Video Article

Structural Characterization of Mannan Cell Wall Polysaccharides in Plants Using PACE

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Abstract

Plant cell wall polysaccharides are notoriously difficult to analyze, and most methods require expensive equipment, skilled operators, and large amounts of purified material. Here, we describe a simple method for gaining detailed polysaccharide structural information, including resolution of structural isomers. For polysaccharide analysis by gel electrophoresis (PACE), plant cell wall material is hydrolyzed with glycosyl hydrolases specific to the polysaccharide of interest (*e.g.,* mannanases for mannan). Large format polyacrylamide gels are then used to separate the released oligosaccharides, which have been fluorescently labeled. Gels can be visualized with a modified gel imaging system (see **Table of Materials**). The resulting oligosaccharide fingerprint can either be compared qualitatively or, with replication, quantitatively. Linkage and branching information can be established using additional glycosyl hydrolases (*e.g.,* mannosidases and galactosidases). Whilst this protocol describes a method for analyzing glucomannan structure, it can be applied to any polysaccharide for which characterized glycosyl hydrolases exist. Alternatively, it can be used to characterize novel glycosyl hydrolases using defined polysaccharide substrates.

Video Link

The video component of this article can be found at https://www.jove.com/video/56424/

Introduction

Polysaccharide analysis by gel electrophoresis (PACE) is a method for the detailed characterization of polysaccharides[1,2,3]. Plant cell wall polysaccharides are notoriously difficult to analyze, and most methods require expensive equipment, skilled operators, and large amounts of purified material. Here, we describe a simple method for gaining detailed polysaccharide structural information, including resolution of structural isomers.

Understanding plant cell wall polysaccharide structure is necessary for those researchers exploring plant cell wall biosynthesis or the role of the plant cell wall in plant development. Recently, however, plant cell wall composition has become interesting to a wider group of researchers due to the focus on using plant biomass (essentially the cell wall) as a feedstock to produce biofuels and biochemicals[4]. As an example, efficient enzymatic saccharification of this material requires a detailed understanding of the polysaccharide structures, so that optimized enzymatic cocktails can be selected[5].

PACE has several advantages over alternative methods which make it ideal for the rapid analysis of complex glycan structures. Firstly, it does not require purification of the polysaccharide in question, as is required for solution state nuclear magnetic resonance (NMR)[6]. Secondly, PACE can resolve structural isomers, unlike mass spectrometry (MS, *e.g.,* matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI)), which can also be challenging to perform by liquid chromatography (LC)[7]. Thirdly, PACE is very sensitive, with low-picomole resolution, unlike thin-layer chromatography (TLC) or paper chromatography. Finally, it does not require expensive equipment or specialist knowledge, as is the case for MS, NMR, and LC.

The PACE method relies on the specificity of glycosyl hydrolase (GH) enzymes, which target certain glycosidic linkages in a mixture of polysaccharides. When the GH enzyme acts on the polysaccharide chain, it reveals a reducing end which can then be chemically derivatized, in this case with a fluorescent label. The unhydrolyzed portion of the sample is therefore rendered undetectable by this method. The labeled oligosaccharides are then separated in a large-format acrylamide gel by electrophoresis. This gives excellent resolution of very similar molecules, for example, the trisaccharides Glc-Man-Man and Glc-Man-Glc will have a different R_f.

PACE has been used extensively to characterize different xylan structures across plant species[8], to identify glycosyltransferase mutants in Arabidopsis[6,9,10,11], to perform glycosyltransferase assays[9], and to characterize novel GH activities[12,13]. We have also recently used it to

characterize yeast cell wall mannan (Mahboubi and Mortimer, in preparation). Here we describe a method for characterizing plant cell wall glucomannan structure, based on previous reports[11,14].

Protocol

1. Harvesting of Plant Material

1. Harvest fresh plant material (~20 mg) and immediately submerge it in 96% (v/v) ethanol and incubate at 70 °C for 30 min; this deactivates any cell wall degrading enzymes present. For dry material, start at step 2.
   CAUTION: Ethanol is flammable.
   Note: A single stem or rosette leaf will provide enough material for analysis. However, fewer errors arise if a larger amount of tissue is pooled and analyzed since this is easier to weigh out and handle.
2. Carefully record tissue type and developmental stage, as polysaccharide structure varies with both. For example, with *Arabidopsis thaliana* (used here), stage the tissue according to the methods from Boyes *et al.*[15]
   Note: In this protocol, we have used the lower half of the inflorescence stem, where the first silique is fully elongated but not yellowing.

2. Preparation of Alcohol Insoluble Residue (AIR)

1. Prepare AIR, following the method of Mortimer *et al.*[9] or another similar method, in order to produce a powder lacking soluble sugars, such as sucrose and starch.

3. Aliquoting and Pre-treatment of AIR

Note: The exact quantity of AIR needed for each sample will depend upon (a) the abundance of the polysaccharide of interest in the material and (b) the average size of oligosaccharides released by the GH. The method adds a single fluorescent molecule to the reducing end of each oligosaccharide, so the number of oligosaccharides determines the sensitivity of the experiment. As a guideline, for glucomannan in Arabidopsis stem digested with GH5/GH26 (as described), 500 µg is recommended[11], whereas for xylan in Arabidopsis stem digested with GH11, 100 µg is recommended as in Mortimer's study[3].

1. Weigh AIR (10 - 15 mg) into a 15-mL centrifuge tube, and add H_2O to a final concentration of 2 mg/mL. Vortex to achieve an even suspension. If this is problematic, then use a glass homogenizer to assist this process.
2. Aliquot 500 µg into microfuge tubes. Dry aliquots using a vacuum centrifuge (see the **Table of Materials**) overnight at 30 °C.
3. **Pre-treat AIR with 1 M NaOH (20 µL) for 1 h at room temperature; this de-acetylates the polysaccharides and swells the cellulose microfibrils, disrupting the biomass structure and allowing GH access.**
   NOTE: This step can be skipped for purified polysaccharides. CAUTION – strong acid/base.
   1. Include an aliquot for a no-enzyme AIR control (treated the same as all samples, except step 4.1. is excluded).
4. Add 200 µL of H_2O and 20 µL 1 M HCl to neutralize. Test that the pH is ~6-7 by removing 1 µL and spotting it onto paper pH indicator strips.
5. Add 50 µL 1 M ammonium acetate buffer, pH 6.0 (or whichever pH is appropriate for the GHs used in the study) and H_2O to give a total volume of 500 µL.
   Note: Ammonium acetate is used since it sublimes upon drying, and so it does not add additional salt to the sample. An excess of salt can lead to poor band-shape and resolution on the PACE gel.

4. Hydrolysis of AIR with Glycosyl Hydrolases (GHs)

Note: As mentioned in the Discussion, the purity of the GHs is critical. Only use heterologously expressed, affinity purified enzymes. Upon receipt of each lot from the manufacturer, hydrolyze defined aliquots of substrate (*e.g.,* 500 µg AIR) with increasing quantities of GH overnight (*e.g.,* 0.5, 1, 2, 5, 10, 15, 20 µL) (3 units/µL). When there is an excess of GH, the PACE fingerprint will look identical. An excess of enzyme is required to deliver a reproducible result because it must be certain that the hydrolysis reaction is approaching the endpoint.

1. Add a pre-determined amount (see above) of mannanases (GH5 and GH26[11]) to the AIR aliquots in buffer from step 3.5, as well as a positive control (30 µg of konjac glucomannan), and a no-AIR negative control (enzyme mix in an empty tube). Vortex, and then spin briefly to collect the reaction mixture in the bottom of the tube.
2. Incubate overnight at 37 °C (or the appropriate temperature for the GH of choice) with gentle agitation (~100 rpm).
3. Stop the reaction by incubating at 95 °C for 20 min.
   Note: Cap closures can be used to seal flip-top microfuge tubes.
   1. Centrifuge using a bench top microfuge at maximum speed for 10 min, and retain supernatant.
4. Resuspend pellet in 250 µL H_2O, centrifuge as above, and retain supernatant.
5. Combine both supernatants, and dry in a vacuum centrifuge (see the **Table of Materials**) at 30 °C (~3 h or overnight without heating).

5. Preparation of Oligosaccharide Standards

1. Prepare 1 mM stock solutions in H_2O of mannose (Man), mannobiose (Man_2), mannotriose (Man_3), mannotetraose (Man_4), mannopentaose (Man_5) and mannohexaose (Man_6), all β,1-4 linked. Aliquot and store at -20 °C until required.
2. Prepare 3 different concentrations of a Man_{1-6} mixture by combining 1 µL (Standard S1), 2 µL (S2) or 5 µL (S3) of all six.

3. Dry in a vacuum centrifuge (see **Table of Materials**) at 30 °C (~1 h).

6. Derivitization of Oligosaccharides

1. Prepare a stock solution of 0.2 M ANTS (8-Aminonaphthalene-1,3,6-trisulfonic acid) in H_2O:acetic acid 17:3. Warm stock to 60 °C to completely dissolve the solid. Store at -20 °C, protected from light, for 2 - 3 months.
2. Prepare a 0.2 M stock solution of 2-picoline borane (2-PB) in DMSO. This is extremely hygroscopic, so immediately resuspend all powder upon receipt in DMSO. Aliquot, and store at -20 °C for 1 - 2 years. Thaw aliquots as required (store at 4 °C for 2 weeks, and then discard).
3. Prepare the derivatization buffer of H_2O:acetic acid:DMSO at 17:3:20.
4. To each sample, add 5 µL of ANTS, 5 µL of 2-PB and 10 µL of derivatization buffer. Spin briefly to collect in the bottom of the tube, vortex thoroughly, and then spin briefly again. See **Figure 1** for reaction description.
5. Incubate samples overnight at 37 °C, protected from light.
6. Dry in a vacuum centrifuge (see **Table of Materials**) at 30 °C (~2 h).
7. Resuspend samples and standard in 100 µL 3 M urea. Store at -20 °C, protected from the light until required.

7. Preparation of PACE gels

Note: Assembly of the gel casting equipment will depend on the brand. Here, we use a vertical electrophoresis system (see **Table of Materials**), equipped with 18 cm x 24 cm glass plates and 1.5 mm spacers.

1. Assemble gel casting equipment per the manufacturer's instructions.
2. Make 10x PACE buffer (1M Tris-Borate, pH 8.2) as follows: add 121.14 g of Tris-Base to ~400 mL of H_2O, and mix to dissolve. Adjust the pH to 8.2 by addition of solid boric acid (approximately 60 g), and then make volume up to 1 L.
3. Make a 10% (w/v) ammonium persulfate (APS) stock in H_2O. Aliquot and store at -20 °C. Thaw aliquots once, store at 4 °C and discard after 2 weeks.
4. **Make and pour the resolving gel. For the above equipment, 1 gel equate to 50 mL. In a 50 mL centrifuge tube, mix H_2O (20.2 mL), 5 mL 10x PACE buffer, 24.6 mL 40% acrylamide/Bis-acrylamide (29:1 acrylamide:Bis). (Caution: toxic).**
   1. Add 200 µL of APS and 20 µL of N,N,N,N′-tetramethyl-ethylenediamine (TEMED). Invert gently to mix (to avoid introducing air bubbles). Pour the gel using a serological or other large volume pipette, to ~4 cm below the top of the glass plates. Pay attention to the possibility of air bubbles getting trapped in the gel. If they do, stop pouring, and tilt/tap the gel to release them.
5. Overlay the gel with isopropanol (Caution**:** flammable) or, carefully, with H_2O. Allow gel to polymerize (20 - 30 mins), then pour off the top layer. If isopropanol was used, then wash 2x with H_2O. Dry any excess liquid using blotting paper.
6. Make and pour the stacking gel. Mix 6.8 mL H_2O, 1 mL 10x PACE buffer, 2.8 mL acrylamide/Bis-acrylamide (**Caution:** toxic), 80 µL APS, and 8 µL of TEMED in a 15 mL centrifuge tube. Invert to mix, and overlay on top of polymerized resolving gel. Gently insert combs, avoiding trapping air bubbles under the comb teeth.
7. Allow gel to polymerize (20-30 min), wrap in moist tissue and then in plastic wrap, and store at 4 °C until required. Store with the combs in place.
   Note: PACE gels can be stored for a maximum of 2 weeks (although less than 1 week is ideal), if they are kept moist.

8. Running a PACE Gel

1. Use a permanent marker to label the well positions on the glass, which will assist in keeping track of the loading order, and in identifying where the wells are once the comb is removed.
2. Remove the comb. Fill the wells with 1x PACE buffer.
3. Use a 10 µL microsyringe to load 2 µL of standards and samples into wells. Avoid using the outermost lanes, which tend to run samples poorly.
4. Assemble the upper chamber of the gel-running apparatus, and place the gel in a cooled (~10 °C) running tank (10 °C) containing 1x PACE buffer. Fill the upper chamber with 1x PACE buffer.
5. Turn on the power, and run the gel at 200 V (constant V) for 30 mins, and then increase voltage to 1000 V for 1 h 40 mins. Protect gels from light (*e.g.,* by wrapping tanks in black garbage bags).
   Note: Check on the gels ~5 min after turning the voltage on, and then another 5 mins after increasing the voltage to 1,000 V. If the power pack is unable to maintain the voltage then there is likely a buffer leak. Remove the gel from the tank. Reassemble the upper chamber, carefully checking placement of all gaskets. A large chip on the upper edge of the glass plate may prevent the plate forming a good seal with the gasket. This can usually be temporarily resolved by adding a drop of 5 % (w/v) molten agarose to the chipped part of the glass, before adding the upper chamber.

9. Visualizing a PACE gel

Note: Use a gel imaging system equipped with long-wave UV bulbs in the transilluminator, and an emission filter for the camera which is suitable for the fluorophore, here 530 nm. Alternatively, a laser scanner may also be used, as described in Goubet[2].

1. Ensure the gel imaging system is dust free by wiping it with moist lint-free tissue.
2. Remove the gel from the PACE tank, and view briefly whilst the gel is still in the glass plates (<80 ms) to determine if the dye front is still on the gel.
3. Open the gel using a wedge tool, and whilst gel is still on one glass plate, use a pizza cutter to remove both the stacking gel and, if the dye front is still on the gel, the bottom of the gel.

4. Put ~5 mL H_2O onto the surface of the transilluminator, and then transfer the gel directly onto the transilluminator. Set the filter to UV 605, and turn on the longwave UV transilluminator (see **Table of Materials**) using the software.
    Note: The ANTS fluorophore used here has an excitation/emission of 353/520 nm.
5. Take several images at various exposure times (*e.g.,* 100 ms to 10 s). Ensure that the UV light is turned off between images by clicking the UV light button (to turn it off) to avoid degrading the fluorescence. Ensure that at least 2 of the images have no saturated bands (the gel imaging software will indicate this).
6. Save files as high-resolution.tif images.
    Note: Images can be analyzed using the software provided with the gel imaging system, or by using freely available image analysis software, such as ImageJ (https://imagej.nih.gov/ij/). See the **Results** section for a discussion of quantitation.

Representative Results

Here, we show an example PACE gel run per the protocol, along with descriptions to assist with data interpretation and troubleshooting, and this is followed by a general guide to successful PACE gel interpretation[13,16]. A representative gel of a standard PACE assay of cell wall mannan content is shown in **Figure 2**, and is described lane by lane.

Standards
Lanes 1, 2 and 3 show a ladder of commercially purchased oligosaccharides (Man_1- Man_6) at 5 (S1), 10 (S2) and 50 (S3) pmol concentration. When receiving a new lot of a commercial oligosaccharide, it is important to check the purity on a gel. Many oligosaccharides, particularly tetrasacccharides and longer, can have significant contamination with oligosaccharides of other degrees of polymerization (DPs), as shown here for Man_6 (marked with *). While quantitating a gel, it is important to have at least 3 concentrations of the standards, since it is necessary for calculating the standard curve. Calculation of the standard curve can also be helpful for ensuring that the derivatization reaction is essentially at completion. Standards of known concentrations allow comparisons between gels, and are a useful control for separation quality and derivatization quality.

Positive control
Lane 4 shows a mannanase digestion of konjac glucomannan. Konjac glucomannan is available commercially, and whilst it does not have the same structure as that, for example, found in Arabidopsis, it serves two purposes. Firstly, it is an important control for the enzymatic digestion. The researcher should establish what a commercial substrate digested with their GHs of choice looks like when digested to completion (*i.e.,* a vast excess of GH is added to the reaction). If in future experiments the pattern changes, this can indicate either a loss of activity or contamination of the GH stock. Secondly, it serves as a control for the derivatization reaction in the presence of the enzyme and buffer salts. If the standards look good, but the positive control is poor, then this may indicate that a component of the hydrolysis reaction is inhibitory to the derivatization.

Wild type (WT) Arabidopsis stem AIR + mannanase cocktail (GH5 + GH26)
Lane 5 shows the PACE fingerprint of a WT Arabidopsis stem (compare with references[11,14]).

csla9 Arabidopsis stem AIR + mannanase cocktail (GH5 + GH26)
Lane 6 shows shows the PACE fingerprint of the stem from an Arabidopsis plant lacking the major stem mannan synthase[11]. Compare to the WT and negative control fingerprints. Whilst the majority of the mannan is absent in this plant, a small quantity remains, as evidenced by the reduced band intensities for all mannanase-derived oligosaccharides This is synthesized by additional mannan synthases (CSLA2, 3)[11].

Negative control - enzyme only
Lane 8 shows bands on the gel that are not specific that derive from contaminants in the mannanase cocktail, and should be excluded from the analysis (marked with *).

Negative control - WT AIR only
Lane 9 shows bands on the gel that are not specific and should be excluded from the analysis (marked with *).

Negative control - *csla9* AIR only
Lane 10 shows bands on the gel that are not specific and should be excluded from the analysis (marked with *).

Pine wood AIR + mannanase cocktail (GH5 + GH26)
Lane 7 shows the PACE fingerprint of pine wood, which contains galactoglucomannan. This clearly has a different pattern of released oligosaccharides to Arabidopsis, due to its different structure.

Negative control - Pine AIR only
Lane 11 shows bands on the gel that are not specific and should be excluded from the analysis (marked with *).

Interpreting a PACE gel is relatively straightforward, but requires awareness of the following points. For robust data, it is recommended to carry out PACE on at least 3 independently grown biological replicates, and it is recommended to perform at least 2 technical replicates on each biological replicate. The controls described are critical for interpretation, as non-specific bands need to be excluded. We also recommend loading samples in a different order on replicate gels, to exclude effects which result from uneven illumination by the transilluminator. Accurate interpretation requires analysis of bands which are not saturated. Since some oligosaccharide fingerprints may contain both very high and low abundance polysaccharides, we recommend analyzing multiple exposures of the same gel. The standards can be used to normalize between different images.

For some types of experiments, it may be desirable to quantify the amount of polysaccharide in the AIR preparation. Whilst it is possible to quantify from a PACE gel, it requires knowledge of the exact molecular identity of each oligosaccharide released by the GHs and where it is located on the PACE gel. This is currently not fully known for mannan, but it has been determined for other polysaccharides e.g. xylan[3]. The standards do provide a way of normalizing between gels, even when quantitation is not being performed, and so will assist in any qualitative or semi-quantitative analysis.

Identifying the structure of individual oligosaccharides can be achieved using cocktails of GHs. Sequential addition of further GHs can reveal details about linkages and substitutions of the released oligosaccharides (e.g., addition of β-1,4-glucosidase that acts on the non-reducing end will reveal which oligosaccharides in the mixture contain that feature). Available enzymes include galactosidases, glucuronidases and glucosidases (see the CAZy database[16] for additional information); see Hogg, D. et al.[17] as an example.

The protocol above can be used to characterize the activity of unknown GHs. In this case, a defined biomass, such as Arabidopsis stem or konjac glucomannan, should be used to screen the unknown GHs[13].
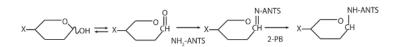

Figure 1: This shows the scheme for the fluorescent derivatization of oligosaccharides with ANTS. Modified from Goubet[2]. Please click here to view a larger version of this figure.
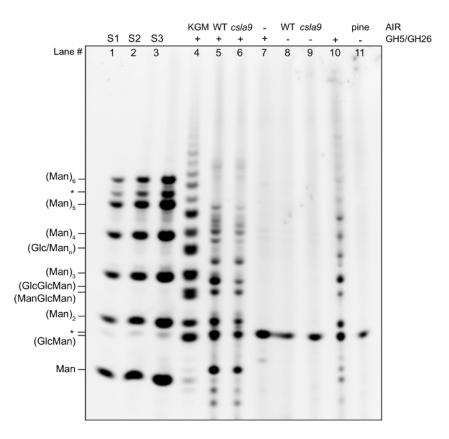

Figure 2: Representative PACE gel showing the mannan fingerprint from Arabidopsis, pine and an Arabidopsis mutant (csla9) which has impaired mannan biosynthesis. AIR was hydrolyzed with a mannanase cocktail, and the released oligosaccharides were derivatized with a fluorophore. The oligosaccharides were separated by gel electrophoresis on the basis of size, shape, and charge. A ladder of manno-oligosaccharides (Man1-6) is shown to assist in identifying relative mobilities of the released oligosaccharides, and a hydrolysis of purified mannan (derived from konjac) is shown as a positive control. * = non-specific bands. Please click here to view a larger version of this figure.

Discussion

PACE is a straightforward method for characterizing polysaccharide structure. It can be applied to any polysaccharide for which there are known GHs with characterized activity, see numerous examples in the literature[1,6,9,11]. Tt has also been applied to the characterization of novel GHs[12,13,18] and glycosyltransferases[9], by making use of defined polysaccharide substrates and acceptors.

Reproducible, interpretable results are dependent on three key steps. First, the GHs used should be free from contaminating activities. This is best achieved by only using heterologously expressed, affinity purified enzymes. Second, for most experiments, it is important that the enzymatic hydrolysis of the substrates is at completion. This will ensure that the same biomass hydrolyzed with the same GH gives the same fingerprint in every experiment. Finally, there should be an excess of fluorophore in the derivatization reaction. This ensures that the available reducing ends are labelled at close to 100%. This will result in high reproducibility of results, as well as data that is quantitative.

Gel and reagent quality are critical to ensuring reproducible data. Poor quality buffers, especially of incorrect pH, air bubbles in the gel, and samples with an excess of salt can all affect resolution and retention factor of the oligosaccharides. However, inclusion of the recommended controls described in the **Protocol** and **Results** section will enable troubleshooting.

PACE is limited by its ability to identify oligosaccharides. For some experiments, a simple fingerprint is all that's necessary. However, to truly quantitate the amount of glucomannan in a sample of AIR, for example, the identity of all the released oligosaccharides is required, which is a time-consuming process. This is more straightforward for less complex polysaccharides such as xylan[3]. Since there are few oligosaccharide standards commercially available, it may not always be possible or desirable to identify all the bands. In this case, PACE can provide very complimentary data to mass spectrometry (*i.e.,* MALDI-CID[9] or ESI-MS[7], and NMR[6]). For these methods, it is often helpful to do a large-scale preparation, separate by size-exclusion chromatography, and then analyze each fraction by both PACE prior to MS or NMR. Whilst it has been reported that bands can be excised and identified by MALDI-CID, in practice we have found that this has a low rate of success (possibly due to interactions of the labeled oligosaccharides with the acrylamide gel when exposed to UV light).

The other major limitation of PACE is throughput. To have good quality, interpretable gels with the appropriate controls, each gel will only have ~10 experimental samples, and a researcher can expect to run ~4 PACE gels per day. Recently, a version of PACE using capillary electrophoresis (CE) has been developed[19], which allows preparation of samples in 96-well plates. It has been used successfully to characterize glycosyltransferase enzyme activities and polysaccharide structures[6,19], although it requires access to a CE machine, which can be expensive to purchase and maintain.

Disclosures

The authors have nothing to disclose.

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