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The coordinated action of glucuronoyl esterase and \(\alpha\)-glucuronidase promotes the disassembly of lignin–carbohydrate complexes

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Glucuronoxylans represent a significant fraction of woody biomass, and its decomposition is complicated by the presence of lignin–carbohydrate complexes (LCCs). Herein, LCCs from birchwood were used to investigate the potential coordinated action of a glucuronoyl esterase (\(Tt\)CE15A) and two \(\alpha\)-glucuronidases (\(Sde\)Agu115A and \(Axy\)Agu115A). When supplementing \(\alpha\)-glucuronidase with equimolar quantities of \(Tt\)CE15A, total MeGlc\(_p\)A released after 72 h by \(Sde\)Agu115A and \(Axy\)Agu115A increased from 52% to 67%, and 61% to 95%, respectively. Based on the combined \(Tt\)CE15A and \(Axy\)Agu115A activities, \(\sim\)34% of MeGlc\(_p\)A in the extracted birchwood glucuronoxylan was occupied as LCCs. Notably, insoluble LCC fractions reduced soluble \(\alpha\)-glucuronidase concentrations by up to 70%, whereas reduction in soluble \(Tt\)CE15A was less than 30%, indicating different tendencies to adsorb onto the LCC substrate.

Keywords: 4-O-methyl D-glucuronic acid; carbohydrate-active enzymes; enzyme adsorption; glucuronoxylan; hemicellulases; lignin–carbohydrate complexes

Hemicelluloses typically account for between 25% and 40% of plant cell walls and are characterized by \(\beta\)-(1→4)-linked carbohydrate backbones that can be substituted by additional monosaccharides and noncarbohydrate groups. In deciduous species, hemicelluloses are mainly represented by acetylated glucuronoxylan, which has a backbone comprised of \(\beta\)-(1→4)-linked xylopyranose (Xyl\(_p\)) units, decorated with 4-O-methyl-D-glucuronic acid (MeGlc\(_p\)A) at a reported frequency of 1 MeGlc\(_p\)A for every 8–15 Xyl\(_p\) [1,2], and acetyl units at C2 and/or C3 positions [3]. Coniferous species also contain xylans, in this case arabinoglucuronoxylans, where the \(\beta\)-(1→4)-linked Xyl\(_p\) backbone is decorated with approximately 1 arabinofuranosyl (Ara\(_f\)) and 2 MeGlc\(_p\)A substitutions for every 14 Xyl\(_p\) residues [4], though without acetylation of the backbone [5]. Detailed characterization of glucuronoxylan structures in Arabidopsis thaliana reveals an even patterning of MeGlc\(_p\)A and acetate groups along the backbone, which is thought to facilitate xylan

Abbreviations
GH, glycoside hydrolase; HPAEC, high-performance anion-exchange chromatography; LCCs, lignin–carbohydrate complexes; PAD, pulsed amperometric detection.
association with the hydrophilic surface of cellulose [6–9] and outward orientation of MeGlcPα substitutions [10,11]. Arabinoglucuronoxylans from spruce (Picea abies) have been shown to display a combination of even patterned and clustered MeGlcPα and Araf substitutions along the xylan backbone [12].

The diversity and recalcitrance of xylan structures are increased through the formation of covalent linkages to lignin. In deciduous and coniferous xylan sources, lignin–carbohydrate complexes (LCCs) include ester linkages between MeGlcPα substitutions of xylan and phenylpropane subunits in lignin [13–17]. For example, roughly 30% of the MeGlcPα residues in xylan from beechwood (Fagus crenata) [18,19] have been estimated to participate in ester-linked LCCs.

Given the complexity of xylan structures and their association with multiple plant cell wall components, several enzyme families are required for their full deconstruction [20]. So far, the combined action of xylan-active enzymes has mostly been studied to promote the complete conversion of xylans to fermentable sugars [21–23]. Enzymes predicted to act on LCCs, including α-glucuronidases (EC 3.2.1.131) belonging to glycoside hydrolase (GH) family GH115 (www.cazy.org) [24,25], and glucuronoyl esterases belonging to family CE15 [13,26–28], could facilitate xylan recovery and be used in higher-value applications [24,25,29,30].

Both bacterial and fungal GH115 α-glucuronidases have been characterized, and apart from BrGH115A from Bacteroides thetaiotaomicron, which targets arabinogalactans [31], all characterized members preferentially release MeGlcPα substitutions in xylans ([23–25,31–37]; summarized in Table S1). GH115 enzymes with resolved structures or structure homology models are reported to adopt a four-domain architecture [31,32], except for SdeAgu115A from Saccharophagus degradans and AxyAgu115A from Amphibacillus xylanus, which adopt a five-domain architecture [24,25]. Despite their structural similarity, AxyAgu115A shows significantly higher activity at alkaline pH and comparatively high activity on complex xylans when compared to SdeAgu115A [25].

Most CE15 glucuronoyl esterases, including TtCE15A from Teredinibacter turnerae, have been characterized using model substrates such as α-glucuronic acid benzyl ester, α-glucuronic acid allyl ester, α-glucuronic acid methyl ester, and α-galacturonic acid methyl ester [26,27,38,39]. It has also been confirmed that TtCE15A does not exhibit significant acetyl esterase activity [38]. In a few cases, CE15 glucuronoyl esterases have been tested using LCC preparations. For example, Arnling Bäåth et al. [13] reported a reduction in molecular weight and increase in carboxylic acid content of LCCs isolated from spruce and birch (Betula pendula) following treatment with the glucuronoyl esterase from Acromonium alcalophilum (AcGE1). Similarly, the glucuronoyl esterase from Cerrena unicolor (CuGE) releases uronic acid-containing xylooligosaccharides from extracted birchwood [40,41]. Furthermore, structural characterization of TtCE15A, OtCE15A, and CuGE showed enzyme interactions with lignin and carbohydrate components of hardwood xylan [38,42–43]. Whereas glucuronoyl esterases were already shown to increase the hydrolytic activity of a commercial enzyme cocktail on milled corn cob [39] and endo-xylanase activity on LCCs from birchwood [40], the impact of glucuronoyl esterases on other accessory enzymes targeting xylan substitutions has not been reported.

Herein, the deconstruction of LCCs from birchwood was investigated using the family GH115 α-glucuronidas SdeAgu115A and AxyAgu115A, together with the CE15 glucuronoyl esterase TtCE15A. In addition to investigating the cooperative action of these enzymes, the combined α-glucuronidase and glucuronoyl esterase treatment could be used to quantify the fraction of MeGlcPα in birchwood xylan extracts that participate in ester linkages to lignin.

**Materials and methods**

**Substrates**

Beechwood xylan and the K-URONIC Acid Kit were purchased from Megazyme (Bray, Ireland), α-glucuronic acid methyl ester was purchased from Carbosynth (Berkshire, UK), and 4-O-methyl-glucuronic acid (MeGlcPα) was purchased from Synthose (Concord, Canada). Organosolv hardwood lignin was provided as a gift from M. Nejad (MSU, USA). Hydrothermally extracted LCCs, fractionated into F1 and F2 fractions, were isolated from birchwood chips (B. pendula) as described previously [16], and received as a gift from M. Lawoko (KTH, Sweden). Briefly, ball-milled acetone-extracted birchwood was subjected to hydrothermal treatment using deionized water at 80 °C for 4 h; the supernatant component was then fractionated using a polyaromatic resin (Amberlite XAD4) into F1 and F2 fractions and lyophilized [44]. Both F1 and F2 fractions contain acetylated xylan, with Xylp representing > 75% of total sugar in the fraction [16]. In the F1 fraction, 14% of Xylp are acetylated at the O-2 position and 22% of Xylp are acetylated at the O-3 position; in the F2 fraction, 5% of Xylp are acetylated at the O-2 position and 7% of Xylp are acetylated at the O-3 position [16]. Arabinose, galactose, glucose, and mannose represent less than 20% of the total sugar in both LCC fractions, and lignin comprises 4% and 6% of F1 and F2 fractions, respectively [16].
 Whereas the F1 fraction was fully soluble in water, the F2 fraction was used as a stable suspension. Given the complete water solubility of the F1 fraction, it was used for both enzyme activity assays and enzyme adsorption assays, whereas the F2 fraction was used only for enzyme adsorption assays.

Quantification of MeGlcPA in lignin–carbohydrate complexes recovered from birchwood

A previously detailed methanolysis protocol was followed to quantify the MeGlcPA content in the F1 LCC fraction [45]. Briefly, 15 mg of LCCs was dried at 80 °C and then treated with 1 mL of 2 M hydrochloric acid (in anhydrous methanol) for 4 h at 100 °C. Samples were subsequently neutralized using 1 mL of 13.5 M pyridine and dried under airflow at room temperature. Dried samples were treated with 1 mL of 2 M hydrochloric acid (in anhydrous methanol) at 121 °C for 1 h, after which the sample was dried under airflow at room temperature and suspended in 1 mL of de-ionized water. The amount of MeGlcPA released was quantified by high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) equipped with a CarboPac PA1 (2 × 250 mm) analytical column and corresponding guard column (2 × 50 mm) (Dionex, Sunnyvale, CA, USA). Briefly, 12.5 µL of sample was injected onto the column and eluted at 0.25 mL min⁻¹ using a gradient elution of sodium acetate, specifically 0–0.1 M sodium acetate over 35 min, followed by 0.1–0.2 M sodium acetate over 10 min, then 0.2–0.5 M sodium acetate over 5 min, and finally 0.5–0 M sodium acetate over 10 min to recondition the column. MeGlcPA eluted at an approximate retention time of 25 min. Data were analyzed using CHROMELEON software (version 7.2; Dionex).

Protein production

Glucuronyl esterase from T. turnerae (TtCE15A; PDB: 6HSW; 49 kDa) was produced as described by Arnling Báath et al. [38]. Briefly, TtCE15A, comprising an N-terminal His₆ tag, was expressed in Escherichia coli BL21 (λDE3) and purified using a 5-mL HisTrap™ Excel column. Xylan-active α-glucuronidases from S. degradans (SdeAgu115A; PDB: 4ZMH; 110 kDa) and A. xylanus (AxyAgu115A; PDB: 6PNS; GenBank: BAM48432.1, 110 kDa) were produced as described previously [24,25]. Similar to TtCE15A, both SdeAgu115A and AxyAgu115A comprised His₆ tags, were expressed in E. coli BL21 (λDE3), and purified using Ni-NTA resin. Cells were sonicated in 50 mM HEPES pH 7.0 binding buffer containing 5% glycerol, 5 mM imidazole, and 300 mM NaCl. The supernatant was then incubated with Ni-NTA resin for 6 h at 4 °C on a vertical tube rotator at 8 r.p.m. After applying the protein sample to a column, the protein was washed with 50 mM HEPES (pH 7.0) containing 5% glycerol, 5 mM imidazole, and 300 mM NaCl. The protein was eluted using 50 mM HEPES (pH 7.0) containing 5% glycerol, 250 mM imidazole, and 300 mM NaCl. A Bio-Gel P10 column was used to exchange the protein into 50 mM HEPES buffer (pH 7.0).

Enzymatic activity toward LCCs

TtCE15A, AxyAgu115A, and SdeAgu115A were tested alone and as combinations of both enzyme classes. Single enzyme reactions comprised 0.5% (w/v) LCCs in 25 mM Britton–Robinson buffer (pH 7.0) with 1 µM of each enzyme (i.e., 7.35 µg of TtCE15A, 16.5 µg of AxyAgu115A, and 16.5 µg of SdeAgu115A). Reactions containing both enzyme types were set up in the same way, except they contained 1 µM TtCE15A plus 1 µM α-glucuronidase, or 10 µM TtCE15A plus 1 µM α-glucuronidase. The latter condition was included to investigate whether rapid separation of carbohydrates and lignin in LCCs is detrimental to the activity of α-glucuronidases due to the exposed lignin. In all cases, the final reaction volume was 150 µL. Reactions proceeded for 72 h at 25 °C in an orbital shaker at 700 r.p.m. with reaction aliquots taken at 6 and 24 h. The samples were then boiled for 6 min to stop further reactions. The earliest time point (6 h) was chosen based on preliminary assays using AxyAgu115A and SdeAgu115A to treat LCCs, where release of MeGlcPA was not observed by HPAEC until 4 h had elapsed [46]. Reaction supernatants were separated by centrifugation at 20 000 g for 5 min and filtered using 0.22-µm filter. The released MeGlcPA was then quantified using HPAEC-PAD as described above. The released lignin was followed by HPLC-UV at 210 nm (Thermo Scientific, Waltham, MA, USA, ICS 5000) equipped with an Aminex HPX87H Column (Bio-Rad, Hercules, CA, USA). For HPLC-UV, the Aminex column was equilibrated in 5 mM H₂SO₄ and conditioned to 50 °C before 12.5 µL of sample was injected onto the column and eluted at a 0.40 mL min⁻¹ using an isocratic elution of 5 mM H₂SO₄. Chromeleon software was used to analyze the obtained chromatograms.

Protein adsorption to LCCs and residual activity

To investigate adsorption of tested enzymes to F1 and F2 LCC fractions, each LCC fraction was suspended in 25 mM Britton–Robinson buffer (pH 7.0) to 1% (w/v) and then incubated with 2 µM TtCE15A, AxyAgu115A, or SdeAgu115A. Enzyme binding to organosolv hardwood lignin and beechwood xylan (2% w/v, pH 7.0) was also evaluated for comparison. After incubation for 24 h at 25 °C with shaking at 700 r.p.m., samples were centrifuged (20 000 g) for 5 min, and the supernatant was filtered through a 0.22-µm filter. Protein content in filtered supernatants was
Results and Discussion

Influence of \textit{Tt}CE15A on \(\alpha\)-glucuronidase activity toward isolated LCCs

The xylan-rich (F1) LCC fraction used in this study was previously extracted from birchwood and characterized in detail [16]. Similar to the previously reported 7.2 \(\mu\)g MeGlc\(p\) A per mg of the F1 LCC fraction [16], acid methanolation of the F1 fraction performed herein confirmed 9.3 \(\mu\)g MeGlc\(p\) A per mg of sample and a MeGlc\(p\) A to Xyl\(p\) mole ratio of approximately 1 : 8.

Treatment of the F1 LCC fraction with either \textit{Axy}Agu115A or \textit{Sde}Agu115A released 20 \(\pm\) 2\% and 22.5 \(\pm\) 0.8\%, respectively, of total MeGlc\(p\) A after 6 h, and 29 \(\pm\) 2\% and 26.9 \(\pm\) 0.1\%, respectively, of total MeGlc\(p\) A after 24 h (Fig. 1). After 72 h, the extent of MeGlc\(p\) A release by \textit{Axy}Agu115A and \textit{Sde}Agu115A had increased to 60 \(\pm\) 1\% and 51.5 \(\pm\) 0.8\%, respectively (Fig. 1). Treatment of the F1 LCC fraction with \textit{Tt}CE15A did not lead to release MeGlc\(p\) A from the substrate, consistent with all MeGlc\(p\) A units in the sample being bound to xylan. Instead, adding equimolar \textit{Tt}CE15A to reactions containing \textit{Axy}Agu115A led to release of 35 \(\pm\) 1\%, 68 \(\pm\) 3\%, and 94 \(\pm\) 4\% of total MeGlc\(p\) A after 6, 24, and 72 h, respectively. This corresponds to an increase in MeGlc\(p\) A release of approximately 34\% after 72 h, compared to treatments with \textit{Axy}Agu115A alone. Comparing the reactions containing \textit{Axy}Agu115A with and without \textit{Tt}CE15A supports the conclusion that 34-40\% of MeGlc\(p\) A present in the LCC substrate is linked to lignin, which is in agreement with the reported amount of xylan that participates in ester linkages in beechwood LCCs [18,19]. Similarly, the addition of \textit{Tt}CE15A increased MeGlc\(p\) A release by \textit{Sde}Agu115A after 6 and 24 h to 31.6 \(\pm\) 0.2\% and 64.0 \(\pm\) 0.8\%, respectively; however, in this case, incubation up to 72 h did not substantially increase these values (66.5 \(\pm\) 13\%) (Fig. 1). \textit{Tt}CE15A was thus able to boost the action of both \(\alpha\)-glucuronidases, though the effect was less pronounced for \textit{Sde}Agu115A than for \textit{Axy}Agu115A. The comparatively low impact of \textit{Tt}CE15A on \textit{Sde}Agu115A performance is consistent with the generally poorer performance of \textit{Sde}Agu115A compared with \textit{Axy}Agu115A [25]. For instance, the different impacts of \textit{Tt}CE15A on \textit{Axy}Agu115A and \textit{Sde}Agu115A performance might be attributed to remaining substitutions on the xylan backbone (e.g., acetyl groups), or relative positioning of MeGlc\(p\) A substituents that could influence \textit{Sde}Agu115A activity.

In addition to underscoring differences in \textit{Axy}Agu115A and \textit{Sde}Agu115A performance, the current analyses shed light on the composition of the LCC substrate. In an earlier study, we showed the beneficial impact of the acetyl xylan esterase from Flavobacterium johnsoniae, \textit{Fj}AcXE, on MeGlc\(p\) A release by \textit{Axy}Agu115A from (2-O-MeGlc\(p\) A)-3-O-acetyl-Xyl\(p\)
positions in acetylated 4-O-(methyl)glucuronoxylans [29]. More recently, we confirmed that AxyAgu115A was able to release >98% of MeGlcA present in extensively deacetylated xylan from hardwood [46]. The LCCs used in the present study have previously been analyzed by 2D HSQC NMR, which confirmed partial acetylation at O-2 and O-3 positions of Xylp residues [16]. Accordingly, when considering these earlier findings together with the observation herein that over 94% of available MeGlcA in the F1 LCC fraction is released by the combined action of TtCE15A on AxyAgu115A, we can conclude that most acetylated Xylp residues in the LCC fraction do not also carry esterified MeGlcA.

**Influence of AxyAgu115A on TtCE15A activity toward isolated LCCs**

Enzymatic release of carbohydrates from LCCs is expected to decrease lignin solubility due to increased hydrophobicity; accordingly, HPLC-UV can be used to follow changes in the soluble lignin content of the F1 LCC after treatment with TtCE15A, AxyAgu115A, and the combined enzyme reactions (Fig. 2). Notably, since SdeAgu115A was less active than AxyAgu115A, SdeAgu115A was not included in these experiments.

The UV absorbance of reaction supernatants containing the F1 LCC decreased by 60% after treatment for 6 h with TtCE15A. This value was not significantly impacted in reactions additionally containing AxyAgu115A. These results support the prediction that xylans do not hinder glucuronoyl esterase access to target linkages [42,43], and indicate that glucuronoyl esterases likely act before α-glucuronidases and do not merely release single MeGlcA residues linked to lignin that remain after α-glucuronidase action. This observation is also supported by crystal structures of GEs with bound MeGlcA-appended xylo-oligosaccharide ligands [42,43].

**Selective adsorption of α-glucuronidases to LCC-derived precipitates**

Increasing the concentration of TtCE15A tenfold decreased the extent of MeGlcA released from F1 LCC in reactions containing AxyAgu115A (Fig. 1) and led to the formation of an observable precipitate. FTIR analysis of the precipitate revealed signature amide I (1650 cm\(^{-1}\)) and amide II (1550 cm\(^{-1}\)) vibrations [48] (Fig. 3), consistent with the presence of protein. Notably, characteristic vibrations for xylan and lignin were not detected in these samples. Given that the protein precipitate only formed in the presence of the LCC substrate, it is conceivable that the amended enzymes adsorbed to the surface of the LCC components, thereby masking corresponding xylan and lignin signals.

To investigate possible preferential binding of the tested enzymes to the LCCs, TtCE15A, AxyAgu115A, and
and SdeAgu115A were each tested for adsorption to F1 and F2 LCC fractions, where the F2 fraction is distinguished by lower water solubility and higher lignin content (i.e., 4% lignin in F1 and 6% lignin in F2) [15]. While the enzymes adsorbed to the F1 LCC fraction to similar extents (23–30% of total protein; Fig. 4), both α-glucuronidases displayed two times higher adsorption to the F2 LCC fraction (57–70% of total protein; Fig. 4). Notably, all tested enzymes bound organosolv lignin to similar extents (<15%); by contrast, the amount of AxyAgu115A and SdeAgu115A bound to glucuronoxylan was approximately three times higher than that measured for TtCE15A (Fig. 4). The comparatively low adsorption of TtCE15A to the F2 LCC fraction, as well as organosolv lignin and glucuronoxylan, might be attributed to the necessary functional association of glucuronoyl esterases with diverse lignin–carbohydrate structures.

**Conclusions**

This study confirmed the activities of the glucuronoyl esterase TtCE15A and two α-glucuronidases in disassembly of LCCs isolated from birchwood. In particular, the release of MeGlcPα by AxyAgu115A improved significantly in combination with TtCE15A, and monitoring the reaction after indicated that 34% of MeGlcPα in the birchwood LCC sample was ester-linked to lignin. It is conceivable that the release of xylan from LCCs is responsible for the reduction in soluble enzyme concentrations in our reactions. Supplementing reactions with known additives that reduce nonproductive associations with lignin (e.g., addition of surfactant or BSA) could possibly curtail this effect, while the addition of xylan-degrading enzymes may reduce the adsorption of α-glucuronidases to precipitated xylan. The combination of α-glucuronidase and glucuronoyl esterase activities on LCCs confirmed through this study sets a precedence of using such enzyme systems for xylan recovery and substrate characterization, and thus motivates the search for new α-glucuronidases and glucuronoyl esterases with faster release of MeGlcPα.

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**Author contributions**

ERM, JL, and LO conceived the study; ERM and LO supervised the study; OR, JAB, and TVV designed experiments; OR and JAB performed experiments; OR and TVV analyzed data; OR and ERM wrote the manuscript; and OR, JAB, TVV, JL, ERM, and LO made manuscript revisions.

**Data accessibility**

Research data pertaining to this article are located at figshare: https://doi.org/10.6084/m9.figshare.13547270.v1.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Substrate preference of GH115 α-glucuronidases based on relative release of MeGlcpA.

**Fig S1.** Gel densitometry of reaction supernatant for evaluating adsorption of enzymes to birchwood LCC F1 [16].

**Fig S2.** Gel densitometry of reaction supernatant for evaluating adsorption of enzymes to beechwood glucuronoxylan.