

Analysis of Xyloglucan Composition in *Arabidopsis* Leaves

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[Abstract] Xyloglucan is one of the main components of the primary cell wall in most species of plants. This protocol describes a method to analyze the composition of the enzyme-accessible and enzyme-inaccessible fractions of xyloglucan in the model species *Arabidopsis thaliana*. It is based on digestion with an endoglucanase that attacks unsubstituted glucose residues in the backbone. The identities and relative amounts of released xyloglucan fragments are then determined using MALDI-TOF mass spectrometry.

Keywords: Xyloglucan, *Arabidopsis*, Cell wall, Hemicellulose, Primary wall, MALDI-TOF

[Background] In many flowering plants xyloglucan is a major component of primary cell walls, where it plays an important role in growth regulation. Sequential extraction protocols offer a way of separating distinct xyloglucan domains (Pauly *et al.*, 1999). Some xyloglucan appears to be trapped inside cellulose microfibrils while another fraction is bound to their surface through hydrogen bonding. The rest of the xyloglucan, possibly the majority of it, occupies the space between microfibrils (Park and Cosgrove, 2015). Part of this later xyloglucan can be extracted through direct endoglucanase digestion of cell wall material. Much of the remaining xyloglucan can then be released through alkaline treatment. Studies of mutants deficient in xyloglucan exoglycosidases have shown that these enzymes, together with Xyloglucan Endotransglycosylases/Hydrolases, act mostly on the enzyme-accessible fraction (Sampedro *et al.*, 2010; Günl and Pauly, 2011; Günl *et al.*, 2011; Sampedro *et al.*, 2012; Sampedro *et al.*, 2017). Digestion of *Arabidopsis* xyloglucan with endoglucanases that attack unsubstituted glucose residues results in a mixture of three and four-glucose subunits that can be quickly and easily analyzed through MALDI-TOF mass spectrometry (Lerouxel *et al.*, 2002; Günl *et al.*, 2010). The area of the ion peaks can be then used to quantify the abundance of the different fragment, although there is evidence of significant differences in response factors (Tuomivaara *et al.*, 2015). This protocol incorporates some changes from our previous versions, such as the use of SDHB (Super-DHB) matrix, which reduces the noise, and the addition of NaCl during extraction to prevent the formation of potassium adducts.

Materials and Reagents

1. Pipette tips (2 µl, 200 µl, 1,000 µl)
2. 1.5 ml microcentrifuge tubes
3. Centrifugal filters, modified PES, 10K (VWR, catalog number: 82031-348)
4. 200 µl PCR tubes
5. Mature *Arabidopsis* leaves (2 or 3 leaves)
6. Liquid nitrogen
7. Ethanol (Merck, catalog number: 1.00983)
8. Type II purified water
9. Acetone (Sigma-Aldrich, catalog number: 179973)
10. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)
11. Cellulase suspension from *Trichoderma longibrachiatum* (Megazyme, catalog number: E-CELTR)
12. Pyridine (VWR, catalog number: 27199.292)
13. Thimerosal (Sigma-Aldrich, catalog number: T5125)
14. Sodium hydroxide (NaOH) (Merck, catalog number: 106469)
15. Acetic acid (AppliChem, catalog number: 141008.1611)
16. 2,5-Dihydroxybenzoic acid (Sigma-Aldrich, catalog number: 39319)
17. 2-Hydroxy-5-methoxybenzoic acid (Sigma-Aldrich, catalog number: 146188)
18. Acetonitrile (Merck, catalog number: 1.00029)
19. Xyloglucan oligosaccharide mixture (Megazyme, catalog number: O-XGHON)
20. Digestion buffer (see Recipes)
21. Super-DHB (SDHB) matrix (see Recipes)

Equipment

1. Variable volume single channel manual pipettes (0.2 to 2 µl, 2 to 20 µl, 10 to 100 µl, 100 to 1,000 µl)
2. Pellet pestles (Sigma-Aldrich, catalog number: Z359947)
3. Bench pillar drill, 250 W
4. Insulation foam block
5. Polycarbonate cover
6. Dry block heater
7. Vortexer
8. Microcentrifuge (Eppendorf, model: 5424)
9. SpeedVac System (Thermo Fisher Scientific, Thermo Scientific™, model: Savant™ SC210 P1)
10. Ultrasonic bath
11. Orbital shaker

12. MALDI target plate (Bruker, model: MTP 384 ground steel TF, catalog number: 8209519)
13. Mass spectrometer (Bruker, model: UltraFlex III MALDI-TOF/TOF)

Software

1. Flex Analysis Version 3.0 (Bruker)

Procedure

A. Cell wall extraction

1. Collect 100 mg of mature *Arabidopsis* leaves (2 or 3 leaves) in a microcentrifuge tube.
Note: We usually collect fully expanded leaves with no signs of senescence from 4 to 5 weeks old plants grown in 16-h days at 22 °C/18 °C light/dark temperature.
2. Grind for a few seconds with a pestle mounted on a bench pillar drill at about 600 rpm, keeping the sample submerged in liquid N₂ (see Figure 1). Do not add liquid N₂ to the sample. Pestles should be changed between samples.



Figure 1. Grinding setup. The bottom half of a plastic 60 ml container was inserted in a block of insulation foam and covered with a perforated piece of polycarbonate. A channel was added to facilitate addition of liquid N₂.

3. Add 1 ml of 80% ethanol. Vortex for 5 sec and heat for 10 min at 50 °C in a dry block heater. Centrifuge for 5 min at 3,000 x g and discard the supernatant.

4. Add 1 ml of 100% ethanol to the precipitate. Vortex for 5 sec and keep at room temperature for 10 min. Centrifuge for 5 min at 3,000 x g and discard the supernatant.
5. Add 1 ml of acetone to the precipitate. Vortex for 5 sec and keep at room temperature for 10 min. Centrifuge for 5 min at 3,000 x g and discard the supernatant.
6. Dry samples in a Speed-Vac for 5 min approximately.
Note: We try to avoid complete drying to facilitate resuspension. Samples can also be air-dried. Dried samples can be stored at -20 °C for later processing.

B. Extraction of enzyme-accessible xyloglucan

1. Add 1 ml of 100 mM NaCl to dried wall residue and resuspend in an ultrasonic bath for at least 20 min. Vortex the sample for 5 sec several times during resuspension. Centrifuge for 3 min at 18,000 x g and discard the supernatant.
2. Add 1 ml of water to cell wall residue, vortex for 5 sec and incubate in an ultrasonic bath for at least 5 min. Centrifuge for 3 min at 18,000 x g and discard the supernatant. Repeat for a total of two times.
3. Add 350 µl of digestion buffer (see Recipes).
Note: Complete resuspension is not necessary as it will be finished during overnight incubation.
4. Pipette a volume of cellulase suspension that contains 6 units of enzyme per sample into a centrifugal filter. Volume will vary depending on enzyme batch. Add 450 µl of digestion buffer. Centrifuge for 5 min at 14,000 x g and discard the filtrate.
5. Add 450 µl of digestion buffer. Centrifuge for 5 min at 14,000 x g and discard the filtrate. Repeat for a total of two times.
6. Add 50 µl of digestion buffer per sample to the retentate and pipette to a clean centrifuge tube.
Note: Steps B4 to B6 can be carried out while cell wall residue is resuspended.
7. Pipette 50 µl of the enzyme solution obtained in step B6 to each of the resuspended cell wall samples from step B3.
8. Incubate overnight in an orbital shaker at 37 °C and 150 rpm.
9. The next day wash one centrifugal filter per sample by adding 500 µl of water, centrifuging for 3 min at 14,000 x g and removing the filtrate. Repeat this process for a total of 3 times.
Note: This step is necessary to remove traces of a polymer, which will otherwise interfere with the MALDI-TOF analysis.
10. Centrifuge the cell wall digestion for 5 min at 18,000 x g.
11. Pipette the supernatant to a pre-washed centrifugal filter. Centrifuge for 10 min at 14,000 x g.
12. Discard the filter and dry the filtrate on a Speed-Vac.
Note: Some residue is usually visible at the bottom of the tube.
13. Store at -20 °C.

C. Extraction of non-accessible xyloglucan

1. Wash the cell wall residue from the accessible xyloglucan extraction (precipitate from step B10) by adding 1 ml of water, centrifuging for 5 min at 18,000 $\times g$ and discarding the supernatant. Repeat for a total of two times.
2. Add 900 μl of 17% NaOH (w/v) and incubate overnight in an orbital shaker at 37 °C.
3. Add 300 μl of acetic acid and centrifuge for 5 min at 18,000 $\times g$.
4. Pipette 500 μl of the supernatant onto a centrifugal filter, centrifuge at 14,000 $\times g$ for 10-15 min and discard the filtrate. Add additional supernatant and repeat until all of it has been filtered.
5. Add 500 μl of digestion buffer to the filter, centrifuge at 14,000 $\times g$ for 15-20 min and discard the filtrate. Repeat for a total of two times.
6. Add 350 μl of digestion buffer to the filter and pipette the retentate to a new microcentrifuge tube.
7. Follow the procedure for extraction of enzyme-accessible xyloglucan starting in step B4.

D. MALDI-TOF analysis

1. Resuspend dried xyloglucan digestion (step B13) in 20 μl of 10 mM NaCl by repeated pipetting. Centrifuge at 18,000 $\times g$ for 2 min.
2. Pipette 2 μl from the top of the solution into a clean 200 μl PCR tube and add 6 μl of SDHB solution (see Recipes). Pipette 2 μl of the mixture onto the MALDI target plate.
3. To prepare a calibration spot mix 2 μl of a 2 mM solution of xyloglucan oligosaccharides (Megazyme) with 6 μl of SDHB solution. Pipette 2 μl of the mixture onto the MALDI target plate.
4. Air dry at room temperature for approximately 30 min.

Note: A dissecting microscope can be used to check the samples. Some crystals should be visible on the plate (Figure 2).

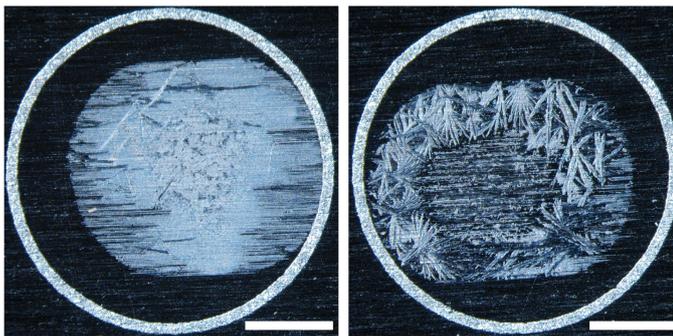


Figure 2. Examples of crystallization on the MALDI plate. Although the sample on the left is cleaner both produced usable spectra. Bars = 1 mm.

5. Load the target plate onto a MALDI-TOF/TOF mass spectrometer.
6. Operate the mass spectrometer in reflectron and positive mode, at an accelerating voltage of 25 kV with a PIE (Pulsed Ion Extraction) of 10 nsec. Set matrix suppression at 450 Da. Average a total of 600 laser shots delivered in 3 sets of 200 shots (at 100 Hz) to 3 different locations.

7. Calibrate the mass range using the calibration spot. The three main peaks correspond to the following sodium adducts: XXXG (C₃₉H₆₆O₃₃Na₁, 1085.3384 Da), XXLG/XLXG (C₄₅H₇₆O₃₈Na₁, 1247.3912) and XLLG (C₅₁H₈₆O₄₃Na₁, 1409.4440). These abbreviations correspond to the standard xyloglucan oligosaccharide nomenclature (Tuomivaara *et al.*, 2015).
8. Compile mass spectra for each sample by plotting the mass over charge (m/z) ratio (x-axis) of all detected ion species against their measured intensities (y-axis) using Flex Analysis Version 3.0 (Bruker).

Data analysis

1. Using Flex Analysis label peaks using SNAP (Sophisticated Numerical Annotation Procedure) with signal to noise threshold set at 3. The program will calculate the area of the peaks from the isotopic envelope for each m/z (Figure 3). Export the data to Excel.

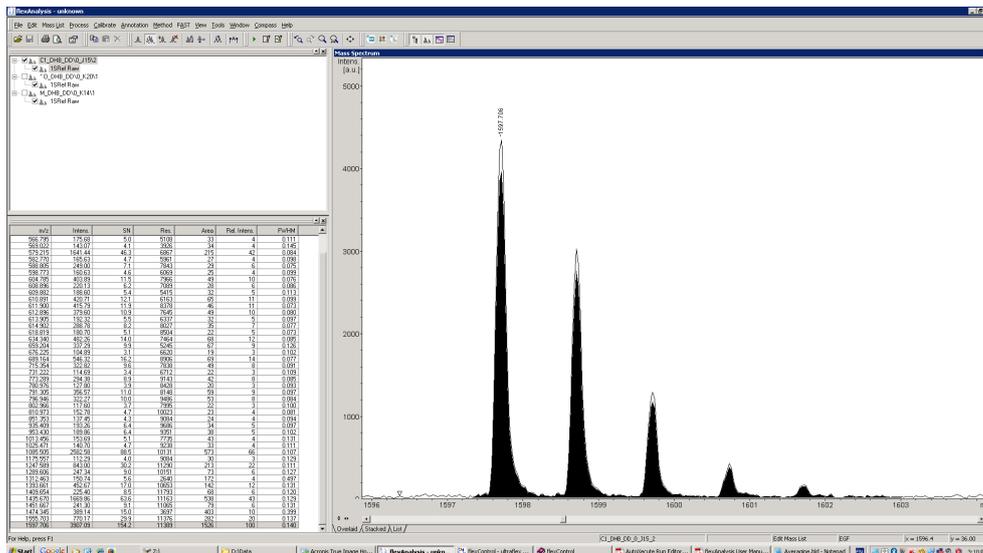


Figure 3. Screenshot of Flex Analysis showing a list of identified peaks with their areas, and a detail of the 1597 m/z peak

2. Manually select the peaks with an m/z corresponding to xyloglucan fragments. Those present in wild-type xyloglucan are shown in Table 1. Sum the total area of the peaks and calculate the proportion of the total area that corresponds to each of the identified xyloglucan fragments. Average at least three independent extractions. Student's t -test or other statistical methods can be used to compare the proportions of xyloglucan fragments in different genotypes. Extraction of non-accessible xyloglucan results in loss of acetyl groups and only non-acetylated fragments will be observed. Changes that are more evident in accessible xyloglucan, compared to non-accessible xyloglucan, are likely caused by xyloglucan metabolism in the wall.

Table 1. Structure and m/z values of xyloglucan fragments present in wild-type *Columbia* leaves. Structure abbreviations correspond to the standard xyloglucan oligosaccharide nomenclature, with acetylation indicated by underlined symbols (Tuomivaara *et al.*, 2015). Adduct composition is indicated as the number of hexoses (H), deoxyhexoses (DH), pentoses (P), acetyl groups (Ac) and sodium atoms (Na). The m/z values of other oligosaccharide adducts can be calculated using the following formula: $162 \times H + 146 \times DH + 132 \times P + 42 \times Ac + 41$.

Structure	XXG	GXXG XLG	X \underline{L} G	XXXG	XXLG XLXG	XX \underline{L} G	XXFG	XLLG	XX \underline{F} G	XL \underline{L} G	XLFG	XL \underline{F} G	
Adduct Composition	H	3	4	4	4	5	5	5	6	5	6	6	
	DH	0	0	0	0	0	1	0	1	0	1	1	
	P	2	2	2	3	3	3	3	3	3	3	3	
	Ac	0	0	1	0	0	1	0	0	1	1	0	1
	Na	1	1	1	1	1	1	1	1	1	1	1	1
m/z	791	953	995	1,085	1,247	1,289	1,393	1,409	1,435	1,451	1,555	1,597	

Notes

An internal standard, such as malto-oligosaccharides or cello-oligosaccharides can be added to the SDHB solution for absolute quantification. Washes with water before extraction will remove a small amount of xyloglucan (Günl and Pauly, 2011). The endoglucanase used in this protocol appears to cut in front of every single unsubstituted glucose. Other endoglucanase with different specificities can produce a different set of fragments when analyzing unusual xyloglucan (Günl *et al.*, 2011). This method cannot separate fragments of identical mass, such as XLXG and XXLG, although in some cases MALDI-TOF/TOF can be used on the same samples to identify the fragments (Sampedro *et al.*, 2010; Sampedro *et al.*, 2012; Sampedro *et al.*, 2017).

Recipes

- Digestion buffer
 - 36.6 μ l acetic acid
 - 29 μ l pyridine
 - 20 mg thimerosal
 - 100 ml of water
 - Store at 4 °C
- SDHB matrix
 - 9 mg ml⁻¹ 2,5-dihydroxybenzoic acid
 - 1 mg ml⁻¹ 2-hydroxy-5-methoxybenzoic
 - 70% acetonitrile
 - 30% water

Store at -20 °C for one month

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