen, 2015) and flanking homology regions matching the *B. subtilis* pectate lyase gene. The resulting expression cassette was inserted into the genomic pectate lyase locus of a *B. subtilis* host by homologous recombination. The *B. subtilis* host strain used was protease deficient derivative (Zhao et al., 2019) of *Bacillus subtilis* ATCC 6051, which had the following protease genes deleted: *nprE*, *aprE*, *wprA*, *bpr*, *vpr*, *mpr*, *epr* and *ispA*. The recombinant *B. subtilis* clone containing the integrated expression construct was selected on chloramphenicol and subsequently cultivated for 48 hours in LB medium at 30 °C. The enzyme-containing supernatant was harvested by centrifuging of the culture broth for 30 minutes at 15 000×*g*.

2.3. Purification of the enzyme

Harvested culture broth was separated from cellular material by passage through an 0.22 µm PES filter (Nalgene), before addition of 3M TRIS to a final concentration of 85 mM. The resulting solution was filtered through an 0.22 µm PES filter, and applied to a 5 ml HisTrap Excel column (GE Healthcare) pre-equilibrated with 50 mM TRIS/HCl, pH 8.0. The loaded column was washed with 2 column volumes of 50 mM HEPES, pH 7.0, to wash out unbound proteins. The bound esterase was eluted with 50 mM HEPES containing 0.75 M imidazole, pH 7.0. The collected fractions were analyzed by measuring A_{280} and A_{280}/A_{260}. The esterase containing fractions were pooled and desalted by gel-filtration into 50 mM HEPES, pH 7.0, containing 100 mM NaCl. The fractions collected in this step contained an electrophoretically homogeneous protein according to SDS PAGE. The identity of the isolated esterase to the expected acetylxylan esterase was confirmed by in-gel tryptic digest followed by MS analysis.

2.4. Monitoring of polysaccharide deacetylation by NMR spectroscopy

Deacetylation of polymeric substrates was followed by ^{1}H NMR spectroscopy. Prior to use, the substrates, enzyme and phosphate buffer were three times freeze dried from heavy water to ensure deuterium exchange of all ionisable proton atoms. The enzyme reactions were carried out at 35 °C directly in narrow NMR tubes (outer diameter 2.5 mm).

The polysaccharide (2 mg) selectively acetylated at position 3 of MeGlcA-glycosylated Xyl*p* residues (Puchart, Mørkeberg Krogh, & Biely, 2019) was dissolved in

160 μl of 15 mM deuterium-exchanged sodium phosphate buffer, pH 6.0, and after thermal equilibration to 35 °C proton NMR spectrum was recorded. Then 40 μg of enzyme was added and the reaction was monitored by NMR spectroscopy.

Aspen acetylglucuronoxylan (2 mg) was dissolved in 150 μl of 100 mM deuterium-exchanged sodium phosphate buffer, pH 6.0, and initial proton NMR spectrum was recorded at 35 °C. Then 6.3 μg of the enzyme was added and the reaction course was followed by proton NMR spectroscopy at 2-min intervals. After 80 minutes ten times higher amount of the enzyme (63 μg) was applied and the reaction was continued for 21 h.

2.5. Acetyl group migration on xylan main chain

The solution of 2-*O*-acetylated xylan prepared as described above, which did not show any sign of acetyl group migration at 35 °C, was heated at 100 °C for several 5-10 min intervals, and after cooling to 35 °C subjected to NMR spectroscopy at this temperature.

2.6. NMR spectroscopy

^{1}H NMR spectra were recorded using automatic chemical shift calibration on AVANCE III HD 400 MHz spectroscope equipped with a broad band BB-(H-F)-D-05-Z liquid N2 Prodigy probe (both from Bruker BioSpin, Rheinstetten, Germany). The following parameters were used: pre-saturation *zgpr* sequence, pre-saturation delay 2 s, r.f. 90° pulse, and acquisition time 2.5 s.

2.7. Deacetylation of acetylated methyl β-D-xylopyranosides

Stock solutions (50 mM) in dimethyl sulfoxide were prepared from the following multiply acetylated derivatives of methyl β-D-xylopyranoside: 2,3,4-tri-*O*-acetyl, 2,3-di-*O*-acetyl, 2,4-di-*O*-acetyl, 3,4-di-*O*-acetyl, 2-deoxy-3,4-di-*O*-acetyl, 3-deoxy-2,4-di-*O*-acetyl, 2-deoxy-2-fluoro-3,4-di-*O*-acetyl, and 3-deoxy-3-fluoro-2,4-di-*O*-acetyl. Working 5 mM solution of each was prepared by 10-fold dilution with 50 mM sodium phosphate buffer, pH 6.0. After thermal equilibration the reaction was initiated by addition of the enzyme (0.187 mg/ml, final concentration) and the reaction was performed at 35 °C and monitored by

TLC. On the TLC plate 8-µl aliquots were spotted for deoxy-analogues and 4-µl aliquots for other substrates.

2.8. Deacetylation of isomeric monoacetates of 4-nitrophenyl β-D-xylopyranoside

Stock solutions (200 mM) in dimethyl sulfoxide were prepared from the all the monoacetates of 4-nitrophenyl β-D-xylopyranoside, namely 2-*O*-acetyl, 3-*O*-acetyl and 4-*O*-acetyl. Working 4 mM solutions in 50 mM sodium phosphate buffer, pH 6.0, were thermal equilibrated and supplied with the enzyme (final concentration: 212 ng/ml for TLC experiment, 51.8 ng/ml for HPLC experiment). The reaction was performed at 35 °C and its course analyzed by either TLC or HPLC.

2.9. HPLC

HPLC analysis was carried out at 35 °C using an MZ-AquaPerfect® C18 5 µm column (150×4 mm; MZ-Analysentechnik, Meinz, Germany) equipped with a spectrophotometric detector operating at 275 nm. The column was eluted isocratically with a solvent system of 60% acetonitrile (v/v) at a flow rate of 1 ml/min. Aliquots (10 µl) of the reaction mixtures were directly loaded at defined time intervals. Each chromatogram was automatically peak-analyzed and area of the remaining substrate (acetylated 4-nitrophenyl β-D-xylopyranoside) and deacetylated product was integrated. Spontaneous deesterification (control) was taken into consideration and the relative proportion of enzyme-catalyzed product formation was used for specific activity calculation.

2.10. TLC

Deacetylation of low molecular weight synthetic compounds was analyzed by TLC which was performed on aluminum-coated silica plates (Merck, Darmstadt, Germany). The plates were developed twice in a solvent system of either ethyl acetate/toluene/isopropanol (for methyl xyloside derivatives) or ethyl acetate/benzene/isopropanol (for 4-nitrophenyl xyloside acetates) 2:1:0.1 (by vol.). The carbohydrates were visualized by orcinol reagent (0.5% orcinol, 5% sulfuric acid in ethanol) after heating.

3. RESULTS

3.1. Amino acid sequence comparison

In this study 414 amino acid long protein encoded by *F. johnsoniae* strain 1WA was recombinantly produced. Its amino acid sequence, not deposited yet, was compared with the previously studied esterase (Razeq et al., 2018), originating from a strain *F. johnsoniae* UW101. Both ORFs are of the same length and code for native proteins that are highly similar (Supplementary Fig. 1S). Signal sequences (21 residues) are identical, but in the secreted proteins there are 6 substitutions, 3 of which are conservative. All the substitutions are distant from catalytically important amino acids, therefore they are expected hardly to affect significantly catalytic properties. Both proteins, showing homology to none CEs classified in CAZy database, contain a putative catalytic triad Ser217, Asp392 and His395 (native enzyme numbering), and belong to a SGNH/GDSL hydrolase superfamily of proteins (CDD 238868). In addition to the residues of serine esterase catalytic triad, other amino acids conserved in the superfamily are Gly258 and Asn294. It is interesting that in the two proteins deposited in GenBank database, having the same number of substitutions and originating from different *F. johnsoniae* strains, some of the amino acid changes occur at the same loci as in our enzyme.

3.2. Action on 3-*O*-acetylglucuronoxylan

The ability of the enzyme to deesterify 3-*O*-acetyl group in a vicinity of 2-*O*-linked MeGlcA side chain was studied using a polymeric substrate, 3-*O*-acetylxylan specifically acetylated only at position 3 on MeGlcA-substituted Xyl*p* residues (Fig. 1, upper formula). In the ^{1}H-NMR spectrum of this polysaccharide there is a single ester peak in the region of acetyl groups (Fig. 2). Its chemical shift corresponds to the 3-*O*-acetyl group in the vicinity of 2-*O*-linked MeGlcA. After addition of the enzyme this singlet completely disappeared, thus confirming the crucial result of the previous report (Razeq et al., 2018) on the ability of *F. johnosniae* UW101 AcXE to deesterify 3-*O*-acetyl group in the vicinity of MeGlcA residue. The disappearance of the acetate signal was accompanied with changes in the skeletal proton signals. The most obvious was a lack of a triplet at 5.04 ppm which was present in the starting

acetylated polymer and assigned to *H*-3 atom linked to *C*-3 carbon atom of Xyl*p* residue whose oxygen *O*-3 was acetylated. It is noteworthy that the intensity of *H*-5 doublet of MeGlcA residue increased upon deacetylation.

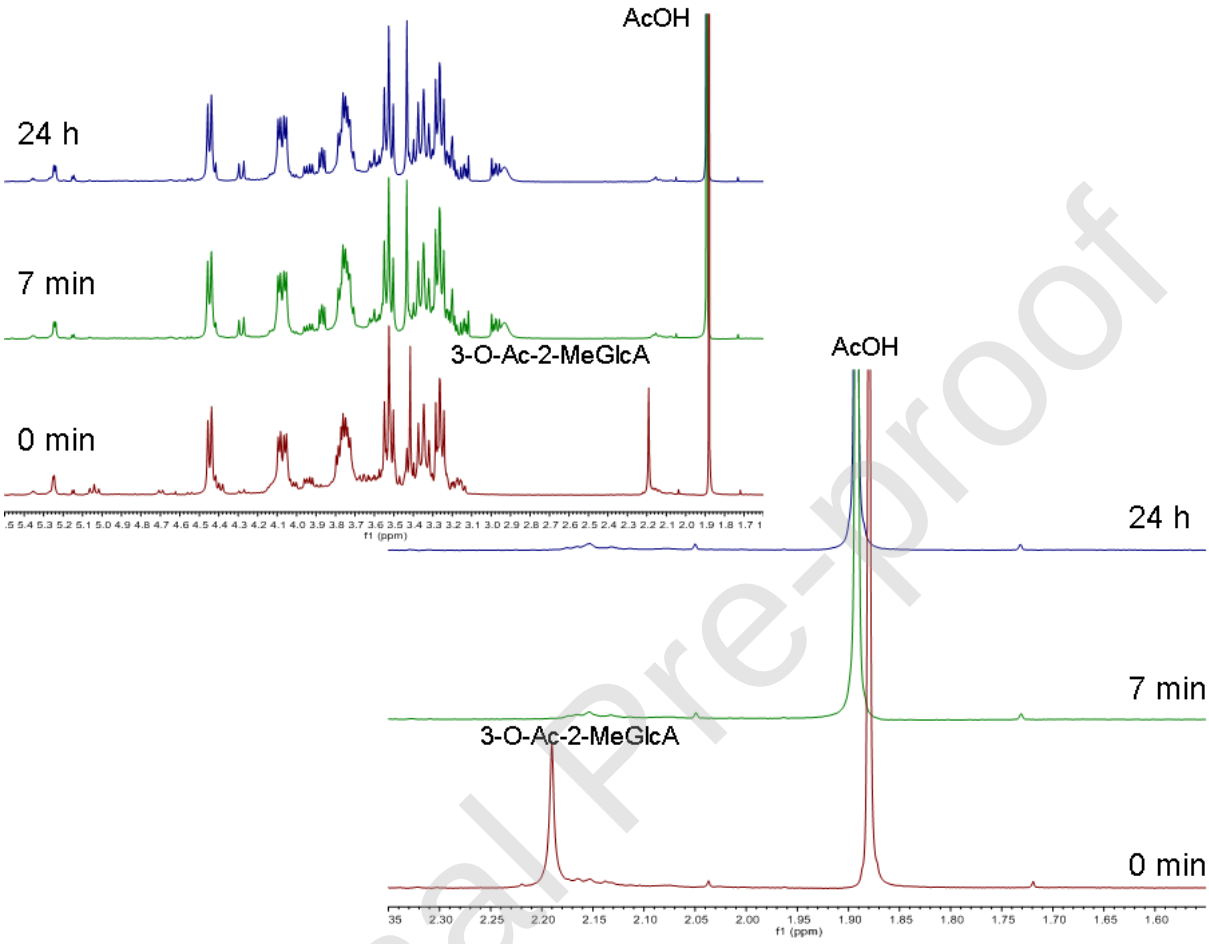


Fig. 2. Deacetylation of 3-*O*-acetylglucuronoxylan by *F. johnsoniae* AcXE monitored by ^{1}H-NMR spectroscopy. The reaction was performed at 35 °C in NMR tube containing 160 µl comprising 15 mM sodium phosphate, pH 6.0, 2 mg of the polysaccharide and 40 µg of the enzyme. The spectra on the right side is extension of the region with signals of the acetyl methyl groups of acetate ester and acetic acid. Note. the starting polysaccharide contained some acetic acid.

3.3. Action of the enzyme on polymeric acetylglucuronoxylan

To learn more about positional preferences for deesterification, the enzyme was applied onto a steam explosion extracted hardwood acetylglucuronoxylan that had been used for the preparation of the 3-*O*-acetylglucuronoxylan. In the native polysaccharide there are four types of acetylated Xyl*p* residues and the signals of these distinct acetyl groups have been assigned previously (Fig. 3, bottom spectrum) (Uhliariková, Vršanská, McCleary, & Biely, 2013). Upon enzyme addition, there was a rapid decrease in the intensity of the peaks corresponding to doubly substituted Xyl*p* residues, namely to 3-*O*-acetylated-2-*O*-(4-*O*-methyl)glucuronylated (2.19 ppm) and 2,3-di-*O*-acetylated (2.07-2.08 ppm) (Fig. 3, the second spectrum from bottom). The decrease in the intensity of the diacetate was accompanied with an increase in the signal of 2-*O*-monoacetate (2.14 ppm), suggesting that the doubly acetylated epitope is, at least preferentially, if not exclusively, deacetylated at position 3.

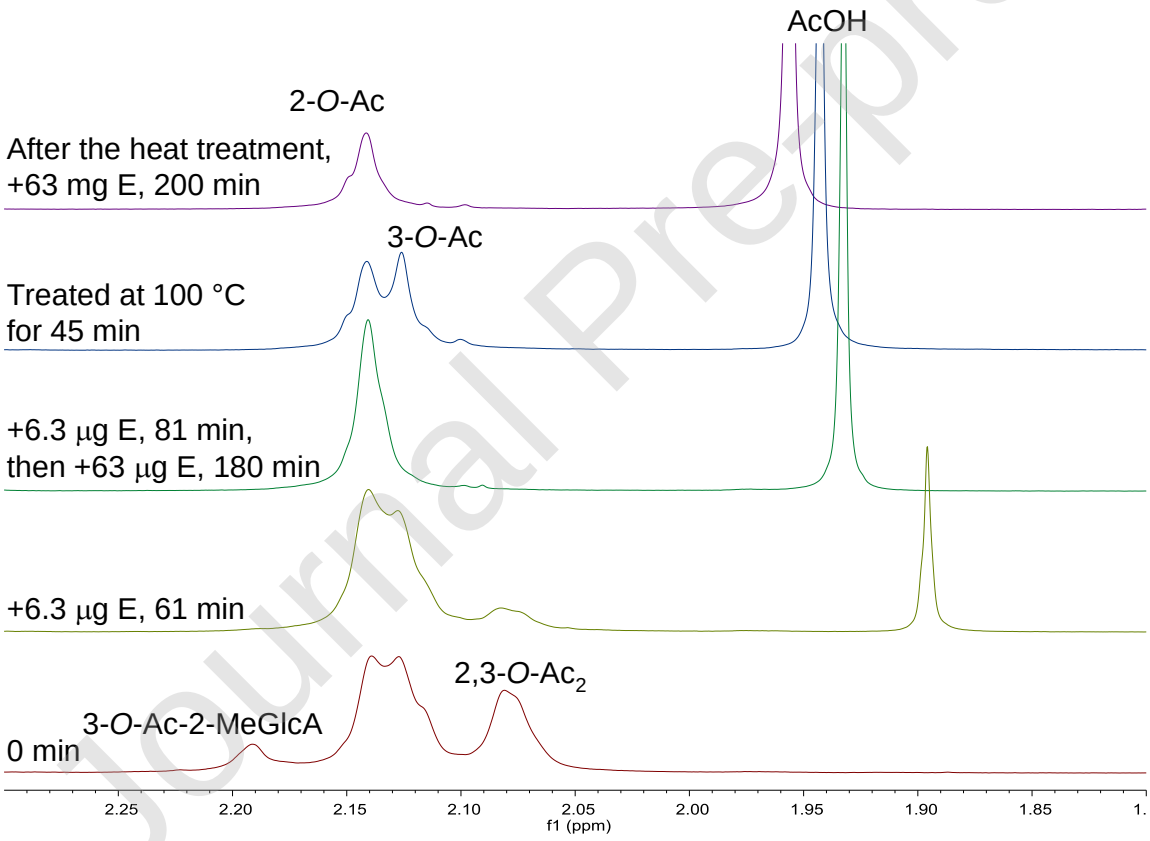


Fig. 3. ^{1}H-NMR spectroscopy monitoring of the deacetylation of aspen acetylglucuronoxylan by *F. johnsoniae* AcXE (the bottom three spectra), and of acetyl group migration upon a treatment at 100 °C (the second spectrum from the top) from position 2 to position 3 in glucuronoxylan exclusively monoacetylated at position 2. The top spectrum shows again the

selective removal of the 3-*O*-acetyl groups from thus obtained singly 2- and 3-*O*-acetylated Xyl*p* residues. The enzyme reactions were performed at 35 °C in NMR tube containing 160 µl of 100 mM sodium phosphate buffer (pH 6.0) containing 2 mg of the polysaccharide and the enzyme amount indicated.

The rates of the disappearance of the signals of 2,3-diacetate and 3-*O*-Ac-2-MeGlcA-Xyl*p* residue were similar and both peaks disappeared after about 80 minutes at the used enzyme concentration. At this time the decrease of the 3-monoacetate signal was negligible, while the signal of the 2-monoacetate clearly increased on account of the 2,3-diacetate 3-*O*-deacetylation. Ten times higher enzyme load of the enzyme and 3 h long incubation were required for complete deesterification of the 3-monoacetate (a peak at 2.13 ppm) (Fig. 3, middle spectrum). However, no change in the signal intensity of the 2-monoacetate was observed during further incubation for 24 h. These results serve as a clear evidence that: i) the enzyme deacetylates 3-monoacetylated Xyl*p* residues extremely slowly when compared with its action on the 3-acetyl group on Xyl*p* residues with the vicinal OH group either substituted with MeGlcA or acetylated; ii) the 2-monoacetate is resistant to the enzyme attack; iii) the acetyl group at position 2 does not migrate to position 3 at 35 °C. The enzyme treatment can lead to two different patterns of glucuronoxylan acetylation (Fig. 4).

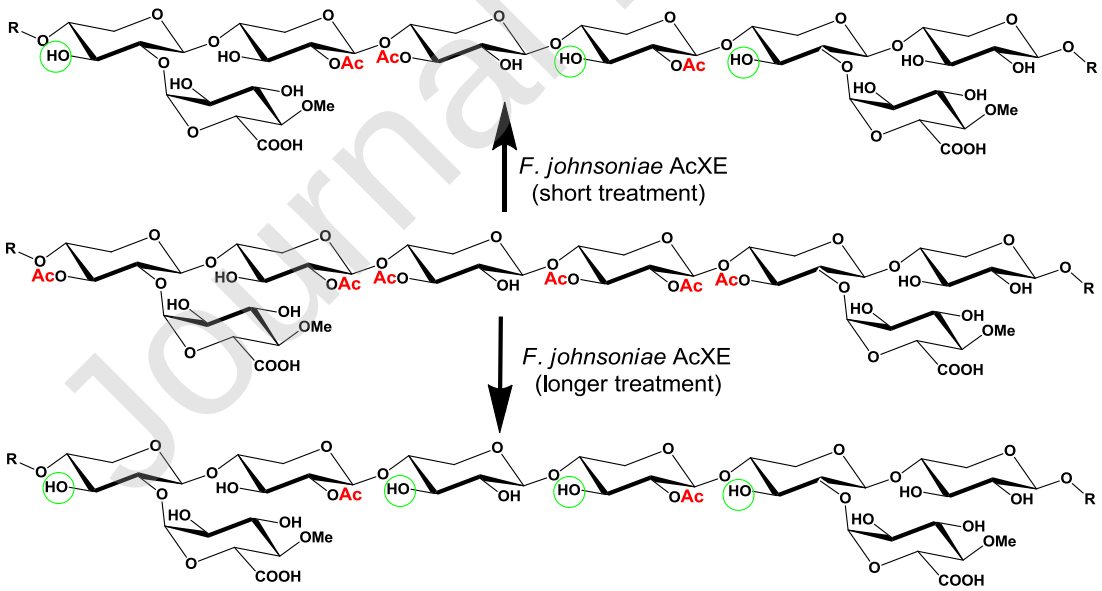


Fig. 4. Scheme of hardwood acetylglucuronoxylan composed of four types of acetylated Xyl*p* residues (middle formula) and its conversion to 2- and 3-monoacetylated polysaccharide

(upper formula) and to exclusively 2-*O*-acetylated polysaccharide (bottom formula) by *F. johnsoniae* AcXE depending on the robustness of the enzymatic treatment.

3.4. Acetyl group migration studied on polysaccharide

The 2-*O*-monoacetylated glucuronoxylan, prepared by the action of *F. johnsosniae* AcXE on steam explosion-extracted polymer, was for the first time used to study acetyl group migration in the polysaccharide. It has previously been shown that such a migration is common at ambient temperature in xylopyranosides (Mastihubová & Biely, 2004a) and quite rapid at increased temperature at the non-reducing-end Xyl*p* residue of methyl xylotrioside (Puchart & Biely, 2015). In the latter compound the equilibrium of all three monoacetates (2-, 3- and 4-monoacetate) was established during a 5 min treatment at 100 °C regardless the position of the acetyl group in the starting monoacetylated isomer. In the case of the 2-*O*-monoacetylated glucuronoxylan, after a 5-min heating at 100 °C less than 10 % of the 2-*O*-acetyl group migrated to position 3. To reach equilibrium between 2-monoacetate and slightly predominating 3-monoacetate, the heating at 100 °C had to be extended for 45-60 minutes (Fig. 3, the second spectrum from the top). It is also noteworthy that during the equilibrium experiments no signal appeared corresponding to 2,3-diacetate or 3-*O*-acetylated-2-*O*-glucuronylated Xyl*p* residue. This serves as an evidence that the acetyl group migration is strictly intracircular, i.e. it does not occur between two xylopyranoside rings.

After reaching the equilibrium between 2-monoacetate and 3-monoacetate (Fig. 3, the second spectrum from the top), the polysaccharide was treated with fresh *F. johnsoniae* AcXE in the same NMR tube. NMR spectroscopy confirmed again the selective 3-*O*-deacetylation. The 3-acetyl group was completely removed after 3 hours, while the 2-monoacetate remained intact even after 24 hours (Fig. 3, top spectrum).

3.5. Action of the enzyme on acetylated methyl xylopyranosides

The enzyme was also tested on fully and doubly acetylated methyl β-D-xylopyranosides. Two of them, 2,4- and 3,4-di-*O*-acetates, were also available in the form of deoxy and deoxy-fluoro-analogues which were earlier used to characterize the mode of action of AcXEs of families CE1, CE4 and CE5 (Biely & Mastihubová, 2004b; Biely, Mastihubová, Côté, & Greene, 2003; Biely, Mastihubová, & Puchart, 2007). The semiquantitative data on the rate and pattern of deacetylation were obtained by TLC analysis

of enzyme-substrate reaction mixtures run in parallel under the same substrate (5 mM) and enzyme concentration (187 μg/ml) (Figs. 5 and 6). Table 1 summarizes the results. In consonance with the behavior of the enzyme on acetylated xylan, the best substrates among the tri- and diacetates with vicinal hydroxyl group were 2,3,4- triacetate and 2,3-diacetate. Both compounds were rapidly deacetylated at position 3. 2,4- and 3,4-diacetates served as much worse substrates, their first deacetylation was very slow and in the case of the 3,4-diacetate deacetylation was not selective. The resulting monoacetates, in general, served as poor substrates resembling the slow deacetylation of 3-monoacetylated Xyl*p* residues in the polymer. The 2-acetate, resistant to the enzyme in the polymer, was, however, also a subject of slow deacetylation, possibly due to acetyl group migration to position 3 or formation of a productive enzyme-substrate complex in a different substrate orientation.

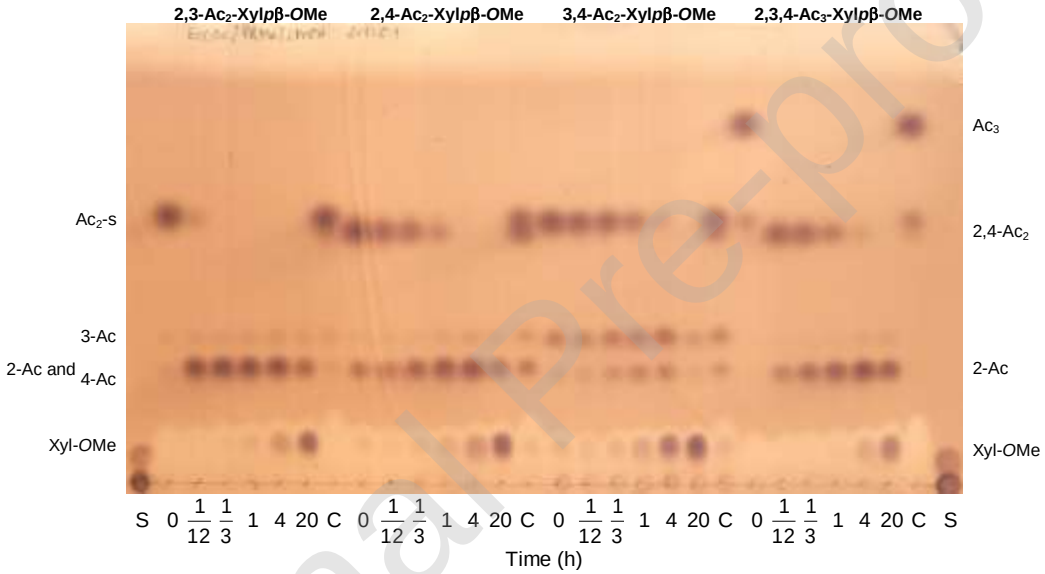


Fig. 5. Time course of deacetylation of unmodified acetylated methyl β-D-xylopyranosides by *F. johnsoniae* AcXE followed by TLC. The reaction was performed at 35 °C using 5 mM substrate and 187 μg/ml enzyme concentration.

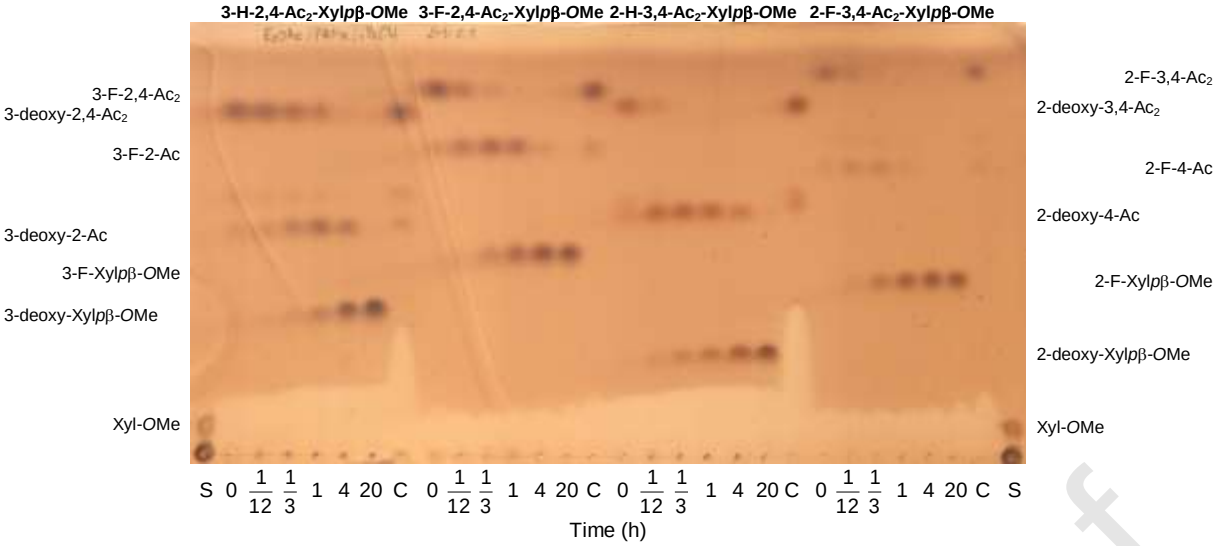
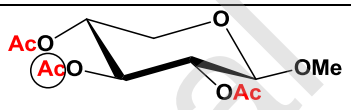
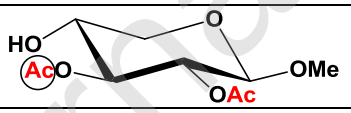
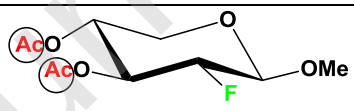
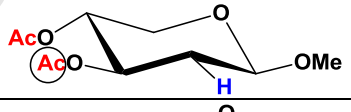
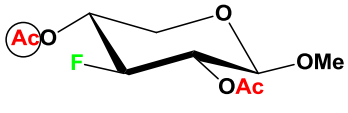
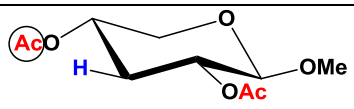
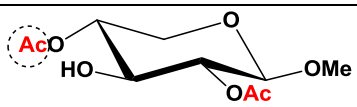
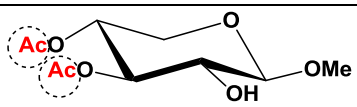


Fig. 6. Deacetylation of deoxy and deoxy-fluoro analogues of methyl β-D-xylopyranoside diacetates by *F. johnsoniae* AcXE followed by TLC. The reaction was performed at 35 °C using 5 mM substrate and 187 μg/ml enzyme concentration.

Table 1. Acetylated methyl β-D-xylopyranosides and their deoxy and deoxy-fluoro analogues used to study regiospecificity of *F. johnsoniae* AcXE. The compounds are listed descendently according the rate of their first deacetylation.

Substrate	Formula and the position of the first deacetylation	Rate of the first deacetylation/ Approximate time required for 50% conversion	Rate of the second deacetylation / Approximate time required for 50% conversion
2,3,4-Ac_3-Xyl*p*β-OMe		Rapid/ <5 min	Slow (position 4) ~30 min
2,3-Ac_2-Xyl*p*β-OMe		Rapid/ <5 min	Very slow ~12 h
2F-3,4-Ac_2-Xyl*p*β-OMe		Rapid/ <5 min Position 3 faster than position 4	Rapid ~10 min
2H-3,4-Ac_2-Xyl*p*β-OMe		Rapid/ <5 min	Slow ~2 h
3F-2,4-Ac_2-Xyl*p*β-OMe		Rapid/ ~5 min	Slow ~1 h
3H-2,4-Ac_2-Xyl*p*β-OMe		Slower/ ~30 min	Slow ~2 h

2,4-Ac_2-Xyl*p*β-OMe		Slow/ ~20 min	Slow ~12 h
3,4-Ac_2-Xylpβ-OMe		Slow ~60 min	Slow ~6 h

The replacement of the free hydroxyl group in methyl β-D-xylopyranoside 2,4- and 3,4-diacetate by either hydrogen or fluorine resulted in a considerable increase in the rate of the first and the second deacetylation. In the analogues lacking the 3-hydroxyl group, the acetyl group migration to adjacent position is excluded, so the second deacetylation of positions 2 is not related to this phenomenon. In general, the regioselectivity of deacetylation of deoxy and deoxy-fluoro methyl xylopyranosides is similar to that of the derivatives with the vicinal hydroxyl group.

3.6. Regiospecificity of the enzyme on monoacetylated 4-nitrophenyl β-D-xylopyranosides

Regiospecificity of the enzyme was also studied using all three monoacetylated 4-nitrophenyl β-D-xylopyranosides as glycosides having a bulkier aromatic aglycone. These compounds have previously been shown to restrict regiospecificity of common AcXEs more than acetylated methyl xylosides (reviewed in Biely, 2012).The best substrate for AcXEs of CE families 1, 4, 5 and 6 was the 2-*O*-acetate. On the basis of deacetylation of acetylglucuronoxylan and methyl xylosides it was expected that *F. johnsoniae* enzyme would deesterify 4- and 3-monoacetylated aromatic xyloside. Both these substances actually served as a substrate, however, surprisingly, 2-monoacetate was deacetylated as well. Moreover, TLC did not reveal significant differences in the reaction efficiency (Supplementary Figure 2S). This was confirmed by HPLC allowing determination of specific activities towards 4-, 3- and 2-monoacetylated β-D-xylopyranoside to be 0.285, 0.188 and 0.128 U/mg, respectively.

4. Discussion

AcXEs play an important role in enzymatic conversion of natural partially acetylated form of hardwood xylan. Such xylan is extracted from wood applying non-alkaline biorefinery processes that avoid saponification of ester linkages. The removal of the acetyl

groups from the polysaccharide main chain is extremely important in view of the extent of its enzymatic saccharification since the deacetylation creates new sites on the polymeric substrate for productive binding to the enzymes hydrolyzing the glycosidic linkages. Almost all so far recognized AcXEs were found to deacetylate singly and doubly acetylated Xyl*p* residues, but they did not recognize as a substrate the 3-*O*-acetyl group on MeGlcA-substituted Xyl*p* residue (Fig. 1) (Koutaniemi et al., 2013; Uhliariková, Vršanská, McCleary, & Biely, 2013). The existence of an esterase liberating the acetyl group shielded by vicinal MeGlcA residue in nature had been predicted for a long time, but has been demonstrated only recently (Razeq et al., 2018). A gene coding for such an enzyme was found in a polysaccharide utilizing locus of the bacterium *F. johnsoniae* UW 101 (Razeq et al., 2018). The positional specificity of the enzyme, however, has not been sufficiently elucidated. Moreover, due to a misinterpretation of the NMR spectra in the above quoted paper (the signals of doubly acetylated Xyl*p* residues have been considered to be the signals of monoacetylated Xyl*p* residues and the signals of monoacetylated residues have been ignored), we have decided to confirm the 3-*O*-deacetylase activity of an enzyme having almost identical amino acid sequence (98.6% identity) from a different *F. johnsoniae* strain. The capacity of the enzyme to remove efficiently the 3-*O*-acetyl group from MeGlcA-glycosylated Xyl*p* residues, the most recalcitrant epitope of hardwood xylan, was confirmed not only on the corresponding 3-*O*-acetylated polysaccharide, but also on native steam explosion extracted hemicellulose. The native polysaccharide also allowed us to estimate relative deesterification rates of the four distinct types of acetyl groups. It should be noted that the changes in the pattern of NMR spectrum observed upon incubation with the esterase studied in this work were essentially the same as those reported earlier (Razeq et al., 2018). The NMR spectra provided information that the new esterase is specific xylan 3-*O*-deacetylase able to liberate all three types of acetyl groups attached to position 3 (Fig. 1), but unable to deesterify position 2. However, the deacetylation of position 3 is affected by a modification of the vicinal position 2. The deacetylation appears to be easier for the enzyme in case the hydroxyl group at the neighboring position 2 is substituted, either MeGlcA-glycosylated or acetylated, because in addition to the unique ability of the enzyme to liberate 3-*O*-acetyl group vicinal to MeGlcA side residue, the esterase equally well deesterifies 3-*O*-acetyl group from doubly acetylated Xyl*p* residues. 3-*O*-Acetylated Xyl*p* residues having free 2-OH group are attacked by at least one order of magnitude slower. Only application of a larger enzyme amounts or longer incubations resulted in glucuronoxylan acetylated exclusively at position 2. This enzyme specificity in combination with a control of the extent of deacetylation provides a tool

for a preparation of acetylglucuronoxylan either 3- and 2-*O*-monoacetylated, or just 2-*O*-monoacetylated (Fig. 3). We should note here that the resistance of the 2-acetyl group to *F. johnsoniae* AcXE can also be seen in the NMR spectra shown by Razeq et al. (2018). Due to an incomplete assignment of the signals by the authors this result is mentioned for the first time in this work.

In exclusively 2-*O*-acetylated xylan, the 2-*O*-acetylation appeared to be very stable, since it did not change due to acetyl group migration during relatively long incubations with the enzyme during NMR measurements. This is in contrast to common acetyl group migration observed at ambient temperature in synthetic monomeric xylopyranosides (Mastihubová and Biely, 2004a). Quite surprisingly, the acetyl group transfer from position 2 to position 3 in the polymer required a significant input of energy, such as a long heating at high temperature. These findings pointed out that xylopyranoid rings in the polysaccharide main chain are not flexible enough to enable conformation changes required for spontaneous acetyl group migration via orthoester intermediates observed on non-reducing-end xylopyranoid rings (Puchart & Biely, 2015). If there is an acetyl group migration on the polymer, it is undoubtedly extremely slow at physiological temperatures. Different situation may, however, occur during non-alkaline wood cooking. The finding of limited acetyl group migration within a polymer might also be relevant to biosynthesis of the acetylated polysaccharide(s). At least some plant xylan acetyl transferases could be specialized for esterification of hydroxyls at position 2 and position 3 (Zhong, Cui, & Ye, 2018).

In contrast to polymeric substrate that was deesterified exclusively at position 3, a lower regiospecificity of the *F. johnsoniae* AcXE was observed on multiply acetylated methyl xylopyranosides. The first deesterification proceeded exclusively at position 3 of 2,3-diacetate as well as 2,3,4-triacetate. However, 2,4-diacetate itself as well as that generated quickly from the triacetate was slowly deesterified at position 4 and yielded 2-monoacetate persisting in the reaction mixtures for a long time. Thus the preference of the enzyme for deacetylation of position 3, eventually 4, may be exploited in preparative synthesis of 2-monoacetylated xylopyranosides from multiply acetylated precursors. The replacement of the 2- or 3-hydroxyl group by hydrogen or fluorine in 2,4- and 3,4-diacetates accelerated the initial deesterification and also facilitated the second deacetylation. The modification of the 2,4-diacetate at position 3 excludes the role of the acetyl group migration to vicinal group in the second slow 2-*O*-deacetylation, which indicates that the enzyme may form productive complexes with xylopyranosides in different orientations. In conclusion we may conclude that the partially acetylated methyl xylopyranosides confirmed the preference of *F. johnsoniae*

AcXE for the 3-*O*-acetyl group, however, the enzyme did not show the same regioselectivity on monoacetylated 4-nitrophenyl xylopyranosides. The substitution of the hydroxyl group at position 2 facilitates greatly the 3-*O*-deacetylation in both polymeric substrate and xylopyranosides. These observations are difficult to be understood and their explanation will await elucidation of the 3D structure of the esterase.

Finally, we should stress the significance of this type of deacetylases in saccharification yields of acetylated plant xylans. In the case of hardwood glucuronoxylan, the 3-*O*-deacetylation of MeGlcA-substituted Xyl*p* residues unblocks 20-30 % of acetylated hardwood xylan to saccharification with glycoside hydrolases. The new deacetylase will also be important for a breakdown of herbaceous arabinoglucuronoxylans since they also contain 3-*O*-acetylated MeGlcA-substituted Xyl*p* residues (Naran, Black, Decker, & Azadi, 2009). The novel *F. johnsoniae* 3-*O*-deacetylase is complementary to other common AcXEs of families CE1, CE5 and CE6 deacetylating mono- and diacetylated Xyl*p* residues, and also to CE4 AcXEs ignoring not only the 3-*O*-acetyl group on MeGlcA-substituted Xyl*p* residues but also diacetylated Xyl*p* residues (Uhliariková, Vršanská, McCleary, & Biely, 2013). Similarly to other AcXEs, the action of the 3-*O*-deacetylase decreases hydrophilicity and solubility of native, partially acetylated polysaccharide. The enzyme allows to obtain selectively 2-*O*-acetylated polysaccharide. This may serve as a source of 2-*O*-acetylated xylooligosaccharides, however, if the acetyl group in the fragments occurs on the non-reducing-end Xyl*p* residues, the acetyl group will migrate to other two positions.

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