The two *Rasamsonia emersonii* α-glucuronidases, *Re*GH67 and *Re*GH115, show a different mode-of-action towards glucuronoxylan and glucuronoxyloligosaccharides

Patricia Murciano Martínez1, Maaike M. Appeldoorn2, Harry Gruppen1 and Mirjam A. Kabel1*

**Abstract**

**Background:** The production of biofuels and biochemicals from grass-type plant biomass requires a complete utilisation of the plant cellulose and hemicellulosic xylan via enzymatic degradation to their constituent monosaccharides. Generally, physical and/or thermochemical pretreatments are performed to enable access for the subsequent added carbohydrate-degrading enzymes. Nevertheless, partly substituted xylan structures remain after pretreatment, in particular the ones substituted with (4-O-methyl-)glucuronic acids (UAme). Hence, α-glucuronidases play an important role in the degradation of UAme-xylan structures facilitating the complete utilisation of plant biomass. The characterisation of α-glucuronidases is a necessity to find the right enzymes to improve degradation of recalcitrant UAme-xylan structures.

**Results:** The mode-of-action of two α-glucuronidases was demonstrated, both obtained from the fungus *Rasamsonia emersonii*; one belonging to the glycoside hydrolase (GH) family 67 (*Re*GH67) and the other to GH115 (*Re*GH115). Both enzymes functioned optimal at around pH 4 and 70 °C. *Re*GH67 was able to release UAme from UAme-substituted xylo-oligosaccharides (UAmeXOS), but only the UAme linked to the non-reducing end xylosyl residue was cleaved. In particular, in a mixture of oligosaccharides, UAmeXOS having a degree of polymerisation (DP) of two were hydrolysed to a further extent than longer UAmeXOS (DP 3–4). On the contrary, *Re*GH115 was able to release UAme from both polymeric UAme-xylan and UAmeXOS. *Re*GH115 cleaved UAme from both internal and non-reducing end xylosyl residues, with the exception of UAme attached to the non-reducing end of a xylotriose oligosaccharide.

**Conclusion:** In this research, and for the first time, we define the mode-of-action of two α-glucuronidases from two different GH families both from the ascomycete *R. emersonii*. To date, only four α-glucuronidases classified in GH115 are characterised. *Re*GH67 showed limited substrate specificity towards only UAmeXOS, cleaving UAme only when attached to the non-reducing end xylosyl residue. *Re*GH115 was much less substrate specific compared to *Re*GH67, because UAme was released from both polymeric UAme-xylan and UAmeXOS, from both internal and non-reducing end xylosyl residues. The characterisation of the mode-of-action of these two α-glucuronidases helps understand how *R. emersonii* attacks UAme-xylan in plant biomass and the knowledge presented is valuable to improve enzyme cocktails for biorefinery applications.

**Keywords:** Biorefinery, α-Glucuronidase, GH67, GH115, *Rasamsonia emersonii*, Xylo-oligosaccharides

*Correspondence: mirjam.kabel@wur.nl
1 Laboratory of Food Chemistry, Wageningen University, Bornse Weilanden 9, 6708 WG Wageningen, The Netherlands Full list of author information is available at the end of the article

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Background
For the production of biofuels and chemicals from plant biomass, a complete utilisation of the cellulose and hemicellulose present is desired. The degradation of these polymers is commonly approached via a physical and/or thermo-assisted chemical pretreatment, followed by enzymatic hydrolysis. In grass-type feedstocks, glucuronarabinoxylan (GAX) is the major hemicellulose. It is constituted of a β-(1 → 4) linked xylopyranosyl backbone, substituted by side groups, such as O-acetyl groups, arabinofuranosyl residues and 4-O-methyl-α-D-glucopyranosyl uronic acids. The occurrence of substituents in the xylan backbone is highly dependent on the feedstock used. In addition, the abundance and distribution of substituents can be affected by the type and severity of the pretreatment performed [1]. For example, O-acetyl groups and arabinosyl residues are released during hydrothermal pretreatments catalysed by alkali or acids [2–4]. However, glucuronic acid (UA) and its 4-O-methyl etherified derivative (UAme) are hardly removed from the xylan backbone during such treatments [1, 5]. Therefore, in commercial enzyme cocktails α-glucuronidases are crucial in addition to endo-xylanases and β-xylanosidases, to achieve a complete hydrolysis of monosaccharides.

Such commercial enzyme cocktails mostly contain enzymes produced by ascomycetes, like Aspergillus species and Trichoderma species. In addition, the ascomycete Rasamsonia emersonii is a candidate for the production of (hemi-)celluloletic enzymes. In this research, α-glucuronidases from R. emersonii were studied.

In fungi, α-glucuronidases are classified based on the Carbohydrate-Active enZymes database [6, 7] in two glycoside hydrolase (GH) families, which are GH67 and GH115. In the genome of 38 different basidiomycetes, only genes encoding GH115 are described and zero encoding GH67, which indicates that GH115 is preferred over to GH67 in basidiomycetes [8]. For ascomycetes, like for various Aspergillus strains, such a distinct choice between GH115 and GH67 is not present. Depending on the strain, genes are present encoding only GH67 or both GH67 and GH115 [8]. In addition to such genome annotations, mainly based on putative functions, it is even more valuable to characterise the mode-of-action of the enzyme proteins corresponding to the annotated genes. The mode-of-action of GH67 α-glucuronidases is well known, because many GH67 α-glucuronidases have been characterised and all are able to release UAme linked to the non-reducing xylosyl end in xylo-oligosaccharides (UAmeXOS) [9–11]. GH67 α-glucuronidases are not able to release UAme from polymeric glucuronoxylan (UAmeXylan) [10, 11]. In contrast with the GH67 α-glucuronidases, the mode-of-action of only a limited number of GH115 α-glucuronidases has been described. Only four α-glucuronidases have been biochemically characterised so far, one isolated from the basidiomycete Schizophyllum commune, one from the ascomycete Pichia stipitis and two from the bacteria Streptomyces pristinaespiralis and Bacteroides ovatus. All four are able to release UAme from UAmeXylan [12–15]. In addition, all four GH115 α-glucuronidases are able to release UAme from UAmeXOS, linked to either internal or to the non-reducing end xylosyl residues [14, 16].

In this research, for the first time, two purified α-glucuronidases, both from the ascomycete R. emersonii, belonging to either GH67 or GH115, are extensively characterised for their mode-of-action towards UAmeXylan and UAmeXOS. Rasamsonia emersonii grows well at temperatures around 45–50 °C and not able to grow at 25 °C. Rasamsonia emersonii is, therefore, catalogued as thermophilic [17]. It is hypothesised, based on the above-described literature findings on the mode-of-action of α-glucuronidases, that the two enzymes studied show a different mode-of-action towards the substrates studied at 65 °C and pH 4.5.

Results and discussion
Purification of the α-glucuronidases ReGH67 and ReGH115. The crude enzyme extracts obtained from Aspergillus niger, having overexpressed ReGH67 or ReGH115, were purified by a 2-step size exclusion chromatography; a subsequent cation exchange step was applied to the SEC 2-fraction of ReGH115 (data not shown). SDS-PAGE of the purified ReGH67 and ReGH115 showed bands of a molecular mass of 100 and 150 kDa, for ReGH67 and ReGH115, respectively, compared to the marker (data not further shown). These masses differed with the predicted masses of the enzymes based on their amino acid sequences, which were 91 and 111 kDa for ReGH67 and ReGH115, respectively. Possibly, ReGH67 and ReGH115 were glycosylated, which could have resulted in the higher molecular mass analysis. In addition, it was shown that no xylanase activity was present in the purified ReGH67 and ReGH115; neither towards 4-O-methylglucuronoxylan (Fig. 1), nor towards linear xylo-oligosaccharides (XOS) (Additional file 1: Figure S1).

Activity of ReGH115 and ReGH67 towards 4-O-methylglucuronoxylan
To date, only four α-glucuronidases belonging to GH115 have been characterised, of which only one originates from an ascomycete, while many GH67 α-glucuronidases have been studied. Therefore, we are in particular interested in the mode-of-action of ReGH115, although
ReGH67 is also of interest as this enzyme originates from the same ascomycete *R. emersonii* as the studied ReGH115.

The activity of the two purified α-glucuronidases was first tested towards BeWX and birchwood xylan (BiWX), which are polymeric xylans constituted of β-(1 → 4) linked xylosyl residues substituted with α-(1 → 2) linked 4-O-methylglucuronic acids (UAme). The UAme substitution pattern of BiWX has been reported to be more blockwise compared to BeWX, which is more random [18]. BiWX and BeWX were incubated with both enzymes for 24 h. ReGH67 was not able to remove UAme from the two UAme xylans (Fig. 1). In contrast, ReGH115 was able to release UAme from both BiWX and BeWX. The release of UAme from the xylan backbone led to aggregation. The latter was seen from the increase in higher molecular mass material (around 112.8 kDa) and from the decrease of lower molecular weight material (around 5.5 kDa) seen by HPSEC analysis of BiWX (Fig. 2). Such aggregation was expected, because the removal of UAme led to larger blocks of unsubstituted xylosyl residues, which is known to allow self-association of these linear xylan blocks [19, 20].

As mentioned earlier in the text, in literature only four GH115 α-glucuronidases are described for their mode-of-action. All four were able to release UAme from UAme xylan, as was observed in our study for ReGH115.

**Optimum temperature and pH**

Activity of ReGH67 and ReGH115 towards 4-O-methylglucuronoxylan. ReGH67 showed a maximum release of UAme from aldouronic acids (AAC) at a pH range from 4 to 6 and at a temperature range from 50 to 70 °C (Additional file 1: Figure S1). In case of ReGH115, the optimum release of UAme from beechwood xylan (BeWX) was observed at pH 4 and at 65–70 °C. Considering these optima, for further hydrolysis experiments a pH of 4.0 at 70 °C was taken for both enzymes to allow best comparison.

**Mode-of-action of ReGH115 and ReGH67 towards AAC**

AAC is a commercially available mixture of various XOS with one UAme linked per xylo-oligomer. The position of the UAme in each oligomer is, however, not specified by the supplier. To enable the analysis of the mode-of-action of ReGH67 and ReGH115 towards characterised UAme XOS, first the exact structures of the UAme XOS present in AAC were determined. Hereto, AAC was labelled under reducing conditions with 2-AA and submitted to RP-UHPLC-MS analysis. The RP-UHPLC-UV absorbance at 254 and 340 nm showed the presence of six peaks (Fig. 3), while the number of peaks detected by total ion current (TIC) was eight. Seven out of the eight peaks detected by TIC were identified based on the recorded MS² spectra, and the exact position of the UAme for each of the XOS present in AAC was determined. The latter determination was performed by a separate analysis, in which the elution of the TIC analysed masses in 2-AA-labelled AAC was detected as single reaction monitoring (SRM). SRM was needed to overcome the problem of co-elution of certain structures, as shown in Fig. 3. Assisted by the separation, the detected parent mass, and the mass fragmentation pattern, seven structures were confirmed in AAC. These seven structures are depicted in...
Fig. 3, including their fragmentation patterns and masses. From Fig. 3, it is clear that UAmeXOS are present having the UAme linked to either the non-reducing, the internal, or the reducing end xylosyl residues. According to the nomenclature for UAmeXOS structures [21], which uses ‘X’ for xylosyl residues and ‘U4m2’ for xylosyl residues attached via their O2 to a 4-O-methylglucuronic acid, the structures were named as 1 = XXU4m2X, 2 = XXU4m2, 3 = U4m2XX, 4 = U4m2, 5 = XU4m2, 6 = XU4m2X and 7 = U4m2X. The numbers correspond to the numbers shown next to the structures in Fig. 3.

Quantification of 2-AA-labelled UAmeXOS in the ReGH67 and ReGH115 digests was achieved using single reaction monitoring (SRM; Fig. 4). It allowed the quantification of selected fragments as described in the “Methods” section. Figure 4 shows that ReGH67 only cleaved UAme from the structures 3 (U4m2XX), 4 (U4m2) and 7 (U4m2X), which are substituted at the non-reducing end xylosyl residue. Hence, the mode-of-action of ReGH67 was comparable to those of many GH67 α-glucuronidases described previously [9–11, 22].

The activity of ReGH115 towards AAc is of particular interest, because its mode-of-action is expected to be a valuable contribution to the so far poorly described α-glucuronidases from the GH115 family. ReGH115 was able to release UAme from all AAc structures, although U4m2XX mostly remained (Fig. 4). Therefore, it was concluded that ReGH115 did not show a distinct preference for a cleavage site in UAmeXOS. UAme is removed from xylose moieties at the reducing, internal, and non-reducing end side positions. So, under the conditions applied, clearly, ReGH115 degraded AAc to a further extent than ReGH67.

In terms of oligosaccharides, a summary of the mode-of-action of the four α-glucuronidases from family GH115 described in literature and of our ReGH115 is given in Table 1. In contrast to the absence of preference of ReGH115, the S. commute GH115 α-glucuronidase prefers to cleave UAme from internal xylosyl residues of UAmeXOS with a DP of 4–6 [16]. The B. ovatus GH115 α-glucuronidase prefers to hydrolyse UAme from either internal or non-reducing end xylosyl residues in UAmeXOS (DP 2–4) [13]. The P. stipitis GH115 α-glucuronidase cleaved the UAme from UAmeXOS in a DP range from 3 to 6 [23]. However, the preference for internal or outer units remains undefined in that research. The S. pristinaespiralis α-glucuronidase was only tested towards one UAmeXOS having a DP of 5. To summarise, in comparison with the published activities of GH115 α-glucuronidases to date (Table 1), our ReGH115 showed activity towards UAmeXylan and UAmeXOS and showed no preference for the position of the UAme in the oligosaccharides tested.

**Conclusion**

Both ReGH67 and ReGH115 showed highest activity around pH 4 and at a temperature between 65 and 70 °C. ReGH67 released only UAme attached to the xylosyl residue located at the non-reducing end of UAmeXOS. ReGH115 was able to release UAme from both UAmexy and UAmeXOS. It showed no preference for the position of the UAme in the UAmeXOS. This analysed mode-of-action is a valuable contribution to the so far poorly described α-glucuronidases from the GH115 family. Also, the knowledge presented is helpful to improve current enzyme cocktails for biorefinery applications.
Fig. 3 RP-UHPLC-UV-MS profiles [UV (340 nm) and total ion current (TIC)] of 2-AA-labelled AAc (a) and MS² fragmentation spectra (b) of the in (a) annotated peaks: 1 XXU₄m₂ (856 m/z), 2,3,6 XXU₄m₂, U₄m₂XX, XU₄m₂X (724 m/z), 4 U₄m₂ (460 m/z), 5,7 XU₄m₂, U₄m₂X (592 m/z). Filled triangle 4-O-methylglucuronic acid.
Methods

Materials used

The aldouronic acid mixture (AAc) containing XOS of a DP of 2–5, having one UA<sub>me</sub> substituent, was supplied by Megazyme (Wicklow, Ireland). Beechwood xylan (BeWX), BiWX and all chemicals used were purchased from Sigma-Aldrich (St Louis, MO, USA), unless otherwise specified. The carbohydrate composition of BeWX

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**Fig. 4** SRM relative abundance of AAc characterised structures during incubation with ReGH67 (filled square and filled diamond) or ReGH115 (x and filled triangle): 1 XXU<sub>m</sub><sup>n</sup>m<sub>2</sub>X (a), 2 XXU<sub>m</sub><sup>n</sup>m<sub>2</sub>XX (b), 3 U<sub>m</sub><sup>n</sup>m<sub>2</sub>XX (c), 4 U<sub>m</sub><sup>n</sup>m<sub>2</sub> (d), 5 XU<sub>m</sub><sup>n</sup>m<sub>2</sub> (e), 6 XU<sub>m</sub><sup>n</sup>m<sub>2</sub>X (f) and 7 U<sub>m</sub><sup>n</sup>m<sub>2</sub>X (g). Experimental duplicates are displayed individually.
Table 1 Performance of GH115 α-glucuronidases described in literature towards polymeric and oligomeric xylan substrates

| α-glucuronidases | ScAgu115 | PsAgu115 | SpAgu115 | BoAgu115 | ReGH115 |
|------------------|----------|----------|----------|----------|---------|
| Organism         | Schizophyllum commune | Pichia stipitis | Streptomyces pristinae spiralis | Bacteroides ovatus | Rasamsonia emersonii |
| Division         | Basidiomycete | Ascomycete | Actinobacterium | Bacteroidete | Ascomycete |
| Mw (Kda)         | 125      | 120      | nt       | 199      | 150     |
| pH optimum       | nt       | 4.4      | nt       | nt       | 4.0     |
| T optimum (°C)   | nt       | 60       | nt       | nt       | 65-70   |
| 3D-structure     | No       | No       | nt       | Yes      | No      |
| Activity on xylan| BeWX     | nt       | nt       | nt       | +       |
|                  | BiWX     | nt       | nt       | nt       | +       |
|                  | SpW      | nt       | nt       | nt       | +       |
|                  | WWX      | nt       | nt       | nt       | nt      |
| Activity on U₄m²Xₙ | nt       | nt       | nt       | +        | +       |
|                  | U₄m²     | nt       | nt       | nt       | +       |
|                  | XU₄m²    | nt       | nt       | nt       | +       |
|                  | U₄m²X    | nt       | nt       | nt       | +       |
|                  | XU₄m²X   | ++       | nt       | ++       | +       |
|                  | XXU₄m²   | nt       | nt       | nt       | +       |
|                  | U₄m²XX   | nt       | nt       | ++       | nt      |
|                  | XXU₄m²X  | nt       | nt       | ++       | nt      |
|                  | XXU₄m²XX | ++       | nt       | ++       | nt      |
|                  | XXU₄m²XX | +        | nt       | ++       | nt      |
|                  | XXU₄m²XX | +        | nt       | ++       | nt      |

Active towards substrate (+), preferential degradation (+++)

nt Not tested, BeWX Beechwood xylan, BiWX Birchwood xylan, SpW Spruce wood, WWX Willow wood xylan
ScAgu115 Chong et al. [16]; Tenkanen and Siika-aho [15]; PsAgu115 Ryabova et al. [14]; Kolenová et al. [23]; SpAgu115 Fujimoto et al. [12]; BoAgu115 Rogowski et al. [13]

* Nomenclature used is according to Faure et al. [21]

and BiWX was 68 and 69 % (w/w) xylan, respectively, and 9 % (w/w) UA_me for both substrates as determined elsewhere [18].

Enzymes’ expression, production and purification

The two α-glucuronidases from R. emersonii (CBS 393.64), ReGH67 (LT555569) and ReGH115 (LT555570) [24], were expressed and produced in A. niger as described previously [25]. Purification of both ReGH67 and ReGH115 was carried out with a multiple-step chromatographic separation approach, as described in detail below, using an AKTA-explorer preparative chromatography system (GE Healthcare, Uppsala, Sweden). As a first step, 2 mL of the crude enzyme mixture (around 2 mg mL⁻¹ protein) was subjected to a self-packed Superdex 200 26/60 column (GE Healthcare), pre-equilibrated in 20 mM Tris–HCl buffer (pH 7.0). After protein application, the column was equilibrated with 20 column volumes of buffer. Elution at 6 mL min⁻¹ was performed with a linear gradient of 0–1 M NaCl in 20 mM sodium acetate buffer (pH 4.0) over 20 column volumes. Elution was monitored at 214 and 280 nm. Fractions (4 mL) were immediately collected and concentrated by ultrafiltration (Amicon Ultra, 10 kDa, Merck Millipore, Cork, Ireland) at 4 °C. The concentrated pools were subjected to SDS-PAGE and analysis of xylanase activity. Fractions close to the expected molecular mass (ReGH67 = 92 kDa; ReGH115 = 111 kDa) were re-submitted to Superdex 200 26/60 fractionation (2nd step). Now, the ReGH67-containing pool was devoid of xylanase activity. The ReGH115-containing pool was subjected to further purification (3rd step), and loaded onto a resource S column (30 × 16 mm i.d., GE Healthcare), pre-equilibrated with 20 mM sodium acetate buffer (pH 4.0). After protein application, the column was washed with 20 column volumes of starting buffer. Elution at 6 mL min⁻¹ was performed with a linear gradient of 0–1 M NaCl in 20 mM sodium acetate buffer (pH 4) over 20 column volumes. Elution was monitored at 214 and 280 nm. Fractions (4 mL) were immediately concentrated by ultrafiltration and subjected to SDS-PAGE. Fractions close to the expected molecular weight (111 kDa) were pooled to obtain ReGH115 and analysed for their protein content.
Enzymatic hydrolysis

BeWX and BiWX were incubated with the purified α-glucuronidases (ReGH67 and ReGH115) in 10 mM NaOAc buffer, pH 4.0 (1 mL, 10 mg substrate dry matter) at 70 °C for 24 h. The enzymes were dosed at 0.05 % (w/w) protein per substrate added. To stop the enzyme incubations, 2 μL of 4 M HCl was added and the sample was centrifuged (10,000 × g, 10 min, 10 °C) prior to analysis. The pH and temperature optima were tested by incubating ReGH67 and ReGH115 with AAc and BeWX in a range of pH 2–7 using 200 mM NaOAc, and the temperatures ranging from 40 to 90 °C (Additional file 1: Figure S1). Incubation time, enzyme dose and stopping of the reaction was performed as described above.

The AAc was used as a substrate to determine the preferential substrate cleavage site of the enzymes over time. Hereto, 200 μL of sample was collected at various hydrolysis times: 0, 5, 10, 15, 20 and 60 min, 2, 8 and 24 h. All samples collected were submitted to HPAEC and, after 2-AA labelling, to RP-UHPLC-MS. The incubation was performed under the same conditions and protein and substrate concentrations as described above for the incubation with BeWX.

Protein analysis

SDS-PAGE was performed using precast 8–16 % bis-acrylamide gradient gels (Bio-Rad, Hercules, CA, USA) in running buffer containing 25 mM Tris–HCl, 10.3 % (w/v) SDS at 100 V. The samples were denatured prior to loading to the gel at 95 °C for 5 min in a loading buffer containing 0.35 M Tris–HCl, 10.3 % (w/v) SDS, 36 % (v/v) glycerol, 5 % (v/v) 2-mercaptoethanol and 0.012 % (w/v) bromophenol blue (pH 6.8). The gels were stained using Coomassie brilliant blue. Protein content was determined according to Bradford [26].

High-performance anion exchange chromatography (HPAEC)

Oligosaccharides and 4-O-methylglucuronic acids released after enzymatic incubation of AAc and BeWX with ReGH67 or ReGH115 were analysed by HPAEC as described elsewhere [1]. Quantification of 4-O-methylglucuronic acid was based on a calibration curve of glucuronic acid (0–50 μg mL⁻¹).

Reverse-phase ultra-high-performance liquid chromatography ultra violet–mass spectrometry (RP-UHPLC UV-MS)

A mixture of AAc, xylose, xylobiose, xylotriose and xylotetraose was labelled with anthranilic acid (2-AA) as described by Ruhaak [27] with some modifications: 50 μL of sample (2 mg mL⁻¹) was dried under vacuum and mixed with 50 μL of a freshly prepared mixture (1:4) of 2-AA (192 mg mL⁻¹) and 2-picoline borane (143 mg mL⁻¹) in DMSO containing 30 % (v/v) of glacial acetic acid.

AAc incubated with ReGH67 or ReGH115 were also labelled following the above-described procedure. All 2-AA-labelled samples were submitted to RP-UHPLC UV-MS analysis. Labelled oligomers were separated on a UHPLC Shield C18 BEH column (2.1 × 150 mm, 1.7 μm particle size; Waters, Milford MA, USA) using an Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, degasser, autosampler and a photodiode array (PDA) detector, and coupled in-line to an LTQ-Velos double ion trap mass spectrometer equipped with a heated ESI probe (Thermo Scientific). The eluents were 0.1 % (v/v) formic acid in demineralised water (A) and 0.1 % (v/v) formic acid in acetonitrile (B) at 50 % (v/v) acetonitrile in demineralised water (C). The flow rate was 300 μl min⁻¹ and the sample injection volume was 10 μL. The elution programme was started at 95 % (v/v) A, 8 % (v/v) B for 25 min, followed by 25–35 min linear gradient to 80 % (v/v) A, 20 % (v/v) B; 35–36 min linear gradient to 50 % (v/v) A, 50 % (v/v) B; 36–41 min 50 % (v/v) A, 50 % (v/v) B; 41–42 min linear gradient to 92 % (v/v) A, 8 % (v/v) B; 42–50 min 92 % (v/v) A, 8 % (v/v) B. Re-equilibration was performed for 17 min at starting condition. The eluate was measured at 254 nm [28]. The compounds eluted were detected by MS in negative mode. Source heater temperature was set at 225 °C and the capillary temperature was 350 °C. Ion source voltage was set at −4.5 kV. The detected mass range was 300–2000 Da. MS² was performed on the most intense ion detected, with normalised collision energy of 30 (arbitrary units). Single reaction monitoring (SRM) was acquired using the above-mentioned MS settings. The main MS² fragments of each individual compound present in AAc were monitored together with the parent mass and retention time of the compound and given in Table 2. The most abundant fragments from each RP18-separated single mass were determined (Table 2). In a separate analysis, these most abundant fragments, which are considered to be the fingerprint of the structure, were selectively monitored. From the latter analysis, the sum of the areas of the two main fragments was used for quantification of the structures. In addition, increasing concentrations of 2-AA-labelled standards (xylose, xylobiose and xylotriose) were analysed. Samples were assumed to be labelled equally efficient as the standards, which showed a linear correlation (R² = 0.99) of their UV response area (340 nm) upon increasing concentrations, as shown in Fig. 5.
Table 2  Single reaction monitoring (SRM) settings of AAc analysed by UHPLC-MS

| Segment 1 (0–16 min) | Scan event name | Parent mass (m/z) | Main MS² fragment (F) | Rt (min) | Segment 2 (16–50 min) | Scan event name | Parent mass (m/z) | Main MS² fragment (F) | Rt (min) |
|----------------------|-----------------|------------------|-----------------------|---------|----------------------|-----------------|------------------|------------------|---------|
|                      |                 | TIC              | 50–1000               | na      |                      | TIC             | 50–1000          | na               | na               |
|                      |                 | SRM 1            | 856                   | 666     | 780                  | SRM 1           | 460              | 270              | 384               |
|                      |                 | SRM 2            | 724                   | 533     | 648                  | SRM 2           | 724              | 534              | 648               |
|                      |                 | SRM 3            | 724                   | 533     | 648                  | SRM 3           | 592              | 402              | 516               |
|                      |                 |                  |                       |         |                      |                 |                  |                  |                    |

Rt retention time in minutes, na not applicable.

Fig. 5 RP-UHPLC-UV (340 nm) response area of various concentrations of 2-AA-labelled xylose (filled diamond), xylobiose (filled circle) and xylotriose (filled square). The concentrations correspond to the amount subjected to labelling.

Additional file

Additional file 1: Figure S1. pH (A) and temperature profiles (C) of the incubated ReGH67 with beechwood xylan. pH (B) and temperature profiles (D) of the incubated ReGH115 with beechwood xylan.

Abbreviations
2-AA: anthranilic acid; AAc: aldouronic acids; BeWX: BirchWood glucuronoxylan; BiWX: BirchWood glucuronoxylan; CAZy: Carbohydrate-Active EnZymes; GH: glycoside hydrolases; HPAEC: high-performance anion exchange chromatography; MALDI-TOF MS: matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry; PAD: pulsed amperometric detection; PDA: photodiode array; SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis; SRM: single reaction monitoring; UAmeXOS: O-methyl-glucuronic acid-substituted xylo-oligosaccharides; RE. Rasmsonia emersonii; RP-UHPLC-UV-MS: reverse-phase ultra-high-performance liquid chromatography–ultraviolet mass spectrometry; XOS: xylo-oligosaccharides.

Authors’ contributions
PM carried out the experiments and data analysis and drafted the manuscript. MMA produced and supplied the enzymes. HG and MK participated in the design and coordination of the study and helped draft the manuscript. All authors read and approved the final manuscript.

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