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Positional specifity of acetylxylan esterases on natural polysaccharide: An NMR study

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ABSTRACT

Background: Microbial degradation of acetylated plant hemicelluloses involves besides enzymes cleaving the glycosidic linkages also deacetylating enzymes. A detailed knowledge of the mode of action of these enzymes is important in view of the development of efficient bioconversion of plant materials that did not undergo alkaline pretreatment leading to hydrolysis of ester linkages.
Methods: In this work deacetylation of hardwood acetylglucuronoxylan by acetylxylan esterases from *Streptomyces lividans* (carbohydrate esterase family 4) and *Orpinomyces* sp. (carbohydrate esterase family 6) was monitored by ^{1}H-NMR spectroscopy.
Results: The ^{1}H-NMR resonances of all acetyl groups in the polysaccharide were fully assigned. The targets of both enzymes are 2- and 3-monoacetylated xylopyranosyl residues and, in the case of the *Orpinomyces* sp. enzyme, also the 2,3-di-*O*-acetylated xylopyranosyl residues. Both enzymes do not recognize as a substrate the 3-*O*-acetyl group on xylopyranosyl residues α-1,2-substituted with 4-*O*-methyl-D-glucuronic acid.
Conclusions: The ^{1}H-NMR spectroscopy approach to study positional and substrate specificity of AcXEs outlined in this work appears to be a simple way to characterize catalytic properties of enzymes belonging to various CE families.
Significance: The results contribute to development of efficient and environmentally friendly procedures for enzymatic degradation of plant biomass.

1. Introduction

Hardwood glucuronoxylans are partially acetylated heteropolysaccharides. Xylopyranosyl (Xyl*p*) residues linked β-1,4-glycosidically form the polysaccharide main chain and this is substituted with α-1,2-linked 4-*O*-methyl-D-glucuronic acid (MeGlcA) residues. The ratio of Xyl:MeGlcA varies, but the most frequently reported values are around 10. In native state, i.e. in plant cell walls, the polysaccharide is partially acetylated [1–3]. More than a half of Xyl*p* residues of the main chain is monoacetylated at position 2 or 3, or 2,3-di-*O*-acetylated. 3-*O*-Acetylation is to a high degree observed on Xyl*p* residues substituted with MeGlcA [4–6]. In summary, the acetic acid in hardwood glucuronoxylans is esterified by hydroxyl groups at positions 2 and 3, however, the acetyl groups occur on the xylan main chain in five structural variants due to substituents on the vicinal hydroxyl groups (see the formula in Fig. 1).

The microbial degradation of acetyl glucuronoxylans involves besides enzymes cleaving the β-1,4-glycosidic linkages of the main chain also α-glucuronidase and enzymes deacetylating the polymeric substrate or acetylated xylooligosaccharides generated by endoxylanases. Regarding the fact that hardwood acetylglucuronoxylan contains five different acetyl groups, a question arises whether the acetylxylan esterases, classified in several CE families [7] and reviewed recently [8], are specialized for one particular acetyl group or behave as more general acetyl esterases. Previous studies of the positional specificity of AcXEs on methyl β-D-xylopyranosides indicated that esterases deacetylate both positions 2 and 3 [9,10]. This positional "non-specificity" was ascribed to the formation of two productive complexes differing in the 180° orientation of the substrate [11,8]. However, all typical acetylxylan esterases exhibited a strong preference for deacetylation of the position 2 in monoacetyl derivatives of 4-nitrophenyl β-D-xylopyranoside [12]. Recent work indicated that AcXEs belonging to families CE1 and CE5 deacetylate in the polysaccharide and oligosaccharides both positions 2 and 3 [13]. It became clear that the catalytic properties of these enzymes should be investigated on natural partially acetylated xylan.

One of the approaches to verify the positional specificity of AcXEs on natural substrates could be the monitoring of the ^{1}H-NMR signal

Abbreviations: AcXE, acetylxylan esterase; Xyl*p*, D-xylopyranose or D-xylopyranosyl; CE, carbohydrate esterase

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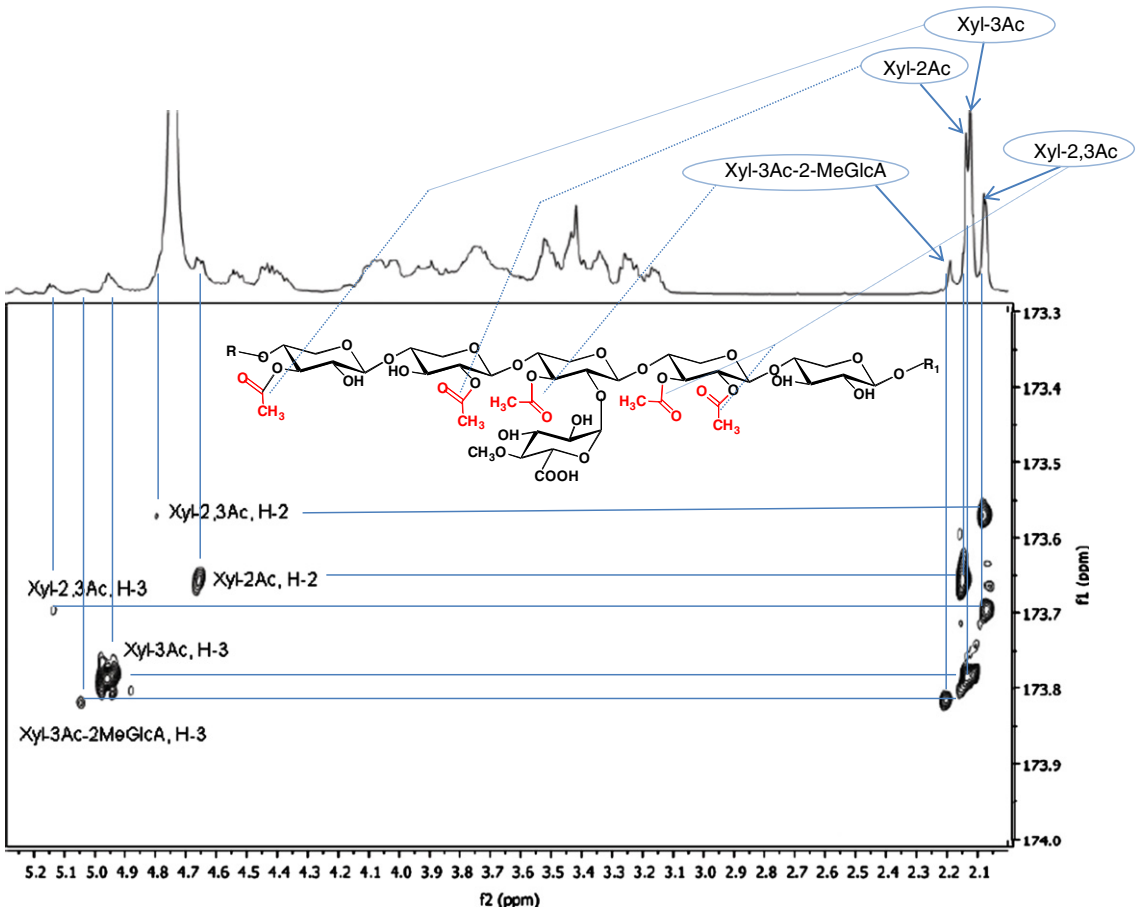

Fig. 1. Five types of acetylation of xylopyranosyl residues in hardwood acetylglucuronoxylans and selective HMBC spectrum of the polysaccharide showing two sets of four distinct signals and their correlation. The two acetyl groups esterifying the same Xyl*p* residue could not be distinguished in both anomeric and acetyl methyl group region. The following designations are used: Xyl-3Ac, 3-*O*-acetylated Xyl*p*; Xyl-2Ac, 2-*O*-acetylated Xyl*p*; Xyl-2,3Ac, 2, 3-di-*O*-acetylated Xyl*p*; Xyl-3Ac-2MeGlcA, MeGlcA 2-*O*-substituted and 3-*O*-acetylated Xyl*p*. The H/C correlations are marked with vertical and horizontal lines (see also Table 2). For better clarity the ^{1}H-NMR signals of the acetyl methyl groups are connected to the formula.

intensity of individual acetyl groups in acetylglucuronoxylans. The corresponding signals in the anomeric region of the spectrum have already been assigned [4–6], however, the complete assignment of the signals of methyl group protons of the acetyl groups is one of the subjects of the present work. These signals of the methyl groups are of much higher intensity that the relevant signals in the anomeric region, and therefore they enable an easy study of the positional specificity of the deacetylases. This is demonstrated here on example of AcXEs from *Streptomyces lividans* [14] and *Orpinomyces* sp. [15]. The results obtained in NMR experiments are complemented with the data on ability of the enzymes to precipitate acetylglucuronoxylan from solution and on their positional specificity on artificial substrates. The precipitation of acetylglucuronoxylan due to deacetylation by *Sl*CE4 and the mode of action of *Sl*CE4 on artificial substrates have been published earlier [12,8]. The *Sl*CE4 showed negligible activity on fully acetylated and 2,3-di-*O*-acetylated MeXyl*p*, and 2,4-di-*O*-acetyl and 3,4-di-*O*-acetyl-MeXyl*p* were deacetylated at positions 2 and 3 several orders faster that the above mentioned compounds with acetyl group at both positions 2 and 3 [16]. Replacement of the free hydroxyl group in 2,4-di-*O*- and 3,4-di-*O*-acetyl derivatives by hydrogen or fluorine resulted in dramatic reduction of the rate of deacetylation [16]. These data pointed out that the *Sl*CE4 requires free vicinal hydroxyl group for efficient deacetylation of position 2 or 3. This conclusion found strong support in structural studies [17]. The enzyme behaved differently on monoacetyl derivatives of 4-nitrophenyl β-D-xylopyranoside, showing a high preference for the 2-acetyl derivative [12]. In this respect the *Orpinomyces* AcXE was examined here for the first time.

2. Materials and methods

2.1. Acetylated carbohydrates

Beechwood acetylglucuronoxylan was isolated from delignified pulp by DMSO extraction [18]. Birchwood acetylglucuronoxylan prepared by steam explosion of birch saw dust was supplied by Dr. Henry Schneider (NRCC, Ottawa, Canada). The steam explosion leads to reduction of the molecular mass of the polysaccharide which makes it more suitable for the NMR study. The ^{13}C-NMR spectrum of this material before and after chemical deacetylation was published earlier [19]. In the present work the chemical deacetylation of the polysaccharide was done in D_2O solution of 0.1 M sodium hydroxide at room temperature overnight. The alkaline solution of the deacetylated polysaccharide was neutralized with 1 M solution of deuterized acetic acid (Aldrich Chemicals, USA) in D_2O before the NMR measurement.

2-*O*-acetyl-, 3-*O*-acetyl and 4-*O*-acetyl 4-nitrophenyl β-D-xylopyranoside were synthetized as described [20]. Di-*O*-acetates (2,3-, 2,4- and 3,4-) of Me-β-Xyl*p* were generous gifts from Dr. P. Kovac (National Institute of Health, Bethesda, MD, USA), Dr. J. Hirsch (Institute of Chemistry, Slovak Academy of Sciences) and Dr. A. Fernandes-Mayorales (Instituto de Quimica Organica General, CSIC, Madrid, Spain).

2.2. Enzymes

AcXE of CE4 family from *S. lividans* (*Sl*CE4) [14] was supplied with Dr. Dieter Kluepfel (Institute of Armand Frappier, Laval, Canada). CE6

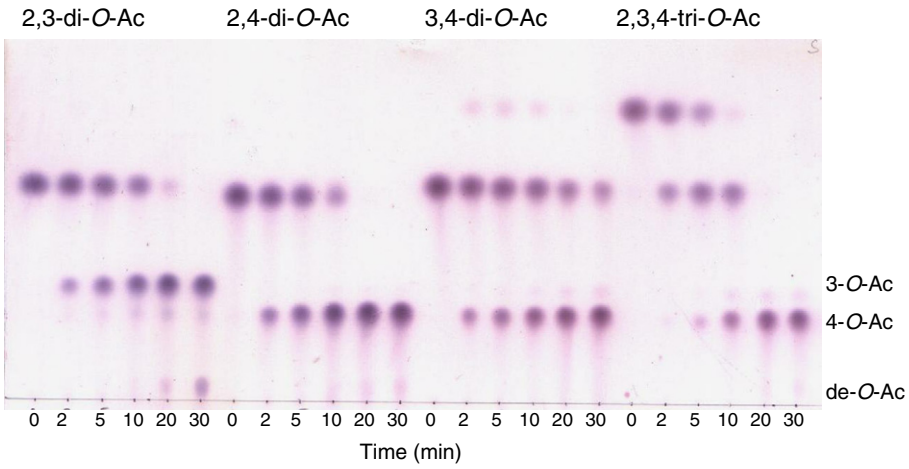

Fig. 2. Deacetylation of Me-Xyl*p* diacetates and triacetate with *Orpinomyces* sp. AcXE followed by TLC.

AcXE from *Orpinomyces* sp. was from Megazyme Int. (Ireland). It is a recombinant enzyme free of other xylanolytic enzymes and, in contrast to the *Sl*CE4, active on 4-nitrophenyl acetate. Before use in the NMR experiment, the enzymes were desalted by membrane filtration with two changes of D_2O followed by twofold lyophilization from D_2O. The auxiliary β-xylosidase was the *Aspergillus niger* GH3 enzyme produced in recombinant *Saccharomyces cerevisiae* as reported [21].

2.3. Action of AcXEs on acetylated carbohydrates

The ability of the AcXEs to precipitate beech acetylglucuronoxylan from solution (0.5%, w/v) in 0.1 M sodium phosphate buffer (pH 6.0) was tested at 25 °C. The positional specificity of the CE6 enzyme was examined on the monoacetates of 4-nitrophenyl β-D-xylopyranoside in the β-xylosidase-coupled assay [21]. The deacetylation of the triacetate and diacetates of Me-β-Xyl*p* at 10 mM concentration in 0.1 M sodium phosphate buffer (pH 6.0) with the enzyme (0.4 mg/ml) at 40 °C was followed by TLC on silica gel (Merck, Germany) in ethyl acetate–benzene–isopropanol (2:1:0.1, v/v). Sugars were detected with N-(1-naphthyl)ethylenediamine dihydrochloride reagent [22]. The positional specificity of the enzymes on birch acetylglucuronoxylan was examined by ^{1}H-NMR. 10 mg of the polysaccharide, twice evaporated from D_2O, was dissolved in 0.65 ml D_2O, the pH of the solution was adjusted to 6.0 with 0.2 M solution of deuterized sodium acetate (Aldrich Chemicals, USA) in D_2O. After recording the ^{1}H-NMR spectrum of the starting polysaccharide, AcXEs desalted and lyophilized twice from D_2O were added (*Sl*CE4 at 0.045 mg/ml and *O*CE6 at 0.3 mg/ml) and the changes in signal intensity of the acetyl groups were monitored in time course.

2.4. NMR measurements

NMR spectra were measured at 25 °C in D_2O on VNMRS 400 MHz Varian spectrometer equipped with 5 mm ^{1}H–^{19} F/^{15} N–^{31}P PFG AutoX DB NB probe head and on VNMRS 600 MHz Varian spectrometer equipped with 5 mm ^{1}H–^{19} F/^{15} N–^{31}P PFG One NMR Probe with an automatic chemical shifts calibration. Advanced techniques from Varian pulse sequence library of 2D homo- and hetero-correlated spectroscopy including sequences with selective excitations were used for the signal assignments.

The following NMR spectra were obtained on 400 MHz spectrometer: ^{1}H-NMR spectra (acquired using PRESAT sequence, presaturation delay 2 s, r.f. 90°pulse, acquisition time 2.5 s, number of transients 32), 2D ^{1}H–^{1}H homonuclear COSY spectrum (relaxation delay 1 s, acquisition time 0.35 s, the data size of 1024 in both, t_1 and t_2), TOCSY spectra (τ_{mix} = 0.08 s and τ_{mix} = 0.15 s, relaxation delay 1 s, acquisition time 0.35 s, the data size of 2048 in $t_1 \times 1024$ in t_2), NOESY spectrum (τ_{mix} = 0.09 s, relaxation delay 1.5 s, acquisition time 0.45 s, the data size 1024 in both, t_1 and t_2), and ^{1}H–^{13}C heteronuclear HSQC spectrum (relaxation delay 0.5 s, acquisition time 0.19 s, the data size 2048 in $t_1 \times 1024$ in t_2, with optimization on $^1J_{CH}$ = 146 Hz) and HMBC spectrum (relaxation delay 0.5 s, acquisition time 0.15 s, optimization on $^nJ_{CH}$ = 8 Hz long range coupling constant, the data size 2048 in $t_1 \times 2048$ in t_2). The selective HMBC spectrum was measured on 600 MHz spectrometer (relaxation delay 0.5 s, acquisition time 0.15 s, optimization on $^nJ_{CH}$ = 8 Hz long range coupling constant, the data size 2048 in $t_1 \times 2048$ in t_2). The spectral widths employed in all 2D NMR experiments were typically 1600 Hz for proton and 14,000 Hz (HSQC spectrum) or 20,000 Hz (HMBC spectrum) for carbon.

3. Results

3.1. Action of Orpinomyces AcXE on acetylated polysaccharide and glycosides

The *Orpinomyces* CE6 AcXE exhibited similarly as other typical AcXEs ability to precipitate beechwood acetylglucuronoxylan from solution as a result of liberation of the acetyl groups. Examination of positional specificity of the enzyme on monoacetates of 4-nitrophenyl

Table 1
^{1}H and ^{13}C NMR data (δ, ppm) of acetylglucuronoxylan at 25 °C in D_2O. The following designations are used: Xyl-(Xyl), non-acetylated Xyl*p*; Xyl-(Xyl-Ac), Xyl*p* linked to acetylated Xyl*p*; Xyl-3Ac, 3-*O*-acetylated Xyl*p*; Xyl-2Ac, 2-*O*-acetylated Xyl*p*; Xyl-2,3Ac, 2,3-di-*O*-acetylated Xyl*p*; Xyl-3Ac-2MeGlcA, 3-*O*-acetylated Xyl*p* 2-*O*-linked with MeGlcA.

Structural unit	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5,5′/C-5	CH_3-CO–	OCH_3
Xyl-(Xyl)	4.44/101.76	3.25/72.67	3.52/73.58	3.76/76.35	3.35, 4.07/62.99	–	–
Xyl-(Xyl-Ac)	4.38/102.74	3.18/72.25	3.49/71.21	3.72/76.50	3.34, 4.03/63.07	–	–
Xyl-3Ac	4.54/101.44	3.45/70.95	4.96/75.27	3.91/75.55	3.44, 4.11/63.02	2.13/20.55	–
Xyl-2Ac	4.66/99.85	4.66/73.44	3.76/71.46	3.83/76.10	3.40, 4.13/62.76	2.14/20.45	–
Xyl-2,3Ac	4.79/99.47	4.79/71.42	5.14/72.96	4.02/75.23	3.50, 4.17/62.52	2.07,2.08/20.29	–
Xyl-3Ac-2MeGlcA	4.68/101.03	3.66/74.82	5.04/73.87	3.96/75.56	3.46, 4.10/62.95	2.19/21.06	–
MeGlcA	5.26/97.13	3.53/72.53	3.81/72.51	3.16/82.23	4.32, −/72.16	–	3,42/60,09

Table 2
The assignment of proton chemical shifts (δ, ppm) of signals of acetyl groups in acetylated glucuronoxylan. The following designations are used: Xyl-3Ac, 3-*O*-acetylated Xyl*p*; Xyl-2Ac, 2-*O*-acetylated Xyl*p*; Xyl-2,3Ac, 2, 3-di-*O*-acetylated Xyl*p*; Xyl-3Ac-2MeGlcA, 3-*O*-acetylated Xyl*p* 2-*O*-linked with MeGlcA.

Structural unit	HMBC		NOESY
	H/C═O	C═O/CH_3	H/CH_3
Xyl-3Ac	H-3 4.96/173.79	173.79/2.13	H-3 4.96/2.13
Xyl-2Ac	H-2 4.66/173.66	173.66/2.14	H-2 4.66/2.14
Xyl-2,3Ac	H-2 4.79/173.57	173.57/2.07	n.d.
	H-3 5.14/173.70	173.70/2.08	
Xyl-3Ac-2MeGlcA	H-3 5.04/173.82	173.82/2.19	n.d.

β-D-xylopyranoside gave quite surprising results. In contrast to other AcXEs capable to precipitate acetylated beechwood xylan from solution and showing strong preference for deacetylation of 2-*O*-acetyl β-D-xylopyranoside [12], the ratio of specific activities of the studied enzyme towards 4 mM 2-*O*-, 3-*O*- and 4-*O*-acetyl derivative was 1.0:0.51:0.048. The action of the enzyme was also examined on di-*O*- and tri-*O*-acetyl derivatives of methyl β-D-xylopyranoside. A visual evaluation of the rate of hydrolysis of the derivatives followed by TLC indicated only a slight enzyme preference for attacking position 2 in 2,4-di-*O*-Ac-MeXyl*p* and 2,3-di-*O*-Ac-MeXyl*p* (Fig. 2). Fully acetylated MeXyl*p* was deacetylated somewhat faster than the above diacetates. The *Orpinomyces* CE6 AcXE obviously does not have any requirement for free vicinal hydroxyl group as the CE4 AcXEs do [16,17,23].

3.2. Assignment of resonances related to the acetyl groups

For a structural analysis of acetylglucuronoxylan used as a substrate of AcXEs, 1D and 2D NMR experiments were used. Analysis of the spectra resulted in a complete assignment of proton and carbon chemical shifts. The data are reported in Table 1. In the anomeric region of the ^{1}H-NMR spectrum the following signals of Xyl*p* residues were present: 2-*O*-acetyl Xyl*p* residue H-1 and H-2, overlapped at 4.66 ppm; 3-*O*-acetyl-Xyl*p* residue H-1 and H-3 at 4.54 ppm and 4.96 ppm, respectively; 3-*O*-acetyl-Xyl*p* residue with 2-*O*-linked MeGlcA H-1 and H-3 at 4.68 ppm and 5.04 ppm, respectively; 2,3-di-*O*-acetylated Xyl*p* residue H-1 and H-2, overlapped at 4,79 ppm, and H-3 at 5.14 ppm. The skeletal proton assignment of Xyl*p* units agrees with literature data [4–6,24,25], however, the analysis of HMBC and NOESY spectra resulted in a different assignment of the acetyl methyl groups signals. On the basis of H/C═O (proton of the backbone/carbon of the acetyl carbonyl group) and C═O/CH_3 (carbon of the acetyl carbonyl group/methyl of the acetyl group) cross peaks observed in the selective HMBC spectrum (Fig. 1), proton and carbon chemical shifts of differently acetylated Xyl*p* residues and the corresponding acetyl groups could be assigned (Table 2). NOESY spectrum (Fig. 3) confirmed this assignment with the H-2/CH_3 cross-peak of 2-*O*-acetyl-Xyl*p* residue which appeared at 4.66/2.14 ppm, and the H-3/CH_3 cross-peak due to 3-*O*-acetyl-Xyl*p* residue at 4.96/2.13 ppm. On the basis of these data, signals of the methyl protons of the acetyl groups at 2.07 and 2.08 ppm were attributed to 2,3-di-*O*-acetyl-Xyl*p* residue. Methyl group signal at 2.13 ppm was assigned to 3-*O*-acetyl-Xyl*p* residue, while those at 2.14 and 2.19 ppm belong to 2-*O*-acetyl-Xyl*p* and to 3-*O*-acetyl-Xyl*p* residue 2-*O*-substituted with MeGlcA, respectively. The data are summarized in Table 2.

The fact that ^{1}H-NMR spectroscopy enables to distinguish positions and environments of acetyl groups in acetylglucuronoxylan prompted us to examine in this way the positional specificity and mode of action of AcXEs on natural substrate dissolved in D_2O. This was done by monitoring changes in intensity of signals observed in the region 5.3–4.35 ppm (H-1 signals and skeletal protons of acetylated Xyl*p* residues) and signals of acetyl group methyl protons unambiguously assigned in this work. Because, H-1 and H-2 signals of 2,3-di-*O*-acetylated Xyl*p* residues (4.79 ppm) were hidden by residual

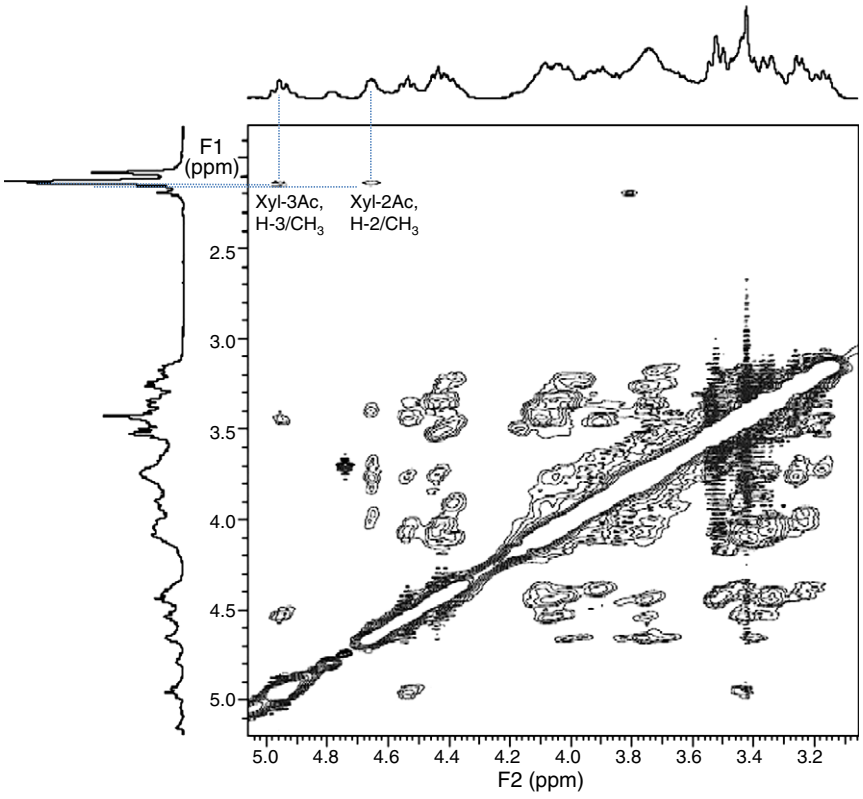

Fig. 3. NOESY spectrum of hardwood acetylglucuronoxylan showing the H-2/CH_3 cross-peak of 2-O-acetyl-Xyl*p* residue and the H-3/CH_3 cross-peak of 3-O-acetyl-Xyl*p* residue. The correlations are marked by vertical and horizontal lines in the upper left corner. The corresponding resonances, derived from a considerably magnified spectrum, are shown in Table 2.

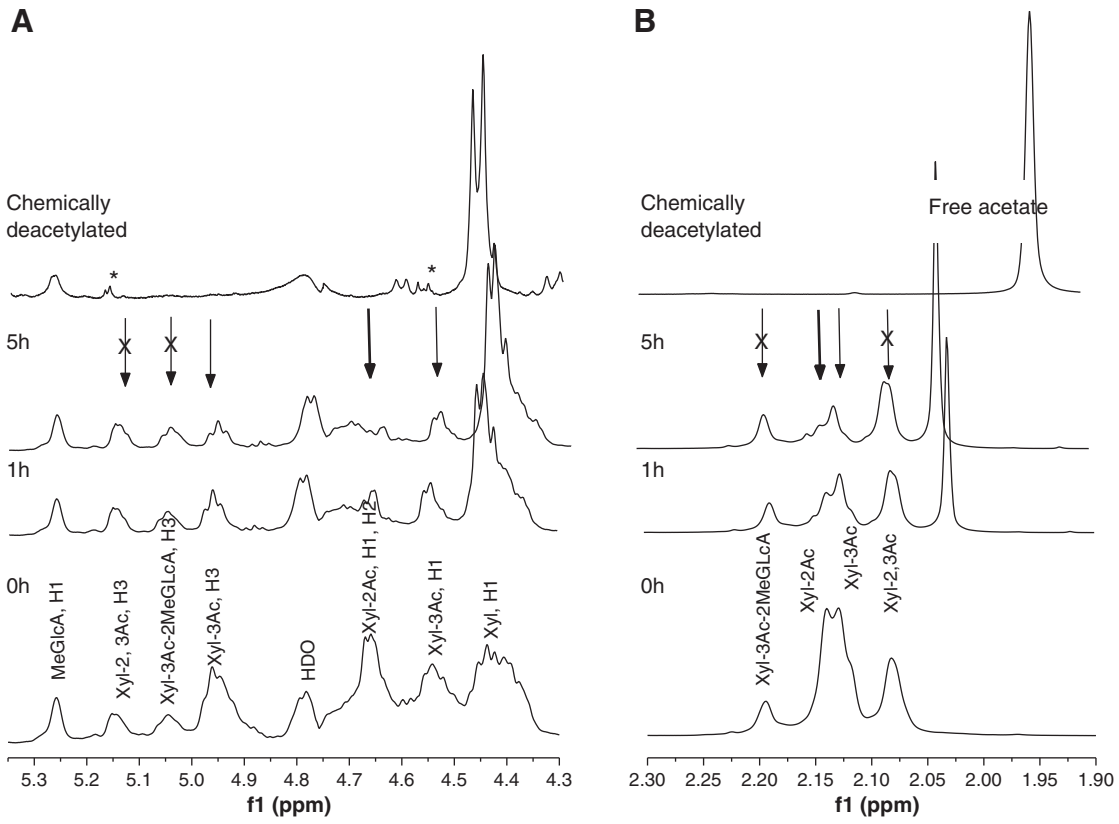

Fig. 4. Monitoring of the action of *Streptomyces lividans* CE4 AcXE on birch acetylglucuronoxylan by ^{1}H-NMR spectroscopy. The changes of signal intensity of acetylated Xyl*p* residues in the anomeric region of the spectrum are shown in Part A, the changes of intensity of the methyl protons of the acetyl groups are shown in Part B. The time of incubation in hours is indicated. The most rapidly decreasing signals are marked with thick arrows, slower decreasing signals with thinner arrows. Signals of protons not influenced by the enzyme are marked with crossed arrows. The spectrum of the chemically deacetylated polysaccharide is shown on the top of Part A, B. The following designations are used: Xyl, non-acetylated Xyl*p*; Xyl-3Ac, 3-*O*-acetylated Xyl*p*; Xyl-2Ac, 2-*O*-acetylated Xyl*p*; Xyl-2,3Ac, 2,3-di-*O*-acetylated Xyl*p*; Xyl-3Ac-2MeGlcA, 3-*O*-acetylated Xyl*p* 2-*O*-substituted with MeGlcA. The H-1 signals of the reducing end Xyl residues, αXyl_{red} at 5.15 ppm and βXyl_{red} at 4.55 ppm, are marked by asterisk.

water signal, its H-3 signal (5.14 ppm) was used for monitoring of the deacetylation reaction. The anomeric region of the spectrum also contains signals of non-acetylated Xyl at the reducing end (αXyl_{red} at 5.15 ppm and βXyl_{red} at 4.55 ppm) which are visible in the ^{1}H-NMR spectrum of the alkali deacetylated glucuronoxylan (Fig. 4).

3.3. Positional specificity of *S. lividans* AcXE

Fig. 4 shows that *S. lividans* AcXE *St*CE4 releases acetyl groups from position 2 and position 3, but only from singly acetylated Xyl*p* residues. The intensity of the resonance corresponding to 2-*O*-acetylated Xyl*p*

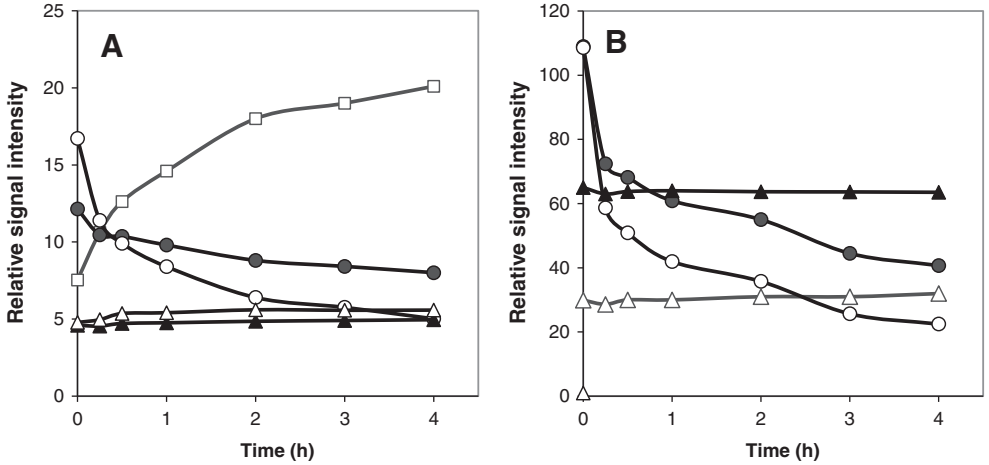

Fig. 5. Time course of the changes in intensity of ^{1}H-NMR signals of acetylated and non-acetylated Xyl*p* residues in the anomeric region of the spectrum (Part A) and signals of the methyl protons of acetyl group (Part B) during incubation of birch acetylglucuronoxylan with *S. lividans* CE4 AcXE. Symbols of xylopyranosyl residues: □, internal non-acetylated; ●, 3-*O*-acetyl; ○, 2-*O*-acetyl; ▲, 2,3-di-*O*-acetyl, △ 3-*O*-acetyl-2-*O*-MeGlcA. The values of the signal of non-acetylated Xyl*p* residues in part A (□) were divided by 2 to make the graph more illustrative.

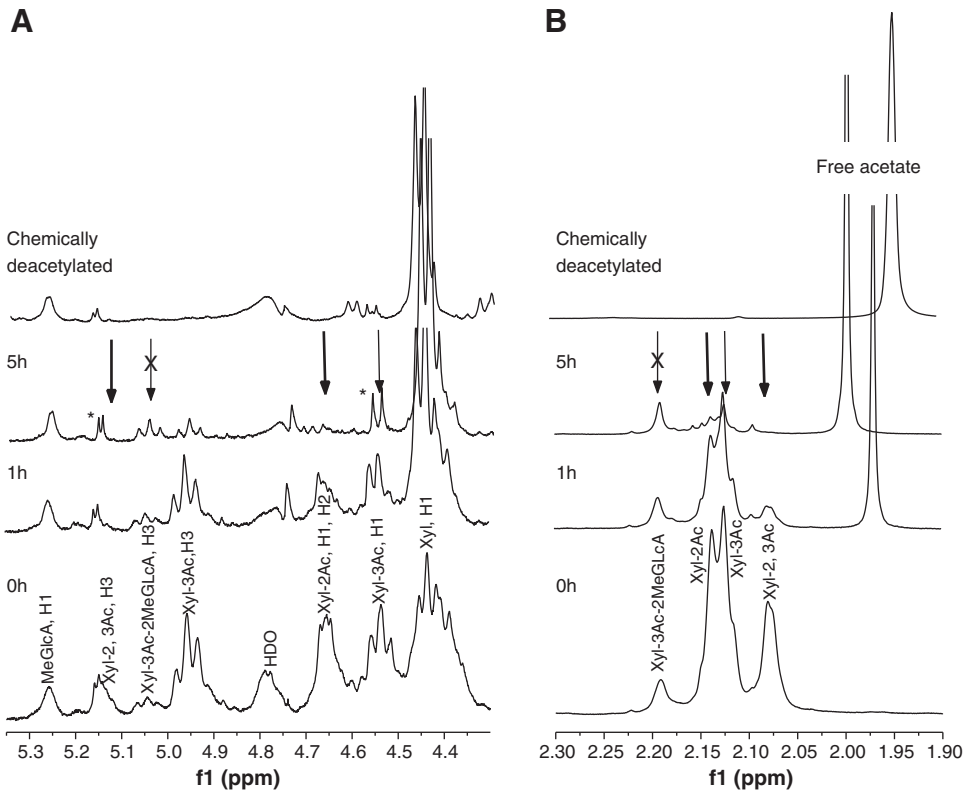

Fig. 6. Monitoring of the action of *Orpinomyces* CE6 AcXE on birch acetylglucuronoxylan by ^{1}H-NMR spectroscopy. The changes of signal intensity of acetylated Xyl*p* residues in the anomeric region of the spectrum are shown in Part A, the changes of intensity of the methyl protons of the acetyl groups are shown in Part B. The time of incubation in hours is indicated. The most rapidly decreasing signals are marked with thick arrows, slower decreasing signals with thinner arrows. Signals of protons not influenced by the enzyme are marked with crossed arrows. The spectrum of the chemically deacetylated polysaccharide is shown on the top of Part A, B. The following designations are used: Xyl, non-acetylated Xyl*p*; Xyl-3Ac, 3-*O*-acetylated Xyl*p*; Xyl-2Ac, 2-*O*-acetylated Xyl*p*; Xyl-2,3Ac, 2,3-di-*O*-acetylated Xyl*p*; Xyl-3Ac-2MeGlcA, 3-*O*-acetylated Xylp 2-*O*-linked with MeGlcA. The H-1 signals of the reducing end Xyl residues, αXyl_{red} at 5.15 ppm and βXyl_{red} at 4.55 ppm, are marked by asterisk.

residues decreased faster than the resonances of 3-*O*-acetylated residues (Fig. 5). A clear sign of the enzyme performance is the remarkable increase of the H-1 signal of completely deacetylated internal Xyl*p* residues at 4.44 ppm. The changes in the intensity of signals in the anomeric region were in accordance with changes of the intensity of signals of methyl protons in the acetyl groups (Fig. 4 and Fig. 5). The enzyme did not attack 2,3-di-*O*-acetylated xylopyranosyl residues and the 3-*O*-acetyl group on xylopyranosyl residues α-1,2-substituted with 4-*O*-methyl-D-glucuronic acid.

3.4. Positional specificity of Orpinomyces AcXE

Action of the CE6 AcXE on acetylglucuronoxylan is shown in Fig. 6 and Fig. 7. The favorite targets of the enzyme are 2,3-di-*O*-acetylated Xyl*p* residues and 2-*O*-acetyl-Xyl*p* residues. A somewhat slower decrease of the signal of 3-*O*-acetyl Xyl*p* residue could be explained by generation of this type of acetylated Xyl*p* from 2,3-di-*O*-acetylated residue after removal of the acetyl group from position 2. However, the enzyme similarly as *Sl*CE4 does not recognize the 3-*O*-acetyl group on Xyl*p*

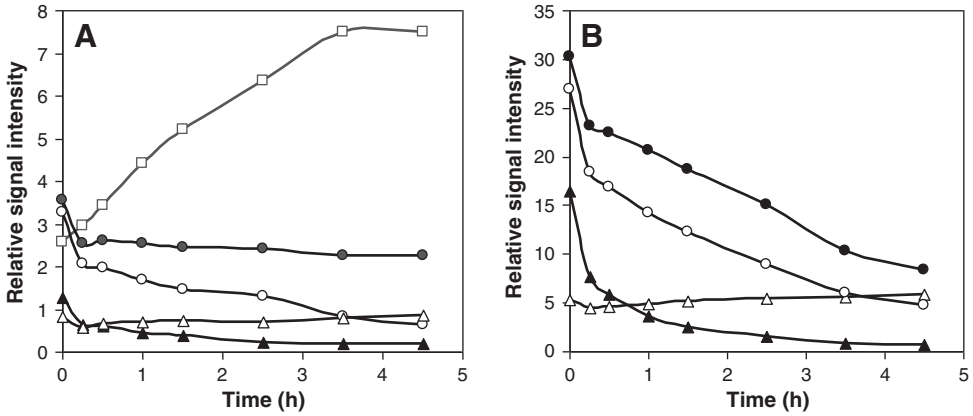

Fig. 7. Time course of the decrease in intensity of ^{1}H-NMR signals of acetylated and non-acetylated Xyl*p* residues in the anomeric region of the spectrum (Part A) and signals of the methyl protons of acetyl group (Part B) during incubation of birch acetylglucuronoxylan with *Orpinomyces* sp. AcXE. Symbols of xylopyranosyl residues: □, internal non-acetylated;●, 3-*O*-acetyl; ○, 2-*O*-acetyl;▲, 2,3-di-*O*-acetyl, △, 3-*O*-acetyl-2-*O*-MeGlcA. The values of the signal of non-acetylated Xyl*p* residues in part A (□) were divided by 2 to make the graph more illustrative.

residues 2-*O*-substituted with MeGlcA. The intensity of the corresponding signal at 2.19 and 5.04 ppm did not change with time. Thus MeGlcA appears to be a serious steric barrier for *O*CE6 because the enzyme does not require a free vicinal hydroxyl group as CE4 AcXEs. The enzyme tolerates well another acetyl group at the vicinal position.

4. Discussion

In accord with the requirement for a free vicinal hydroxyl group of the CE4 enzymes for deacetylation of position 2 or 3 [16,17,23], *Sl* CE4 does not recognize as its targets the doubly acetylated xylose residues and also the 3-*O*-acetylated residues substituted by MeGlcA. The intensity of the corresponding resonances in the anomeric region of the spectrum and in the region of signals of the acetyl methyl group did not change during several hours (Fig. 5). The fact that the *Sl*CE4 deacetylates both positions 2 and 3 in monoacetylated Xyl*p* residues is in contrast to the high selectivity of the enzyme for position 2 in monoacetates of 4-nitrophenyl xylopyranoside [12]. However, *Sl*CE4 was shown to be capable of deacetylating positions 2 and 3 in 2,4- and 3,4-di-*O*-acetyl-methyl β-D-xylopyranoside [16]. A question remains therefore to be answered whether the deacetylation of both positions is a consequence of acetyl group migration from position 3 to position 2 prior the nucleophile attack on the ester carbonyl group [11,8], or whether the polysaccharide can form with the enzymes productive complex also in orientation by 180° reversed to that in which the position 2 is deacetylated [8]. The formation of such two productive complexes was suggested for acetylated MeXyl*p* when the 3D structure of *Trichoderma reesei* CE5 AcXE was established [11]. The stereochemistry of the position 2 and 3 on Xyl*p* residue in both orientations is the same [8]. Since the formation of the reverse productive complex can be excluded with CE4 chitin deacetylases hydrolyzing the amide of $GlcNH_2$ exclusively at the position 2 [26–28], we hypothesize that the deacetylation of both positions 2 and 3 by *Sl*CE4 takes place at the same orientation of the substrate in the enzyme–substrate complex. In the case the enzyme facilitated acetyl group migration would not be involved in the mechanism of deacetylation of the two positions, the enzyme should be able to form productive complexes with both 2-*O*- and 3-*O*-acetylated Xyl*p* residues at the same orientation of the xylan main chain.

Similar considerations concern the mode of action of *O*CE6. In acetylglucuronoxylan the enzyme attacks preferentially position 2 in both mono- and di-*O*-acetylated Xyl*p* residues. The rate of deacetylation of position 3 stays only slightly behind, perhaps due to generation of the 3-*O*-acetylated Xyl*p* by preferential removal of the acetate from the 2-position of the 2,3-di-*O*-acetylated residues. This mode of action is in accord with substrate specificity of the enzyme on acetylated derivatives of MeXyl*p* and monoacetyl derivatives of 4-nitrophenyl Xyl*p*. Anyway, the ability of the CE6 enzyme, belonging to the serine-type esterases [15,29], to deacetylate both position 2 and 3 remains difficult to be explained similarly as in the case of *Sl*CE4, an aspartate metalloenzyme [14,30]. Thus, the formation of two different spatially distorted enzyme-substrate complexes with the same xylan main chain orientation cannot be excluded also in the case of the CE6 AcXE. More detailed studies of the reaction mechanisms and particularly three-dimensional structures of the enzymes with appropriate ligands are required to answer these questions.

5. Conclusions

AcXEs from *S. lividans* and *Orpinomyces* sp. belonging to CE family 4 and 6, respectively, efficiently deacetylate hardwood acetylglucuronoxylan. The monitoring of the deacetylation by ^{1}H-NMR spectroscopy showed that targets of both enzymes are acetyl groups at positions 2 and 3 on singly acetylated Xyl*p* residues. The *Orpinomyces* sp. AcXE deacetylates efficiently also the doubly acetylated Xyl*p* residues. Both enzymes do not attack the 3-*O*-acetyl group on Xyl*p* residues α-1,2-substituted with MeGlcA. The ^{1}H-NMR spectroscopy approach to study positional and substrate specificity of AcXEs outlined in this work appears to be a simple way to characterize catalytic properties of enzymes belonging to various CE families. Particularly clear data are generated by following the changes in the signals of the acetyl methyl groups. A detailed knowledge of the mode of action of polysaccharide deacetylating enzymes is important in view of the development of efficient bioconversion of plant materials that did not undergo alkaline pretreatment leading to hydrolysis of ester linkages.

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