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Glucuronoyl esterases are active on the polymeric substrate methyl esterified glucuronoxylan

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ABSTRACT

Alkali extracted beechwood glucuronoxylan methyl ester prepared by esterification of 4-*O*-methyl-D-glucuronic acid side residues by methanol was found to serve as substrate of microbial glucuronoyl esterases from *Ruminococcus flavefaciens*, *Schizophyllum commune* and *Trichoderma reesei*. The enzymatic deesterification was monitored by ^{1}H NMR spectroscopy and evaluated on the basis of the decrease of the signal of the ester methyl group and increase of the signal of methanol. The results show for the first time the action of enzymes on polymeric substrate, which imitates more closely the natural substrate in plant cell walls than the low molecular mass artificial substrates used up to present.
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1. Introduction

The discovery of the enzyme called glucuronoyl esterase in the cellulolytic system of the wood rotting fungus *Schizophyllum commune* using synthetic esters of uronic acids [1] suggested that the enzyme could have important biotechnological implications. There are reports that one of the covalent linkages between hemicellulose and lignin in plant cell walls are ester linkages between 4-*O*-methyl-D-glucuronic acid (MeGlcA) and lignin alcohols [2–13] which could be potential substrates of the esterase. After the genes coding for glucuronoyl esterase were found to be widely distributed in both prokaryotic and eukaryotic microorganisms, a new family of carbohydrate esterases, CE15, was established [14,15]. However, only a few CE15 enzymes have been characterized in terms of catalytic activity up to now [1,14,16–18], and only two members of the family have had their 3D structure determined [19–21]. To general disappointment, there is no report that would demonstrate the physiological role of glucuronoyl esterases in microbial degradation of plant cell walls or lignin–carbohydrate complexes. However, glucuronoyl esterases certainly play an important role in this process, since they appear to be inducible constituents of plant cell wall degrading enzyme systems, they are frequently bi- or multimodular enzymes containing carbohydrate binding modules (CBM) [14,17] and they are also parts of cellulosomes, like the enzyme from *Ruminococcus flavefaciens* [22,23]. The activity of glucuronoyl esterases was demonstrated only on low molecular mass artificial substrates [1,14,16–18]. The enzymes are active on synthetic alkyl and alkylaryl esters of MeGlcA and glucuronic acid [24–27], and also on their aryl α- or β-glycosides [24], which indicates that they could recognize as substrates also esters of β-glycosidically linked MeGlcA. Such residues occur in arabinogalactans [28], however, their esterification has not been reported. The enzymes were found to be inactive on similar esters of D-galacturonic acid which excludes their participation in enzymatic hydrolysis of pectin substances [24]. Cloning of microbial GE genes in plants, in order to find out more about their physiological role, had deteriorating effect on plant development in *Arabidopsis* [29], and led to changes in the cell wall composition in aspen [30].

In this work we provide the first experimental evidence that glucuronoyl esterases recognize methyl esters of MeGlcA as a part of high molecular mass polysaccharide. The methyl ester of beechwood glucuronoxylan was prepared by alkali-catalyzed esterification. The modified polysaccharide and its deesterification

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by enzymes was studied by NMR. We show that monitoring of the decrease of the ester signal or increase of the signal of methanol could be used as basis for an alternative assay of glucuronoyl esterase.

2. Materials and methods

2.1. Enzymes

Glucuronoyl esterase from *Trichoderma reesei* was a recombinant enzyme obtained by homologous cloning of its catalytic domain as described earlier [14]. *S. commune* glucuronoyl esterase was prepared by cloning in *Pichia pastoris* as reported [27]. Both enzymes were shown to be electrophoretically homogeneous in the original papers [14,27]. Glucuronoyl esterase from *R. flavefaciens* was a recombinant enzyme prepared by cloning of the carboxy-terminal domain of a multi-domain protein CesA [22,23] in *Escherichia coli* as follows: An 1.5 kb DNA band coding for CesA amino acid residues from 352 to 768 plus a Met coding codon at the beginning, *Nde*I and *Not*I restriction sites on the 5′ and 3′ ends, respectively, were synthesized by Mr. Gene GmbH (Regensburg, Germany). Cloning of the 1.5 kb DNA fragment into PET24b, transformation *E. coli* BL21 (DE3), induction for the target protein expression, and extraction of proteins from *E. coli* cells were done according to the Novagen pET system suggested protocols. Purification of the active enzyme was done using column chromatograph on an AKTA Protein Purification System and the enrichment of the active enzyme was traced by activity against 4-O-methyl-D-glucuronic acid methyl ester hydrolysis followed by thin layer chromatography [1]. Purity of the target enzyme was accessed using SDS–PAGE followed by Coomassie Brilliant Blue staining (Fig. S1).

2.2. Preparation of glucuronoxylan methyl ester and other substrates

Beechwood glucuronoxylan, isolated by alkaline extraction from delignified holocellulose [31], was converted to its methyl ester a by modification of the procedure previously used for methyl esterification of galacturonic acid residues of pectin [32]. Briefly, glucuronoxylan was dissolved in water, converted into the tetrabutylammonium (TBA) salt using cation exchanger Amberlite IRN 77 (TBA^+ form) and lyophilized. The TBA salt (1000 mg) was dissolved in dry DMSO (6 ml) under stirring at 50 °C. After cooling, methyl iodide (1.8 ml) was added and the reaction mixture stirred for 2 days at 30 °C. After addition of 3 M NaCl (6 ml), the mixture was poured into acetone (600 ml). The formed precipitate was filtered, washed with acetone, suspended in deionized water and dialyzed. Lyophilization of the retentate afforded the esterified polysaccharide in about 90% yield from which only one third was found to be soluble in water. The water-soluble fraction of the glucuronoxylan methyl ester used as polymeric substrate of glucuronoyl esterases. Its average molecular weight, determined by high pressure gel permeation chromatography using two coupled HEMABIO columns (100 and 300, 8 × 250 mm) (Tessek, Praha, Czech Republic) and refractometric detection, was found to be 37.5kDa. The columns were eluted with 0.1 M aqueous solution of $NaNO_3$. The set of pullulans was used as standards. The polymeric character of glucuronoxylan methyl ester was also obvious from a TLC analysis which confirmed the absence of fragments shorter than decasaccharide. The structural features of the starting polysaccharide and its methyl ester is shown in Fig. 1. 4-Nitrophenyl 2-*O*-(methyl 4-*O*-methyl-D-glucopyranosyluronate)-β-D-xylopyranoside [33] was a generous gift from Dr. Ján Hirsch (Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia).

2.3. NMR measurements

NMR measurements were performed in D_2O at 25 °C on VNMRS 600 MHz Varian spectrometer equipped with HCN ^{13}C enhanced salt tolerant cold probe. Chemical shifts were referenced to internal standard 3-methyl silylpropionic acid sodium salt (TSP, δ 0 ppm). ^1H NMR spectra were acquired using presat sequence. Advanced techniques from Varian pulse sequence library of 2D homo- and hetero-correlated spectroscopy were used for the signal assignments.

2.4. Monitoring of glucuronoyl esterase action

The deesterification of glucuronoxylan methyl ester was monitored by ^1H 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 ^1H NMR spectrum of the starting polysaccharide, appropriate amount of tested glucuronoyl esterase lyophilized twice from D_2O was added and the spectra recorded in time intervals. Changes in signal intensity of the MeGlcA ester methyl group and MeOH in time course were monitored. The rate of deesterification was evaluated on the basis of signal heights. The dependence of the rate of

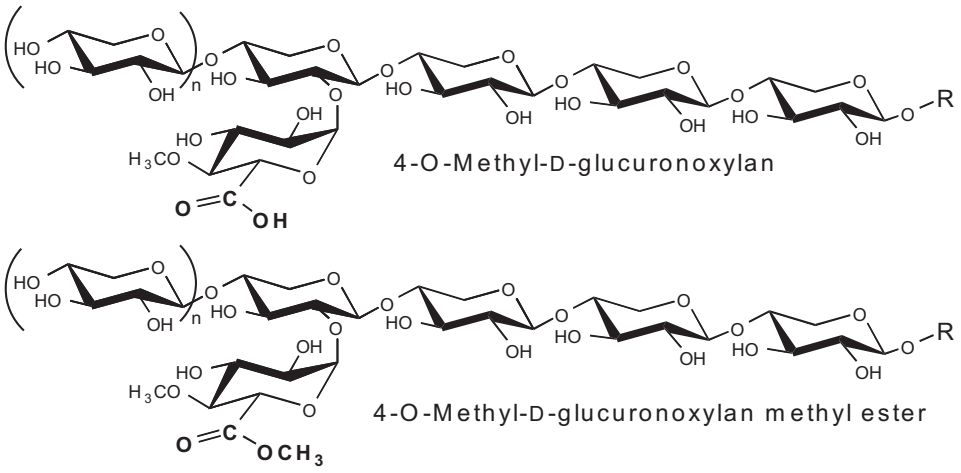

Fig. 1. Scheme of glucuronoxylan and its methyl ester.

deesterification on *T. reesei* glucuronoyl esterase concentration, and the comparison of the action of *R. flavefaciens* enzyme on low and high molecular mass substrates was examined in small volume cuvettes with reaction volume of 0.25 ml.

3. Results

3.1. ^{1}H NMR spectra of glucuronoxylan methyl ester

The ^{1}H NMR spectrum of glucuronoxylan methyl ester in D_2O (Fig. 2) shows the presence of six anomeric signals between 4.48 ppm and 5.28 ppm, assigned to H-1 of MeGlcA (δ 5.28), α-anomer of the reducing-end Xyl residue ($Xyl_{red\alpha}$, δ 5.19), internal Xyl*p* unit substituted at *O*-2 with MeGlcA (Xyl_{MeGlcA}, δ 4.64), β-anomer of the reducing-end Xyl residue ($Xyl_{red\beta}$, δ 4.59), internal non-substitued Xyl*p* residues (Xyl_{int}, δ 4.48) and non-reducing-end Xyl*p* residues (Xyl_{nred}, δ 4.46). Since the internal non-substituted Xyl*p* residues are most abundant, its H-1 signal (δ 4.48) shows high intensity similarly as the signals of other hydrogens of these Xyl*p* residues: H-5_{eq} at 4.12, H-4 at 3.80, H-3 at 3.57, H-5_{ax} at 3.39 and H-2 at 3.30 ppm. These signals did not change due to esterification of MeGlcA residues with methanol. The only signal of glucuronoxylan shifted to a higher ppm in the ester (from 3.47 to 3.46 ppm) is the singlet corresponding to the OCH_3 group at position 4 of MeGlcA. The ^{1}H spectrum of the ester also shows a new singlet at 3.85 ppm which corresponds to protons of the methyl ester group $COOCH_3$ (Fig. 1, Table 1). The integration of anomeric signals at δ 4.48, 4.64, 5.28 ppm indicated a molar ratio of non-substitued Xyl*p* units to 2-*O*-substitued Xyl*p* units and methyl ester of MeGlcA, 5:1:1. Thus the proton NMR data indicated the presence of five Xyl units per one residue of the uronic acid methyl ester. Since the average ratio of Xyl to MeGlcA in alkali extracted beechwood glucuronoxylan is around 10:1 [31], this result serves as evidence that the water-soluble fraction of the glucuronoxylan methyl ester corresponds to a more densely branched portion of the polysaccharide.

The comparison of the ^{1}H NMR spectra of glucuronoxylan and its methyl ester pointed clearly to the possibility to follow the deesterification of the polysaccharide on the basis of the decrease in intensity of the signal of the methyl ester group at 3.85 ppm and also the signal of the 4-*O*-methyl group of esterified MeGlcA residues at 3.47 ppm on account of the increase of the 4-*O*-methyl

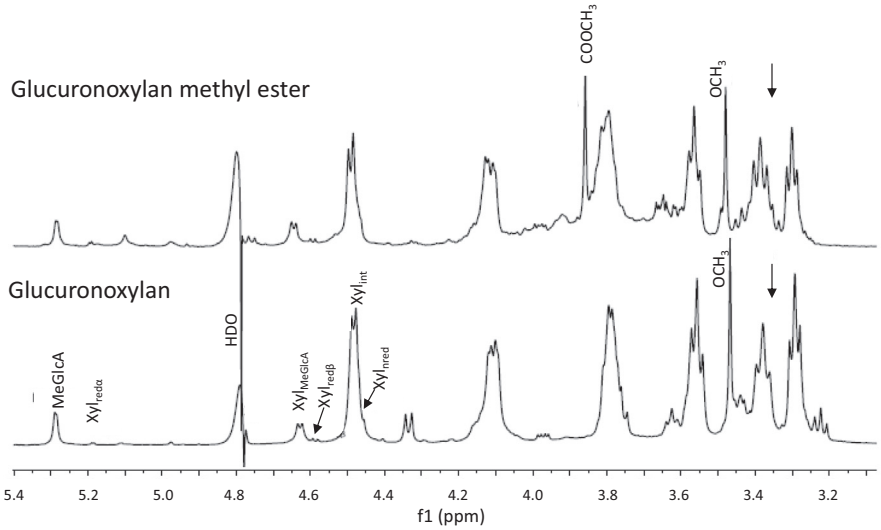

Fig. 2. ^{1}H NMR spectrum of glucuronoxylan and its methyl ester. Arrows mark the position of the methanol signal which is absent in both spectra. The signals of glucuronoxylan methyl ester are assigned in Table 1.

Table 1
Chemical shifts (δ/ppm) of the glucuronoxylan methyl ester in D_2O.

Unit	H_1/C_1	H_2/C_2	H_3/C_3	H_4/C_4	H_5/C_5	4-OCH_3 (MeGlcA)	$COOCH_3$ (ester)	
MeGlcA	5.28	3.61	3.81	3.35	4.75	3.47	3.85	*3.83
	100.92	73.80	74.72	84.15	72.30	62.72	56.07	*55.79
							C=O	C=O
							174.89	*173.52
Xyl_{MeGlcA}	4.64	3.40	3.65	3.79	3.39,4.11			
2-O-subst. Xyl*p*	104.07	80.11	75.21	78.81	65.24			
Xyl_{int}	4.48	3.30	3.57	3.80	3.39,4.12			
Internal Xyl*p*	104.54	75.56	76.49	79.18	65.78			
$Xyl_{red\beta}$	4.59	3.26	3.55	3.78	4.06,3.39			
Red. end β-anomer	99.28	n.a.	76.54	79.40	66.13			
$Xyl_{red\alpha}$	5.19	3.55	3.76					
Red. end α-anomer	94.91	74.28	74.73					
Xyl_{nred}	4.46	3.29	3.44	3.63	3.32,3.98			
Xyl*p* residue on non-red. end	104.60	n.a.	78.48	71.97	68.11			
		74.93?						
Signals of unknown components	4.76	4.13						
	102.93	72.82						

* Minor resonances visible in ^{1}H–^{13}C 2D spectra only.

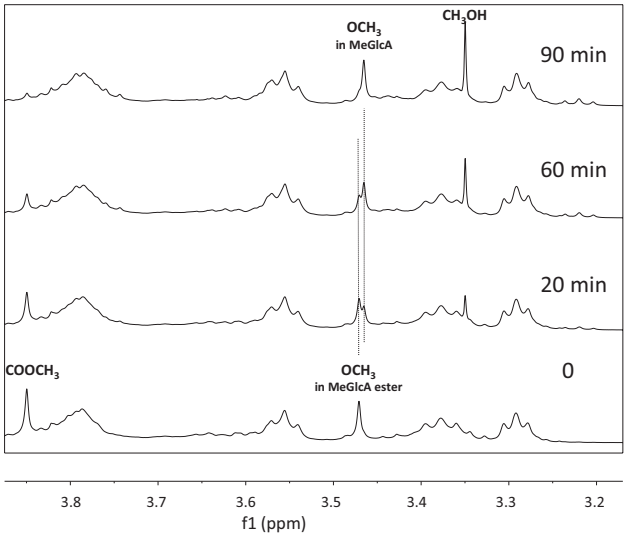

Fig. 3. Monitoring of deesterification of glucuronoxylan methyl ester by *R. flavefaciens* glucuronoyl esterase by ^{1}H NMR spectroscopy. Decrease in intensity of the signals of the protons of the methyl ester group ($COOCH_3$) and the methyl ether group (OCH_3 at 3.85 ppm) were accompanied by increase of intensity of proton signals of the methyl group in deesterified MeGlcA (OCH_3 at 3.7 ppm) and appearance of signals of methanol (3.35 ppm).

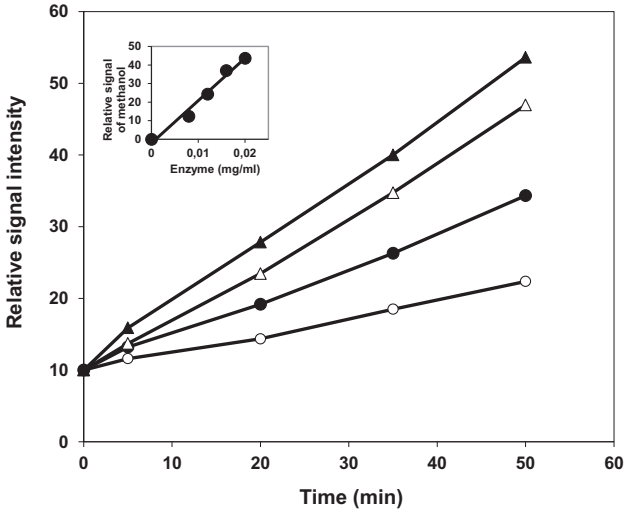

Fig. 5. Change in the intensity of ^{1}H NMR signals of methanol during incubation of glucuronoxylan methyl ester with different amounts of *T. reesei* glucuronoyl esterase. Enzyme concentration: ○, 0.008; ●, 0.0012; △, 0.0016; ▲, 0.02 mg/ml. The insert shows the dependence of the increase of the NMR signal of methanol after a 50 min incubation of 1% glucuronoxylan methyl ester solution with different concentrations of *T. reesei* glucuronoyl esterase.

group of deesterified MeGlcA residues at 3.46 ppm. In addition, release of methanol should show up with a new singlet at 3.35 ppm.

3.2. ^{13}C NMR spectra of glucuronoxylan methyl ester

To complete the characterization of the glucuronoxylan methyl ester, its ^{13}C chemical shifts were also assigned using ^{1}H–^{13}C correlated 2D experiments. According the HSQC spectrum, the C-4 signal of the internal xylose units was downfield shifted to 79.18 ppm, thus confirming the involvement of this carbon in (1 → 4) linkages. The C-2 and C-4 signals of 2-*O*-substituted xylose units were also downfield shifted, the former to 78.81, and the latter to 80.11 ppm. Signals at δ 5.28/100.92 (H-1/C-1), 3.61/73.80 (H-2/C-2), 3.81/74.72 (H-3/C-3), 3.35/84.15 (H-4/C-4) and 4.75/72.30 (H-5/C-5) were attributed to MeGlcA methyl ester (Table 1). The H-5 signal of MeGlcA methyl ester was not visible in the proton spectrum due to saturation of water signal.

3.3. Glucuronoxylan methyl ester – substrate of glucuronoyl esterases

All glucuronoyl esterases examined, regardless of bacterial or fungal origin, effectively deesterified glucuronoxylan methyl ester. Fig. 3 shows the monitoring of the action of *R. flavefaciens* glucuronoyl esterase. The changes in the intensity of the signals of the ester group $COOCH_3$ and 4-*O*-methyl ether group OCH_3 in time correspond to polysaccharide deesterification resulting in release of methanol (singlet at 3.35 ppm). Similar results were obtained with glucuronoyl esterases from *S. commune* and *T. reesei* (Fig. S2). When the intensity of the ester and methanol signals are plotted versus time (Figs. 4 and S3), the rate of deesterification appears to be constant for a relatively long time, up to about 70–80% of substrate deesterification.

It was of interest to compare the performance of glucuronoyl esterases on polymeric substrate and 4-nitrophenyl 2-*O*-(methyl 4-*O*-methyl-D-glucopyranosyluronate)-β-D-xylopyranoside. The experiment with *R. flavefaciens* glucuronoyl esterase was done also in D_2O solutions following the changes of decrease of the ester signal and increase of the methanol signal by ^{1}H NMR. The

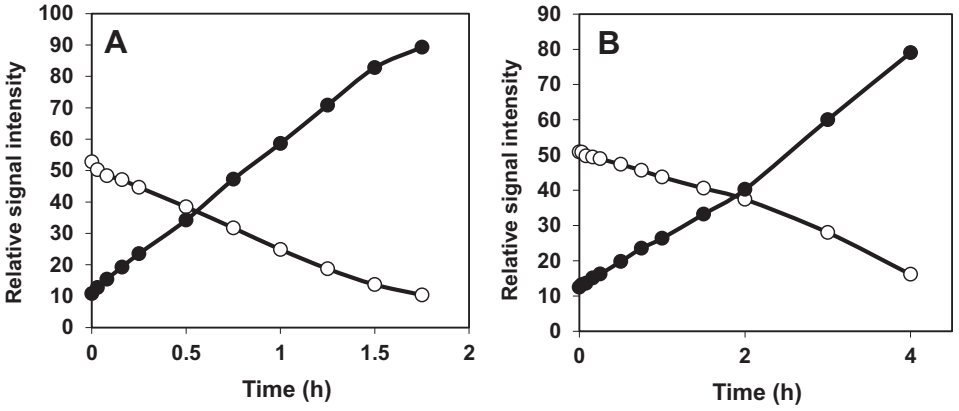

Fig. 4. ^{1}H NMR monitoring of the deesterification of glucuronoxylan methyl ester by glucuronoyl esterase from *R. flavefaciens* (0.0015 mg/ml) (Panel A) and *S. commune* glucuronoyl esterase (0.01 mg/ml) (Panel B). Symbols: ○, signal of $COOCH_3$; ●, signal of methanol (see Fig. 3).

concentration of the low molecular mass substrate was selected to be equimolar to the molar concentration the uronic acid ester in 1% solution of the glucuronoxylan methyl ester. This corresponded to about 4.7 mM (0.2% w/v) solution of 4-nitrophenyl 2-*O*-(methyl 4-*O*-methyl-D-glucopyranosyluronate)-β-D-xylopyranoside. The deesterification of 4-*O*-methyl-D-glucuronosyl residues in both substrates proceeded at a similar rate, pointing to the same or a very similar specific activity of the enzyme for high and low molecular mass substrates (Figs. S4 and S5).

From the NMR experiments with enzymes it became obvious that the changes of signal intensities can be explored for analytical purposes, for a novel glucuronoyl esterase assay. Substrates of glucuronoyl esterases are not commercially available and the preparation of the glucuronoxylan methyl ester is much easier than the synthesis of MeGlcA esters. Fig. 5 shows how the rate of deesterification of the polymeric substrate depends on concentration of *T. reesei* glucuronoyl esterase. The rate is proportional to the amount of the enzyme in the NMR test-tube, although the linearity between the enzyme amount and the rate of methanol release was found lower than with time (see insert in Fig. 5).

4. Discussion

In this work the first evidence is presented that glucuronoyl esterases could attack ester linkages on a polymeric substrate. Methyl ester of glucuronoxylan does not occur in nature, but imitates the natural substrate present in plant cell walls more closely than synthetic substrates used up to now. This result together with earlier reported ability of the enzymes to hydrolyze esters of uronic acids with bulkier alcohols than methanol [1,16,25,26], and their preference for uronic acid esterified with bulkier alcohols [26], suggests that glucuronoyl esterases could attack also ester linkages between MeGlcA carboxyl and more complex alcohols or structural arrangements, including linkages between hemicellulose and lignin.

Unfortunately, esters of glucuronoxylan with larger alcohols to be tested as glucuronoyl esterase substrates have not been isolated from plant cell walls, and esters with alkyl, aryl or alkyl aryl alcohols bulkier than methanol are difficult to be prepared due to dramatic decrease in the polysaccharide solubility. Therefore, esterification of MeGlcA residues with larger alcohols preserving the solubility of the polysaccharide remains a challenge for organic chemists.

The results pointing to a similar rate of deesterification of low and high molecular mass methyl esters of MeGlcA is in consonance with the fact that glucuronoyl esterase active sites are exposed to the protein surface, providing access also to large substrates [20,21,26]. The published 3D structures [20,21] did not suggest any interaction of enzymes with sugar residues of the xylan main chain. In plant cell walls the ester linkages connect two types of large molecules and could actually occur on a phase boundary between highly hydrated hemicellulose and hydrophobic lignin. Indirect evidence supporting the physiological role of the glucuronoyl esterases has been so far the effect of the expression of fungal enzymes on development and structure of plant cell walls of transgenic plants [29,30]. The biotechnological importance of glucuronoyl esterases is stressed by reports that cross-links between lignin and hemicellulose are the main contributors to plant cell wall recalcitrance [34].

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.07.019.

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