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PROGRESS REPORT

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Andrew Mort – Principal Investigator

We have made progress on several projects to do with determining the structure of pectins. These include: 1) Devising a new sensitive method to determine the degree of methyl esterification (DOM) of pectins; 2) Solubilization of all of RGI from cotton cell walls; 3) Solubilization of RGII from cotton cell walls; 4) Characterization of xyloglucan from cotton cell walls; and 5) Investigation giving an indication of a cross-link between extensin and pectin.

1. DOM of pectins.

Our paper is now in press describing the quantitative reduction of methyl esterified galacturonic acid and to galactose measurement of the degree of conversion to galactose to determine the degree of methyl esterification in any pectin. In the last progress report, we described how the reduction will be used to determine the distribution of methyl esters in pectins, but mentioned some details which remained to be worked out.

We have checked that in the reduced samples, lithium in ethylenediamine can be used to destroy all of the remaining galacturonic acid residues (two successive treatments are necessary for complete destruction). The oligogalactans produced are then representative of contiguous methyl esterified galacturonans in the original samples. The identity and size of each oligogalactan is easy to confirm by liquid secondary ion mass spectrometry (LSIMS). LSIMS is similar to FAB/MS except that cesium ions instead of xenon atoms are used to bombard the sample. The use of cesium ions allows higher energies to be used for ionization. The oligogalactans can be separated by NH_2 normal phase chromatography and quantitated by a refractive index monitor.

We have succeeded in specifically breaking the galactosyl linkages in the reduced polymer thus releasing oligogalacturonans terminated in a galactose residue by using HF at -10° for 15 min. Quantitation of these oligomers is more difficult because the methods for separating them

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preclude the use of a refractive index monitor. We have taken two approaches. The first involves the use of a pulsed amperometric detector (PAD); however, the response of the detector is not uniform on a molar, or mass basis to different species. Second approach: After labeling of oligosaccharides with 2-amino pyridine the response per mole is uniform. However, during the labeling, a large excess of 2-aminopyridine is mixed with oligomers. Direct injection of the mixture does not allow detection of singly charged species because they elute along with the non-attached 2-aminopyridine on ion exchange columns. We developed a rapid quantitative method to remove the non-attached 2-aminopyridine from derivatized oligogalacturonides which consists of passing the reaction mixture through a small cation exchange cartridge in the sodium or ammonium form. 2-Aminopyridine is tightly bound, but the derivatized sugars wash through. The derivative which we had been using, the glycosylamine of the 2-aminopyridine was found to be unstable once the excess derivatizing reagent was removed. We have now switched to forming the reductively aminated aduct which is extremely stable. We now have two possible ways to proceed with the quantitiation of the oligogalacturonide fractions: 1) Label with reductive amination to 2-aminopyridine for quantitation by UV absorption or fluorescence , or 2) Standardize the PAD using the aminated derivatives and then use the PAD for quantitation without derivatization. We know already that we can separate the oligogalacturonides we produce using the Dionex PA-1 column and PAD.

2. Solubilization of RGI from cotton cell walls.

After digestion of cotton cell walls with a purified endopolygalacturonase from *E. carotovora*, about 80% of the galacturonic acid of the wall is solubilized. The galacturonic acid so released is predominantly as dimers, trimers, and tetramers of galacturonic acid. A small amount appears from its sugar composition to be RGII, and a very small amount to be RGI. Treatment of the residue from the endopolygalacturonase digestion with strong alkali (24% KOH + 0.1% $NaBH_4$) solubilized the remaining galacturonic acid, rhamnose, galactose,

arabinose and xylose. From this range of sugars one could surmise that all of the xyloglucan and RGI were solubilized by the alkali treatment. The xyloglucan and RGI did not separate by ion exchange or gel filtration chromatography. Treatment of the extract with "purified" cellulase from Worthington degraded the xyloglucan predominantly into its component hepta-, octa-, and nonasaccharides. There was no evidence of glycosidase activity (no monosaccharides were produced). The RGI remained high molecular weight as judged by gel filtration chromatography.

Preliminary experiments treating the RGI obtained after the cellulase treatment with lithium in ethylenediamine, showed that most of the sidechains on the RGI are a single galactose residue.

3. Solubilization of RGII.

As mentioned above, after endopolygalacturonase treatment of the walls, a fraction whose composition resembles the RGII characterized by Albersheim's group, was solubilized. It showed extremely well defined peaks on HPLC gel filtration indicating that it has a very well defined size. After partial acid hydrolysis, as described by Spellman *et al.* [Spellman, M. W., McNiel, M., Darvill, A. G., and Albersheim, P. (1983) *Carbohyd. Res.* **122**, 131-153], we detected ions at the correct masses by LSIMS for those containing the heptasaccharide they characterized.

4. Characterization of xyloglucan from cotton cell walls.

After digestion of the xyloglucan – RGI complex with cellulase as described in Section 2, the xyloglucan became digested into characteristic oligosaccharides. In conjunction with Dr. Ziad El Rassi, an assistant professor of analytical chemistry at Oklahoma State University (whose specialty is HPLC), we have devised a way to make a quantitative "fingerprint" of any xyloglucan. After the digestion with cellulase, the fragments were labeled by reductive amination with 2-aminopyridine (see Section 1), and then separated by reverse phase chromatography using a water-acetonitrile gradient. Baseline resolution of the major hepta-,

octa-, and nonasaccharide fragments was obtained, and also of minor fragments such as tetra-, penta-, and hexasaccharides. Each peak was identified by collection followed by LSIMS. This HPLC procedure (using retention times rather than mass spectroscopy to identify the peaks) should also allow others to characterize any xyloglucan very rapidly.

5. Indications of a covalent cross-link between extensin and pectin.

A graduate student in the group has started a project to look for polysaccharide linkages to extensin. He has found that after treating walls with HF at -73°, 80% of the arabinose was solubilized from the walls along with a small amount of pectin. Many previous experiments indicate that at this low temperature, only arabinofuranosyl linkages are cleaved ( ). Much of the arabinose in this linkage is on the hydroxyproline residues of extensin. Prior to the HF treatment, very little of the protein could be released by proteolysis with trypsin. After the HF treatment, about 25% of the hydroxyproline was solubilized by trypsin. At the same time, as the protein was solubilized, a considerable amount of pectin was too. More of the hydroxyproline could be released by subsequent treatments with endopolygalacturonase and cellulase (Worthington). In all cases, pectin became solubilized along with protein. In future experiments, we will be trying to determine if the pectin is covalently linked to the protein, and if it is, how.

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We now have a Dionex Carbohydrate Analysis System set up and have found in our very recent (2 days ago) experiments that as Hotchkiss and Hicks claimed, oligogalacturonides of high (at least 40) degree of polymerization can be separated from each other. Having this capability will allow us to determine how specifically we can actually cleave homogalacturonans at rhamnose residues and thus go on to find the range of lengths of uninterrupted galacturonan sequences. For quantitation in the experiments and others we will try to calibrate the PAD of the Dionex System with known samples labeled with 2-aminopyridine.

We plan to finish and publish our experiments on developing the methods for determining methyl ester distribution in pectins. What remains to be done is to identify all of the peaks we see off a PA-1 column for the fragments we obtain for the -10° HF treatment of the reduced pectin and to calibrate the PAD for these fragments. Identification of the materials in the peaks should be straightforward by NMR and mass spectrometry. We can then apply the methods to our cotton cell wall pectins.

A graduate student in the lab, Mr. Jinhua An, will finish and publish the work on characterizing the xyloglucan of cotton and then proceed to determining the frequency of sidechains on solubilized RGI and the structures of its major sidechains.