Title

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Structural Analysis of cell wall polysaccharides using PACE

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i. Summary

The plant cell wall is composed of many complex polysaccharides. The composition and structure of the polysaccharides affect various cell properties including cell shape, cell function and cell adhesion. Many techniques to characterize polysaccharide structure are complicated, requiring expensive equipment and specialized operators e.g. NMR, MALDI-MS. PACE (Polysaccharide Analysis using Carbohydrate gel Electrophoresis) uses a simple, rapid technique to analyze polysaccharide quantity and structure (Goubet et al. 2002). Whilst the method here describes xylan analysis, it can be applied (by use of the appropriate glycosyl hydrolase) to any cell wall polysaccharide.

ii. Key words

electrophoresis, polysaccharides, glycosyl hydrolases, cell walls, fluorescent labelling, 8-Aminonaphthalene-1,3,6-trisulfonic acid, xylan

1. Introduction

2. Materials

All solutions should be prepared with ultrapure water (18 MΩ at 25 °C) and all chemicals should be analytical grade. All reagents should be prepared and stored at room temperature unless otherwise indicated. Follow local safety guidelines when disposing of waste materials.

2.1. Equipment

1. Speedvac equipped with a vacuum pump and cold trap (e.g. Savant concentrator with refrigerated vapor trap, Thermo Scientific). When used, do not heat rotor above 45 °C.

2. Heatblock set at 37 °C, 95 °C.

3. Gel casting apparatus and running apparatus (Hoefer SE660) equipped with a circulating cooling bath (e.g. Hoefer RCB-20). (**Note 1**)

4. High voltage power supply which can provide 1000 V and 250 mA with either constant voltage or constant current (e.g. Owl Hi-voltage TLQ EC1000XL, Thermo Scientific).

5. Hamilton microsyringe (10 µl volume).

6. Pizza cutter

7. Gel visualization and imaging set up. This should either include a UV-transilluminator equipped with long-wave UV bulbs, a filter set suitable for ANTS (we use a Syngene Chemi XRQ Imaging System) or a laser scanning system (e.g. Typhoon, GE Healthcare).

8. Software for densitometry such as GeneTools (available from Syngene) or the freely available Image J (http://imagej.nih.gov/ij/).

2.1. Preparation of alcohol insoluble residue (AIR) from fresh plant material.

Alcohol insoluble residue (AIR) is prepared from plant tissue as described in (Mortimer et al. 2010).

2.2. Glycosyl hydrolases (GH)

1. GH11 Xylanase was obtained from Megazyme (**Note 2**).

2. Digestion buffer (10x), 1 M ammonium acetate, pH 6.0 (adjust pH with acetic acid). Store at 4 °C, stable for ~3 months (**Note 3**).

2.2. Oligosaccharides for use as standards

Xylose (X), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4), xylopentaose (X_5) and xylohexaose (X_6) are available from Megazyme International (Co. Wicklow, Ireland). Store at -80 °C as 100 mM stocks in water. Working stocks (0.5 mL aliquots) can be diluted to 1 mM in water and stored at -20 °C. For quantitative gels, make 3 different concentration ladders of standards to be run in 3 different lanes. Standard 1 (S1): mix 1 µl of all 6 xylo-oligos, S2: mix 2 µl of all 6 xylo-oligos, S3: mix 5 µl of all 6 xylo-oligos. For qualitative gels, only one ladder is required (S2 is recommended). These should be dried in the speedvac and then fluorescently labelled as described below.

2.3. Fluorescent labelling components

1. 2-Picoline-borane (2-PB; Sigma, MO). Make up a 0.2 M stock in dimethyl sulfoxide (DMSO). Since it is very hygroscopic, upon opening immediately resuspend the whole amount in DMSO and store in 1 mL aliquots at -20 °C. Stable for at least 1 year.

2. ANTS (8-Aminonaphthalene-1,3,6-trisulfonic acid, disodium salt; ThermoFisher Scientific, MA), 0.2 M in 17:3 water: acetic acid. Add ANTS to water, warm to 60 °C to dissolve and add the required volume of acetic acid. Store protected from the light at -20 °C. Stable for 4-6 weeks (-80°C storage may increase stability).

3. DMSO buffer (30 µl acetic acid, 170 µl H_2O, 200 µl DMSO) – make fresh each time.

4. 3 M Urea – store at 4 °C for up to 6 months.

2.4. PACE gel components

1. Forty percent acrylamide/Bis-acrylamide (29:1 acrylamide:Bis) solution (Sigma. MO). (**Note 4**)

2. 10 x resolving buffer (1M Tris-base borate buffer). Prepare ~ 1.5 M Tris-base, and add solid boric acid until the pH is 8.2 (approximately 60 g/L). Dilute solution until Tris final concentration is 1 M.

3. Ammonium persulfate (APS; 10% w/v) in water. Make fresh or use a frozen stock.

4. N,N,N,N´-tetramethyl-ethylenediamine (TEMED).

5. Isopropanol.

3. Methods

3.1. Glycosyl hydrolase digestion of AIR

1. Accurately weigh 20-30 mg AIR into a 15 mL falcon tube (**Note 5**). Resuspend in water to obtain a suspension of 5 mg/mL and vortex/sonicate to ensure even dispersion (a Dounce homogenizer can also be employed). Use a pipette to accurately aliquot the correct amount into individual 1.5 mL microfuge tubes (in this protocol 20 µL ie 100 µg), and then dry in a speedvac.

2. Pre-treat dried 100 µg aliquots of Arabidopsis stem AIR with 20 µl of 4 M NaOH for 1h at room temperature (**Note 6**). Neutralize with 1M HCl (check pH with pH strips). Add 50 µl digestion buffer and make the volume up to 500 µl (**Note 7**).

3. Add 3 µl of GH11 (**Note 8**) and incubate overnight at room temperature. Include the following controls (i) AIR no enzyme and (ii) enzyme no AIR. Boil the samples at 95 °C in a heatblock for

30 minutes to deactivate the enzyme. Use microcentrifuge lid locks to prevent loss of sample. Dry samples in speedvac (**Note 9**).

3.2 Fluorescent labelling of oligosaccharides

1. To each dried sample or standard, add 5 µl of 0.2 M ANTS solution, vortex and then add 5 µl 2-PB solution and 10 µl of DMSO buffer. Vortex to mix again, and then centrifuge briefly.

2. Incubate at 37 °C for overnight.

3. Dry the samples in the speedvac. Resuspend the dried samples in 100 µl 3M urea, and store at -20 °C for up to 2 weeks (longer at -80 °C) (**Note 10**).

3.3. Preparation of ANTS gel

1. Set up the gel casting apparatus according to the manufacturer's instructions (e.g. http://www.hoeferinc.com/index.php/literature/user-manuals/). To make two resolving gels in an 18 x 24 cm casting set up with 0.75 mm spacers, mix 20.2 mL H_2O, 5 mL 10 x resolving buffer (1 M Tris-Borate), and 24.6 mL of acrylamide in a 50 mL Falcon tube. Add 200 µl 10% APS and 20 µl of TEMED, and invert tube a few times to mix. Quickly pour the gel using a large volume pipette (e.g. serological pipette), filling the glass plates to within ~ 4 cm of the top. If air bubbles get trapped whilst pouring, tilt the gel or tap the glass to dislodge them.

2. Gently overlay the gel solution with isopropanol. Once the gel has polymerized (~ 20 mins), pour off the isopropanol and rinse with water.

3. For the stacking gel, mix 6.8 mL H_2O, 1 mL 10 x resolving buffer, and 2.8 mL of acrylamide in a 15 mL Falcon tube. Add 80 µl of APS and 8 µl of TEMED and invert a few times to mix. Rapidly pour stacking gel, and then insert combs, taking care to avoid trapping air bubbles under the comb teeth (**Note 11**).

3.4. Separation of oligosaccharides by PACE

1. Fill running chamber with 1 x resolving buffer and allow to cool (maintain at 10 °C). This chamber buffer should be changed every 1-2 months, depending on frequency of gel running.

2. Gently remove the comb, and fill the wells with 1 x resolving buffer (0.1 M Tris Borate).

3. Load 2 µl of samples or standards per well using a microsyringe. Avoid using the outermost lanes, as these lanes tend to spread on long gels, resulting in the loss of the outermost samples. If preferred, use a mixture of electrophoresis dyes run in the outer lane to visualize the gel progress (e.g. bromophenol blue and thorin1 at 1 mg/mL).

4. Assemble the upper chamber of the gel running apparatus, and place the gel in the cooled lower chamber according to the manufacturer's instructions (e.g. http://www.hoeferinc.com/index.php/literature/user-manuals/). Gently fill the upper chamber with 1 x resolving buffer, without disturbing the samples.

5. Protect gels from excess direct light e.g. by wrapping tank with a black plastic garbage bag.

6. Turn on the current, and run for 30 min at 200 V (maintain constant voltage). Then increase voltage to 1000 V for 1 h 40 min (maintain constant voltage) (**Note 12**).

3.5. Visualization of PACE gels

1. Turn on G-box camera (to allow it to cool) and the computer, and open GeneSys software. Clean transilluminator with water and lint-free tissue (e.g. Kimwipe). Ensure correct filter is in place.

2. View gel briefly (~80 ms) whilst still within glass plates to determine if the dye front is still on the gel. Open gel using wedge tool, and whilst gel is still on one glass plate, use a pizza cutter to remove both the stacking gel and the bottom of the gel containing any free ANTS dye.

3. Put a small amount of water on the surface of the transilluminator, and, using gloves, place gel directly on the surface of the transilluminator.

4. Take a number of images of varying exposures from short (100 ms) to long (10 s). Ensure at least 2 do not have any saturating bands (the software can indicate this) (**Note 13**). Do not leave UV light on between images as this will degrade the fluorescence.

5. Save images as 16 bit Syngene files (.sgd). This is important as it will record information about the image e.g. exposure time and system set up. Images can be exported as 8 bit tifs for editing in Photoshop, Powerpoint etc.

3.6 Interpretation of results

1. The control lanes are critical to interpreting the gels. Bands that are present in either the enzyme only lane or AIR only lane, as well as the samples should be excluded as "background" (see Figure 1).

2. In order to obtain robust results, it is recommended to repeat PACE analysis on at least 3 independently grown biological replicates. It is also recommended to perform at least 2 technical replicates on each biological replicate.

3. The simplest interpretation of data can be through a "fingerprint" e.g. by comparing a wild type plant to a mutant or by comparing the action of two different GHs (see Figure 1).

4. In order to identify bands, either run a standard alongside (e.g. the known products of digestion of glucuronoxylan by GH11 are Xyl, Xyl_2 and $MeGlcAXyl_4$, and commercial standards exist) or use further hydrolysis to obtain the oligosaccharide identity e.g. addition of a xylosidase will hydrolyze Xyl_2 to Xyl and $MeGlcAXyl_4$ to $MeGlcAXyl_3$ and Xyl. Addition of a GH115 glucuronidase will hydrolyze $MeGlcAXyl_4$ to Xyl_4 etc.

5. All band identities are required in order to quantitate the gel.

3.7. Quantitation of PACE gels

1. After opening the image analysis software of choice, use the 3 different concentrations of standards to produce an in-gel standard curve. It is recommended to use mono- or di-saccharide sugars for this, since commercially available longer oligosaccharides tend to be less pure.

3. See Figure 2 for a description of how to calculate the proportion of xylan branches.

4. Notes

1. Whilst a standard commercial gel set up could be used (such as that used for SDS-PAGE), the resolution provided by the 18 x 24 cm gels allows for detailed analysis. Thinner gels (0.75 mm spacers) are also recommended.

2. The purity of the GH is critical to the success of the experiment. Prior to usage, it is recommended to test a new GH batch on a range of commercially purified polysaccharide substrates (e.g. homogalacturonan, rhamnogalacturonan I, glucomannan, cellulose, xyloglucan, wheat arabinoxylan) to check for contaminating activities. All GHs used should be heterologously expressed and affinity-tag purified (using fresh affinity resin). Prozomix (www.prozomix.com) make good quality GHs. As an alternative to commercially produced GHs, it is possible to express and purify published enzymes such as the Pichia-expressed GHs described in (Bauer et al. 2006). The choice of GH depends on the nature of the experiment, but here a GH11 has been used since its digestion products have been well characterized e.g. (Brown et al. 2007).

3. Alter the pH conditions to suit the GH used. Ammonium acetate is preferred because it sublimes in the speedvac, reducing the salt content of the final sample.

4. Not all acrylamides are made equal, and often cheaper “own-brand” acrylamides don’t polymerize consistently in the gel rigs. In my experience, de-gassing the acrylamide did not alter this.

5. A static eliminator (e.g. AD-1683. A&D Co. Ltd., Saitama, Japan) can reduce the difficulties in weighing this material.

6. This protocol will describe quantities and methods for analyzing the xylan from the basal stem of 6-week old Arabidopsis plants. The quantity of starting material needed will vary depending on the species, tissue, polysaccharide of interest and GH used. Each reducing end released by the GH will be available for the addition of one fluorescent molecule, so some optimization is required. The severity of the pre-treatment step can also be optimized depending on the polysaccharide of interest.

7. If adjusting the pH is proving problematic, add some (~ 250 µl) water first, as this makes adjusting the pH easier, and results

8. It is recommended to perform the hydrolysis so that the reaction reaches a stable end point to enable comparison between samples. Therefore when testing a new batch of hydrolase, compare the effects of different protein quantities on the final fingerprint pattern. For a particularly recalcitrant sample, it can be beneficial to add a further 1-2 µl of GH for a second incubation of 1-2h the following morning.

9. If your samples contain a large amount of particular matter (undigested cell wall; denatured protein etc.), it is recommended to briefly centrifuge the sample and collect the supernatant in a fresh tube. Wash the pellet with a small volume of water, centrifuge again, and combine with the first supernatant. The supernatant can then be dried and derivatized as described.

10. If you intend to produce quantitative data from the gels, then it is recommended that you run the samples within a week of labelling.

11. We prefer to make gels 24h ahead of running to ensure consistent polymerization and therefore sample separation. Gels can be stored for 1-2 weeks prior to use by covering the top in moist, lint-free tissue, wrapping in a plastic bag and storing at 4 °C. Do not allow the tissue to dry out. Lint-free tissue is key, since most tissues contain optical brighteners which fluoresce in the presence of UV light.

12. If a constant voltage cannot be maintained (especially at higher voltages), this is likely due to a leak in the seal between the upper chamber and the top of the glass plates. Check gasket is properly placed or for damage to glass plates which is preventing a seal being formed. Emergency seals of chips in the glass can be achieved by using a drop of melted 3% (w/v) agarose.

13. If the gel is curling up, or if you are getting background from tissue lint or other dust, it is possible to buy low-fluorescence glass plates and image the gel within the plates. These plates are higher in cost. Ensure the free ANTS has run off the bottom of the gel prior to imaging, as it is extremely bright.

5. References

Bauer S, Vasu P, Persson S, Mort AJ, Somerville CR (2006) Development and application of a suite of polysaccharide-degrading enzymes for analyzing plant cell walls. Proc Natl Acad Sci U S A 103 (30):11417-11422. doi:10.1073/pnas.0604632103

Brown DM, Goubet F, Wong VW, Goodacre R, Stephens E, Dupree P, Turner SR (2007) Comparison of five xylan synthesis mutants reveals new insight into the mechanisms of xylan synthesis. Plant J 52 (6):1154-1168. doi:10.1111/j.1365-313X.2007.03307.x

Goubet F, Jackson P, Deery MJ, Dupree P (2002) Polysaccharide analysis using carbohydrate gel electrophoresis: A method to study plant cell wall polysaccharides and polysaccharide hydrolases. Anal Biochem 300 (1):53-68. doi:DOI 10.1006/abio.2001.5444

Mortimer JC, Miles GP, Brown DM, Zhang Z, Segura MP, Weimar T, Yu X, Seffen KA, Stephens E, Turner SR, Dupree P (2010) Absence of branches from xylan in Arabidopsis gux mutants reveals potential for simplification of lignocellulosic biomass. Proc Natl Acad Sci U S A 107 (40):17409-17414. doi:10.1073/pnas.1005456107

6. Figure Captions

Figure 1: Example PACE gel showing a GH11 digestion of wild type (WT) and *gux* Arabidopsis stem (mutants described in Mortimer et al. 2010, along with a better experimental gel). This gel has been specifically chosen as example of a gel with common issues, which are marked up with numbers. (1) Issue: more oligosaccharides released than expected. This indicates incomplete hydrolysis of the xylan by the GH11. Solution: increase quantity of GH added or increase incubation time. (2) Issue: bands are saturated. Solution: take images with a shorter exposure. (3) Non-specific bands are identified. Solution: in our experience, it is difficult to track down all sources of non-specific bands. The controls allow for their exclusion from any analysis.

Figure 2: Calculating xylan branching frequency from a GH11 xylanase digestion of Arabidopsis stem AIR. Three different concentrations of standards are run within the gel to allow an in gel standard curve to be generated. The Syngene software can then use this to calculate the quantity of oligosaccharides in the sample bands (one fluorescent molecule = one labelled reducing end). Since the identity of all bands are known, the ratio of xylose backbone to [Me]GlcA sidechains can be calculated.

7. Acknowledgements

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8. Figures

Figure 1

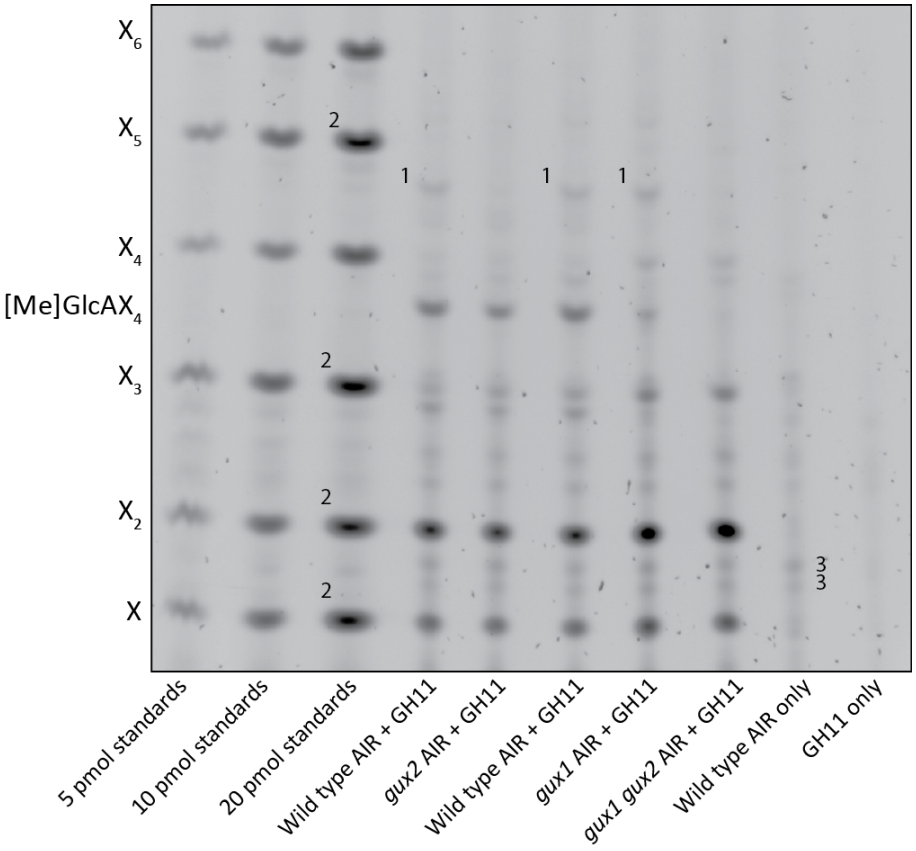

Figure 2