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JoVE (Journal of Visualized Experiments)

1 Alewife Center,

Cambridge, MA 02140, USA

Dear Editor,

**Re: Sequencing of plant cell wall heteroxylans using enzymic, chemical (methylation) and physical (mass spectrometry, nuclear magnetic resonance) techniques by Ratnayake S & Bacic A.**

The manuscript has been revised accordingly to the editorial and reviewers’ comments, with the changes that address each of the comments typed in red.

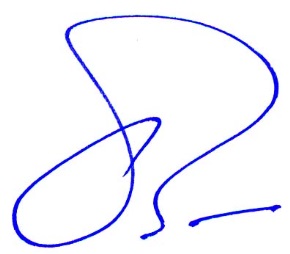
The major concerns of all three reviewers can be summarized as follows:

* insufficient explanation of how each analysis contributes to the delineation of the structure of the heteroxylan; and
* lack of mass fragmentation analysis on the oligosaccharides (including structural isomers) separated by chromatography system.

We believe both these major concerns have now been addressed, as well as most of the others raised by the reviewers - see responses below in red. Furthermore, results of an additional fragmentation analysis of the per-O-methylated fluorophore tagged RE oligosaccharides have been included in the manuscript.

We trust you will now find the revised manuscript acceptable for publication.

Regards,



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**Editorial comments:**  
  
1) All of your previous revisions have been incorporated into the most recent version of the manuscript. In addition, Editor may have made formatting changes and minor copy edits to your manuscript. On the JoVE submission site, you can find the updated manuscript under "file inventory" and download the microsoft word document. **Please use this updated version for any future revisions and track all changes using the track changes function in Microsoft Word**.  
  
2) The following commercial language was removed from the protocol: "Eppendorf tube" was replaced with "microcentrifuge tube".  
Au Response: OK

3) Grammar/Formatting:  
-1.1.4/2.4/2.6/4.2.2/6.4/7.9/7.13/7.14/ – Please correct the run on sentence.

Au Response: Done.  
  
4) Additional detail is required for 2.6 – What temperature is the water bath? “Warm” is not specific.

Au Response: A specific temperature value (40C) has been added.  
  
5) Please adjust the highlighting to identify 2.75 pages or less of text (which includes headings and spaces) that should be visualized to tell the most cohesive story of your protocol steps. We suggest filming only one of the MS analyses. Also note that some of your protocol steps may be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step. Please see JoVEs instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

Au Response: Done, Some protocol steps have been combined as requested. The color of the text has been changed to red to highlight protocol steps.

6) All figure legends should have a title and a brief description in the legend (Figure 1 does not.)

Au Response: Done.  
  
7) Discussion: What is the significance of this technique with respect to other methods?   
Au Response: this has been addressed in the revised “Discussion”.

8) JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.  
Au Response: Done.

9) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

Au Response: Done  
  
10 ) Please disregard the comment below if all of your figures are original.  
If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Au Response: Permission to re-use the following figures has been obtained:

1. Figure 1 has been taken from Ratnayake *et al* (2014)2, Figure 1.
2. The ESI-MS3 spectra has been taken from Ratnayake *et al* (2014)2, part of Figure 5.
3. Figure 4 has been taken from Ratnayake *et al* (2014)2, Supplemental Figure S3.

Permission correspondence is attached as supplementary files.

**Reviewers' comments:**  
  
**Reviewer #1:**

*Manuscript Summary:*   
Structural study of plant polysaccharide is important for the understanding about cell wall biosynthesis, structure and function, and their degradation. However, it has been a great challenge due to structural heterogeneities in plant polysaccharides that are delineated according to species, tissue types and developmental phases. The present study describes the structural studies of xylooligosaccharides that were enzymatically released from the wheat arabinoxylan primarily using mass spectrometry, and further studied with nuclear magnetic resonance spectroscopy. The major impact of this study lies on the modification of oligosaccharide reducing end by either tagging a fluorophore or reduction to alditol for characterizing the internal or reducing end glycosyl sequence. The detailed description of the fluorophore tagging and its product cleanup will be valuable since this method is increasingly used in characterizing plant derived oligosaccharides. Moreover, the derivatized oligosaccharides can be separated on a chromatography system to distinguish isomeric structures.   
  
*Major Concerns:*  
There is a major drawback on the work flow of this methodological paper that the mass fragmentation analysis was not performed on the oligosaccharides separated by chromatography system, and instead the per-O-methylated oligosaccharides that introduced to an ESI-ITMS by direct diffusion were analyzed. Mass fragmentation analysis on the per-O-methylated oligosaccharides has been widely described and the method is known. In addition, the deduced structures can be a mixture of isomers. Therefore, this work can be more valuable if examples on the fragmentation analysis of those column separated oligosaccharides are shown. Additionally, the discussion can also be more informative if the results on fragmentation analysis of the fluorophore tagged and per-O-methylated oligosaccharides are compared.

Au response: Endoxylanase digestion of wheat heteroxylan resulted in xylo-oligosaccharides comprising up to 12 pentosyl units (DP 12, unpublished data). Structural heterogeneity of these xylo-oligosaccharides includes a large number of isomeric structures. There is no single chromatographic system that can separate these complex xylooligosaccharides and this is the underlying motivation for developing and describing the approach, a combination of enzymic, chemical and physical, we have outlined in this manuscript. The online RP C-18 column allowed classes of oligosaccharides to be fractionated which could be identified from the QTOF-MS extracted ion chromatogram analyses which recognises the individual oligosaccharides (Figure 2 B&C). The complete characterization of these individual oligosaccharides can then be achieved by the isolation and fragmentation of interested oligosaccharides using ESI-MSn experiments as described.

The results of an additional fragmentation analysis of the per-O-methylated fluorophore tagged RE oligosaccharides have now been included in the "Representative Results' to demonstrate the utility of this use.

*Minor Concerns:*  
P2 L55: primary wall and secondary wall of grasses?

Au response: Correct and this is already implied in the sentence. No change required.

P2 L98: How much oligosaccharides were tagged with 2AB since 'quantitatively convert' was mentioned?

Au response: 1 mg was used and is now included. All those chains with a reducing end will be reduced. We have removed “quantitatively” as we are not able to make this measurement.

P3 L100: Sodium cyanoborohydride will release poisonous cyanide gas when it is in contact with water. Thus, a safety precaution should be highlighted.

Au response: a safety message is included.

P3 L106: Is mixing needed?

Au Response: yes and now added to protocol.

P4 L134: How is the cleanup procedure for the 2AB-tagged oligosaccharides? Sections 1.1.5 described the cleanup of 2AB-tagged polymeric xylans using 4 volume ethanol. Are the cleanup procedures different for oligosaccharide compared to the polymer?

Au Response: Thank you for picking up this error. The cleanup procedure for oligosaccharides has been revised (Section 3.2) as it is different to that required for the polymer.

P4 L135: (Section 1.1.1-1.1.5)

Au Response: The cleanup procedure for oligosaccharide has been revised.

P4 L136: Method for per-O-methylation of xylooligosaccharides should be included.

Au Response: this is exhaustively described in Pettolino et al (2012)- reference “1” and this has now been referenced.

P8 L296: Product ion m/z 391 can be derived from linear Xyl4-Xyl-ol or (Ara)Xyl-Xyl-Xyl-Xyl-ol or Xyl-(Ara)Xyl-Xyl-Xyl-ol. Why they are not determined as the possible structures?

Au Response: The structure (Ara)Xyl-Xyl-Xyl-Xyl-ol cannot be excluded based upon the MSn analyses, however, it is not a possibility as the specificity of the endoxylanase enzyme does not generate oligosaccharides without an unsubstituted pentosyl residue at the non-reducing end.

The linear structure Xyl4-Xyl-ol is also a formal possibility, however, this oligosaccharide would be further digested by the endo-xylanase to Xyl(1-3)-Xyl-ol oligosaccharides. Therefore it would not be detected and thus was not considered as a possible structure.

The structure Xyl-(Ara)Xyl-Xyl-Xyl-ol is possible and is included in Figure 3.

P10 L395: 'The complete structural characterization…….' At this point, the column separated oligosaccharides were not analyzed further with mass fragmentation analysis to identify the possible structure. Thus, the 2-AB tagging cannot be considered as the method for complete structural characterization. Please rephrase the sentence.

Au Response: “complete” has been removed as suggested.

Table 1: H-1 for terminal alpha-GlcpA in proton NMR should be around 5.3ppm

Au Response: Correct but as terminal alpha-GlcpA was not detected in our structure it has been removed from Table 1.  
  
*Additional Comments to Authors:*  
N/A  
  
**Reviewer #2:**

*Manuscript Summary:*   
The article describes a series of techniques used to determine the structure of heteroxylans present in plant cell walls. In the article the authors give details of their version of the procedure of Mazumder and York that uses MSN analysis of per-O-methylated oligosaccharides and NMR to determine branching patterns of the heteroxylan. The article also uses 2 aminobenzamide to label the reducing end of the heteroxylan and oligosaccharides produced by enzymatic digestion to aid in the identification of the reducing end structures and improve reversed phase chromatography of the oligosaccharides. NMR analysis is used to identify specific sugars and linkages.   
  
*Major Concerns:*  
The article would benefit from a more narrative approach to the results section. It would be very helpful to the reader for the authors to explain how each analysis contributes to the delineation of the structures. One example is that it is not clear what the LC-MS analysis contributes. If the results section presented each technique with a preamble about its purpose and followed by showing what was concluded about the heteroxylan structure the reader would likely be better able to use these techniques. The current version indicates the identity of fragments but doesn't address what the presence of these species indicates about the structure of the heteroxylan. The MSn and NMR data are discussed with respect to the final structure but it would be good to include the MALDI-TOF and LC-MS data in the discussion.   
Au Response: As suggested, and within the constraints of the JoVE guidelines, a more descriptive interpretation of the spectra to explain the presence of structural species and the deduced structures with an additional fragmentation analysis (2AB labelled neutral RE oligosaccharide derived from W-sol AXs) have been included in the manuscript.

The method allows assignment of the structures at the RE on the heteroxylan (an important and distinguishing structural feature) and sequences the oligosaccharides released by endo-xylanase digestion. The order of these internal region oligosaccharides in the polymer cannot be determined from this (or any other) approach except by doing shorter enzyme hydrolysis times that result in larger stretches of oligosaccharides that can be further sequenced using the combination of approaches described in this manuscript.

[**Editorial recommendation:** Please keep JoVE’s manuscript requirements in mind as you address the above comment and similar comments. Results should be discussed in the "Representative Results' section, not in the Discussion.]  
  
*Minor Concerns:*  
HexA1 used without definition.

Au Response: defined on page 6- “hexuronic acid”.

Figure 2A should indicate in the legend that peaks are sodium adducts.

Au Response: the adducts are a mixture of M+ H+/Na+/NH4+ adducts & these have been indicated as appropriate in the figure legend.

Define EIC (extracted ion current)

Au Response: defined in Figure 2 legend.

Line 287 Open solid line is confusing and perhaps solid line is sufficient.

Au Response: The word ‘open’ has been deleted  
  
*Additional Comments to Authors:*  
N/A  
  
  
**Reviewer #3:**

*Manuscript Summary:*   
The manuscript describes methods for sequencing of plant wall heteroxylans polysaccharides by a combination of enzymatic digestions, permethylation and MS and NMR techniques. The authors use two methods 1. Tag with 2AB the polysaccharide and then digest it with enoxylanse and perform MS and NMR analysis, 2. Reduce, then digest polysaccharide with endoxylanse and then tag and perform same analysis. In the two methods 1 is suppose to give information on reducing end oligosaccharide as 2AB-oligos, the other will give the reducing end oligosaccharide as xylitol residue at the reducing end since it was initially reduced. Also newly released internal residues in example 2 will be all be labeled with 2-AB, whereas in example 1 only reducing end oligo is labeled with 2-AB. The differences between labeled and unlabeled MS spectra and fragmentation of these oligos will reveal information on both reducing end and internal structure of plant wall heteroxylans. MS analysis after permethylation has given diagnostic fragments that allows for sequence information. H-NMR analysis gives alpha beta configuration and D and L information.  
  
*Major Concerns:*  
The authors explained the methodology well and the idea behind labeling with 2-AB before and after xylanase digestions. They perform MS analysis after permethylation and also LC-MS using C18 column and H-NMR. They had also previously performed glycosyl composition. However, they failed to explain the significance of the data. The manuscript seems to be description of method, and then list of MS spectra and subsequent assignment of m/z ions to oligosaccharides and fragment ions formed during MSn analysis. The results were stated but not explained or discussed. It seems to be list of protocols used, and data obtained with no discussion of obtained data to xylan structures and biosynthesis.  
Au Response: We agree but the manuscript was prepared according to the JoVE’s manuscript requirements as this is a methodology paper. However, a more descriptive interpretation of the spectra to explain the presence of structural species and the deduced structures with an additional fragmentation analysis (2AB labelled neutral RE oligosaccharide derived from W-sol AXs) has been included in the manuscript.

[**Editorial recommendation:** Please keep JoVE’s manuscript requirements in mind as you address the above comment and similar comments. Results should be discussed in the "Representative Results' section, not in the Discussion.]  
  
*Minor Concerns:*  
There are no minor revisions, the manuscript will need to explain the results better, and assignment of M/z is not enough.

Au Response: changes have been made throughout the text to improve the flow/descriptions and a more descriptive interpretation of the spectra has been included.

Apparently the method used has already been published by other authors as stated in the introduction. What is novel about this paper? What did the authors do that was different or significant?  
If this is a method paper then the novelty should be discussed?

Au Response: the novelty is the order in which the combination of enzymic/chemical/physical techniques have been applied to enable comprehensive sequencing of internal and reducing end oligosaccharides derived from a polysaccharide by enzymic digestion  
  
*Additional Comments to Authors:*  
N/A