STRUCTURAL STUDIES OF COMPLEX CARBOHYDRATES OF PLANT CELL WALLS

Progress Report for the Period
June 15, 1992 - June 14, 1993

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Introduction.

Abstracts of papers published, in press, or in preparation reporting work supported by this grant during the past year follow and summarize the progress made in this research project. [N.B. At the recommendation of the 1989 site visit review committee, this present grant is an amalgamation of two research grants, one on the structure/function of cell walls and the other on methods to determine the structure of complex carbohydrates.]

Publications preceded by an asterisk indicate work also supported by DOE Grant # DE-FG09-85ER13425 (The Structures and Functions of Oligosaccharins); such abstracts are given in that grant's progress report.

1. O'Neill, M.A., P.D. Robison, K.J. Chou, A.G. Darvill, and P. Albersheim. 1992. Evidence that the acidic polysaccharide secreted by *Agrobacterium radiobacter* (ATCC 53271) has a seventeen glycosyl-residue repeating unit. *Carbohydr. Res.* 226:131-154.

The extracellular anionic polysaccharide produced by the bacterium *Agrobacterium radiobacter* (ATCC 53271) contains D-galactose, D-glucose, and pyruvic acid in the molar ratio 2:15:2. Analysis of the methylated polysaccharide indicated the presence of terminal non-reducing glycosyl, 3-, 4-, 6-, 2,4-, and 4,6-linked glucosyl residues, 3-linked 4,6-O-[(S)-1-carboxyethylidene] glucosyl residues, and 3-linked galactosyl residues. Partial acid hydrolysis of the methylated polysaccharide, followed by reduction with NaB²H₄ and then O-ethylation gave a mixture of alkylated oligoglycosyl alditols that were separated by reversed-phase h.p.l.c. and analyzed by ¹H-n.m.r. spectroscopy, g.l.c.-m.s., and glycosyl-linkage composition analysis. Smith degradation of the polysaccharide gave three diglycosyl alditols that were separated by semi-preparative, high-pH anion-exchange chromatography, and were analyzed by ¹H-n.m.r. spectroscopy, g.l.c.-m.s., and glycosyl-linkage composition analysis. The polymer obtained by NaBH₄ reduction of the periodate-oxidized polysaccharide was methylated, and the noncyclic acetals were hydrolyzed with aq. 90% formic acid to generate a mixture of partially O-methylated mono- and diglycosyl alditols. The partially O-methylated oligoglycosyl alditols were O-ethylated. The resulting alkylated oligoglycosyl alditols were separated by reversed-phase h.p.l.c. and then characterized by ¹H-n.m.r. spectroscopy, g.l.c.-m.s., and glycosyl-linkage composition analysis. The results from the studies described here provide strong evidence that the acidic polysaccharide secreted by *A. radiobacter* (ATCC 53271) has a heptadecasaccharide repeating unit.

2. Hisamatsu, M., W.S. York, A.G. Darvill, and P. Albersheim. 1992. Structure of Plant Cell Walls XXXV. Characterization of seven xyloglucan oligosaccharides containing from seventeen to twenty glycosyl residues. *Carbohydr. Res.* 227:45-71.

The complete primary structures of seven oligosaccharide subunits of the xyloglucan secreted by suspension-cultured *Acer pseudoplatanus* cells were determined. The oligosaccharides, ranging in size from 17 to 20 glycosyl residues, were generated by treatment of the

xyloglucan with an *endo*- β -(1 $\rightarrow$ 4)-glucanase. The oligosaccharide components of a fraction obtained by Bio-Gel P-2 chromatography of enzyme-treated xyloglucan were further purified by normal-phase h.p.l.c. and then converted to the corresponding oligoglycosyl alditols by reduction with NaBH₄. The oligoglycosyl alditols, after purification to near homogeneity by reversed-phase h.p.l.c., were structurally characterized by ¹H-n.m.r. spectroscopy, fast-atom bombardment mass spectrometry (f.a.b.-m.s.), and analysis of their glycosyl-residue and glycosyl-linkage compositions. Novel structural elements of xyloglucans were observed in this study, including β -D-xylopyranosyl and α -L-arabinofuranosyl-(1 $\rightarrow$ 3)- β -D-xylopyranosyl sidechains. The results also extend our list of correlations between ¹H-n.m.r. resonances and specific structural features of xyloglucans and thus enhance our ability to determine the structures of xyloglucans from various sources.

- *3. Darvill, A., C. Augur, C. Bergmann, R.W. Carlson, J.-J. Cheong, S. Eberhard, M.G. Hahn, V.-M. L6, V. Marfà, B. Meyer, D. Mohnen, M.A. O'Neill, M.D. Spiro, H. van Halbeek, W.S. York, and P. Albersheim. 1992. Oligosaccharins - oligosaccharides that regulate growth, development and defence responses in plants. *Glycobiology* 2:181-198.
4. Zhang, G.F. and L.A. Staehelin. 1992. Functional compartmentation of the Golgi apparatus of plant cells. Immunocytochemical analysis of high-pressure frozen- and freeze-substituted sycamore maple suspension culture cells. *Plant Physiol.* 99: 1070-1083.

(NOTE: This grant provided the monoclonal antibodies to L.A. Staehelin for these studies as part of our collaborative research.)

The Golgi apparatus of plant cells is engaged in both the processing of glycoproteins and the synthesis of complex polysaccharides. To investigate the compartmentalization of these functions within individual Golgi stacks, we have analyzed the ultrastructure and the immunolabeling patterns of high-pressure frozen and freeze-substituted suspension-cultured sycamore maple (*Acer pseudoplatanus* L.) cells. As a result of the improved structural preservation, three morphological types of Golgi cisternae, designated *cis*, medial, and *trans*, as well as the *trans* Golgi network, could be identified. The number of *cis* cisternae per Golgi stack was found to be fairly constant at approximately 1, whereas the number of medial and *trans* cisternae per stack was variable and accounted for the varying number of cisternae (3-10) among the many Golgi stacks examined. By using a battery of seven antibodies whose specific sugar epitopes on secreted polysaccharides and glycoproteins are known, we have been able to determine in which types of cisternae specific sugars are added to *N*-linked glycans, and to xyloglucan and polygalacturonic acid/rhamnogalacturonan-I, two complex polysaccharides. The findings are as follows. The β -1,4-linked D-glucosyl backbone of xyloglucan is synthesized in *trans* cisternae, and the terminal fucosyl residues on the trisaccharide side chains of xyloglucan are partly added in the *trans* cisternae, and partly in the *trans* Golgi network. In contrast, the polygalacturonic/rhamnogalacturonan-I backbone is assembled in *cis* and medial cisternae, methylesterification of the carboxyl groups of the galacturonic acid residues in the polygalacturonic acid domains occurs mostly in medial cisternae, and arabinose-containing side chains of the polygalacturonic acid domains are added to the nascent polygalacturonic acid/rhamnogalacturonan-I molecules in the *trans* cisternae. Double labeling experiments demonstrate that xyloglucan and polygalacturonic acid/rhamnogalacturonan-I can be synthesized concomitantly within the same Golgi stack. Finally, we show that the xylosyl residue-linked β -1,2 to the β -linked mannose of the core of *N*-linked glycans is added in medial cisternae. Taken together, our results indicate that in sycamore maple suspension-cultured

cells, different types of Golgi cisternae contain different sets of glycosyl transferases, that the functional organization of the biosynthetic pathways of complex polysaccharides is consistent with these molecules being processed in a *cis* to *trans* direction like the *N*-linked glycans, and that the complex polysaccharide xyloglucan is assembled exclusively in *trans* Golgi cisternae and the *trans* Golgi network.

- * 5. Hahn, M.G., A. Darvill, P. Albersheim, C. Bergmann, J.-J. Cheong, A. Koller, V.-M. L6. 1992. Preparation and characterization of oligosaccharide elicitors of phytoalexin accumulation. In: *Molecular Plant Pathology (Vol. 2): A Practical Approach* (S. Gurr, M. McPherson, D.J. Bowles, eds.). IRL Press, pp. 103-147.
6. Lerouge, P., M.A. O'Neill, A.G. Darvill, and P. Albersheim. 1993. The purification of commercially available *endo*- α -L-arabinanase and α -L-arabinosidase for use in the structural analysis of pectic polysaccharides. *Carbohydr. Res.* 243:373-378.

Commercially available glycanases have considerable potential for use in the structural analysis of complex carbohydrates. However, these preparations often contain mixtures of both *endo*- and *exoglycanases* that can limit their use in structural studies. For example, we have found that arabinosyl-containing oligosaccharides released by treating rhamnogalacturonan I (RG-I), a plant cell wall pectic polysaccharide, with a commercially available fungal *endo*- α -1,5-L-arabinanase, were hydrolyzed by a contaminating α -L-arabinofuranosidase. Fungal arabinases have been purified to apparent homogeneity by fast protein liquid chromatography (FPLC) although their use for the fragmentation of plant cell wall polysaccharides was not reported. We now report that both an *endo*arabinanase and an α -L-arabinosidase can be obtained, by FPLC, in a level of purity suitable for the structural analysis of RG-I and arabinans. The activities of the *endo*arabinanase and arabinosidase were monitored colorimetrically and by using a method based on high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD).

7. Lerouge, P., M.A. O'Neill, A.G. Darvill, and P. Albersheim. 1993. Structural characterization of *endoglycanase*-generated oligoglycosyl side chains of rhamnogalacturonan I. *Carbohydr. Res.* 243:359-371.

Rhamnogalacturonan I (RG-I) has been isolated from the walls of suspension-cultured sycamore cells (*Acer pseudoplatanus*), and additional structural features of the polysaccharide elucidated has been further structurally characterized. Treatment of RG-I with a purified *endo*-1,5- α -L-arabinanase released a series of arabinose-containing oligosaccharides with degrees of polymerization (DPs) between 2 and 20. These oligosaccharides were shown, by glycosyl-linkage composition analysis, to contain terminal, 5-, and 3,5-linked *Araf* residues. These results provide evidence that a branched arabinan is attached to the backbone of RG-I. RG-I was freed of 95% of its arabinosyl residues by treating the polysaccharide with a combination of *endo*-1,5- α -L-arabinanase and α -L-arabinosidase. No galacturonic acid was released by these enzymes, which is evidence that the arabinosyl-containing portions of the side chains do not contain galactosyluronic acid. The galactose-containing portions of the side chains of RG-I were not fragmented by an *endo*-1,4- β -D-galactanase. However, approximately 85% of the galactose and small amounts of galacturonic acid were released by digestion of arabinose-depleted RG-I with a combination of *endo*- and *exo*- β -D-galactanases. The galacturonic acid may have been released by small amounts of an *exo*- α -galactosyluronidase contaminating the galactanases. Treatment of RG-I with this mixture of *endo*- and *exoglycanases* resulted in a relatively size-homogeneous, almost side chain-free, backbone composed of the *O*-acetylated diglycosyl repeating unit -4)- α -D-GalpA-(1,2)- α -L-Rhap. A combination of ^1H -n.m.r. spectroscopy

copy and periodate oxidation established that the repeating unit contained a single *O*-acetyl substituent on C-2 or C-3 of the galactosyluronic acid residue.

8. Spiro, M.D., K.A. Kates, A.L. Koller, M.A. O'Neill, P. Albersheim, and A.G. Darvill. 1993. Purification and characterization of biologically active 1,4-linked α -D-oligogalacturonides after partial digestion of polygalacturonic acid with *endopolygalacturonase*. *Carbohydr. Res.* 247:9-20.

A procedure is described for generating and purifying biologically active (1 $\rightarrow$ 4)-linked α -D-oligogalacturonides (oligogalacturonides). Oligogalacturonides are generated by treatment of polygalacturonic acid (PGA) with a homogeneous fungal α -(1 $\rightarrow$ 4)-endopolygalacturonase (EPG). Oligogalacturonides with a dp greater than seven were selectively precipitated in the presence of sodium acetate and ethanol. Oligogalacturonides with a dp less than eleven and modified oligogalacturonides remained soluble. Oligogalacturonides with dp between 10 and 15 were purified from the resolubilized NaOAc/ethanol-precipitated material using Q-Sepharose fast-flow anion-exchange chromatography followed by semipreparative high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Approximately 70 mg of each homogeneous oligogalacturonide (dp between 10 and 15) was obtained from 10 g of EPG-treated PGA. The tridecagalacturonide fraction was selected for chemical and structural characterization and was shown to be homogeneous by glycosyl-residue composition analysis, HPAEC-PAD, FABMS, and ^1H NMR spectroscopy. The purified tridecagalacturonide elicited phytoalexin accumulation in soybeans and induced flower formation and inhibited root formation in tobacco thin-cell-layer explants.

9. York, W.S., L.K. Harvey, R. Guillen, P. Albersheim, and A.G. Darvill. 1993. Structural analysis of tamarind seed xyloglucan oligosaccharides using β -galactosidase digestion and spectroscopic methods. *Carbohydr. Res.* In press.

The borohydride-reduced forms (oligoglycosyl alditols) of two isomeric octasaccharides ($\text{Glc}_4\text{Xyl}_3\text{Gal}$) that are released from xyloglucans of various plant species upon treatment with a fungal *endo*-(1 $\rightarrow$ 4)- β -glucanase were isolated and structurally characterized. A mixture of oligosaccharides that is released from tamarind seed xyloglucan by the *endo*-(1 $\rightarrow$ 4)- β -glucanase was digested with a commercially available β -galactosidase (*Aspergillus niger*). The β -galactosidase selectively hydrolyzed the galactosyl residue of one of the two isomeric octasaccharides present in the mixture. A homogeneous preparation of the β -galactosidase-resistant octasaccharide was prepared by high-resolution gel-permeation chromatography of the enzyme-digestion products. Spectroscopic characterization of the oligoglycosyl alditol prepared by reduction of this octasaccharide confirmed the previously proposed structure that had been based on analysis of the mixture of isomeric octasaccharides. The availability of large amounts of the pure, reduced octasaccharide and of a pure, reduced pentasaccharide (Glc_3Xyl_2) made it possible to completely assign their ^1H and ^{13}C NMR spectra. In addition, the borohydride-reduced form of the β -D-galactosidase-susceptible octasaccharide isomer was purified by high pH anion-exchange chromatography of the *endo*-(1 $\rightarrow$ 4)- β -glucanase-released octasaccharides from rapeseed xyloglucan (no β -galactosidase treatment), and its ^1H and ^{13}C NMR spectra were assigned. Additional correlations between specific structural features of xyloglucan oligoglycosyl alditols and the positions of specific resonances in their NMR spectra were deduced and added to the extensive list that we have compiled. The effects of recording the NMR spectra of the xyloglucan oligoglycosyl alditols in the presence of borate salts, which could lead to incorrect structural assignments, are also described.

10. York, W.S., J.U. Thomsen, and B. Meyer. 1993. The conformations of cyclic β -(1 $\rightarrow$ 2)-D-glucans: Application of multidimensional clustering analysis to conformational data sets obtained by Metropolis Monte Carlo calculations. *Carbohydr. Res.* In press.

Sets containing up to 1.3×10^6 energetically accessible conformations of linear (1 $\rightarrow$ 2)- β -D-glucan oligosaccharides were obtained by Metropolis Monte Carlo (MMC) calculations performed with the GEGOP (GEometry of GlycOProteins) program. Quantitative analyses of the data sets (which were expressed in terms of the glycosidic dihedral angle coordinates) were obtained by two different clustering methods: (i) the three-distance hierarchical clustering method (3-DM), published by Jure Zupan, and (ii) a nonhierarchical clustering method (Population-Density Projection, PDP) which, through a segmentation analysis of two-dimensional projections of the population-density surface, establishes a partitioning of conformational space into a set of "cluster regions", followed by a clustering step where each conformation of the data set is assigned to one of these regions. Computer programs (MCLUST and PDPCLUST) were developed to perform the 3-DM and PDP analyses, respectively. The two types of analysis provided very similar sets of conformational families (clusters), which could be expressed as combinations of distinct conformations of the glycosidic torsional angles (ϕ , ψ) centered at (50°, 10°) for conformation A, (40°, 160°) for conformation B, (55°, -160°) for conformation B', and (170°, 10°) for conformation C. The analysis provided the populations of the families, along with relative rates for transitions between families. Examination of the frequencies of the A, B, and C glycosidic bond conformations with respect to their relative positions in the sequence revealed the tendency of the (1 $\rightarrow$ 2)- β -D-glucan to adopt conformational repeating structures of the general form $[A_nB]$, where $n = 3$ or 6. These repeating structures combine in an energetically cooperative fashion to give low-energy cyclic conformations having, for example, C_5 symmetry $[AAAB]_5$ for the eicosamer, and C_3 symmetry $[AAAAAAB]_3$ for the heneicosamer.

11. York, W.S., G. Impallomeni, M. Hisamatsu, P. Albersheim, and A.G. Darvill. 1993. Eleven newly characterized xyloglucan oligoglycosyl alditols: the specific effects of sidechain structure and location on ^1H NMR chemical shifts. In preparation.

Eleven previously uncharacterized oligosaccharides, each containing from seventeen to twenty residues, were isolated from the xyloglucan produced by suspension-cultured *Acer pseudoplatanus* cells and characterized by ^1H NMR spectroscopy, fast atom bombardment mass spectrometry, and matrix-assisted laser-desorption mass spectrometry. The complex mixture of xyloglucan oligosaccharides released by *endo*-(1 $\rightarrow$ 4)- β -glucanase (*Trichoderma reesei*) treatment of cell walls was similar to that released by digestion of the soluble xyloglucan present in the culture medium. The oligosaccharides were converted to oligoglycosyl alditols by borohydride reduction and purified by a combination of gel-permeation (Bio-Gel P-2) chromatography, normal-phase HPLC, reversed-phase HPLC, and high-performance anion-exchange (HPAE) chromatography. Eleven new oligoglycosyl alditols (along with several others that had been previously characterized) were isolated and characterized, allowing additional correlations between xyloglucan structure and specific chemical shift effects in the ^1H NMR spectra to be determined. The correlations between structural and spectral features deduced in this study will facilitate the structural determination of a wide range of xyloglucans and their subunit oligosaccharides.

12. Puhlmann, J., E. Bucheli, M.J. Swain, N. Dunning, P. Albersheim, A. Darvill, and M.G. Hahn. 1993. Generation of monoclonal antibodies against plant cell-wall polysaccharides. I. Characterization of a monoclonal antibody to a terminal α -(1 $\rightarrow$ 2)-linked fucosyl-containing epitope. In preparation.

Monoclonal antibodies (McAbs) generated against rhamnogalacturonan I (RG-I) purified from suspension-cultured sycamore maple (*Acer pseudoplatanus*) cells fall into three recognition groups. Four McAbs (group I) recognize an epitope that appears to be immunodominant and is present on RG-I from maize and sycamore maple, pectin and polygalacturonic acid from citrus, gum tragacanth, and membrane glycoproteins from suspension-cultured cells of maize, tobacco, parsley, bean, and sycamore maple. A second set of McAbs (group II) recognizes an epitope present in sycamore maple RG-I but does not bind to any of the other polysaccharides or glycoproteins recognized by group I. Lastly, one McAb, CCRC-M1 (group III), binds to RG-I and more strongly to xyloglucan (XG) from sycamore maple but not to maize RG-I, citrus polygalacturonic acid, or to the plant membrane glycoproteins recognized by group I. The epitope to which CCRC-M1 binds has been examined in detail. Ligand competition assays using a series of oligosaccharides derived from or related to sycamore maple XG demonstrated that a terminal α -(1 $\rightarrow$ 2)-linked fucosyl residue constitutes an essential part of the epitope recognized by CCRC-M1. Oligosaccharides containing this structural motif compete with intact sycamore maple XG for binding to the antibody, whereas structurally related oligosaccharides, which do not contain terminal fucosyl residues or in which the terminal fucosyl residue is linked α -(1 $\rightarrow$ 3) to the adjacent glycosyl residue, do not compete for the antibody binding site. The ligand binding assays also indicate that CCRC-M1 binds to a conformationally dependent structure of the polysaccharide. Other results of this study establish that some of the carbohydrate epitopes of the plant extracellular matrix are shared among different macromolecules.

13. Zablackis, E., J. Huang, B. Müller, A.G. Darvill, and P. Albersheim. 1993. Characterization of the cell wall polysaccharides of *Arabidopsis thaliana* leaves. In preparation.

The cell wall polysaccharides of *Arabidopsis thaliana* have been isolated, purified and characterized. The primary cell walls of all higher plants that have been studied are composed of cellulose, the three pectic polysaccharides homogalacturonan, RG-I and RG-II, the two hemicelluloses xyloglucan and glucuronoarabinoxylan, and structural glycoproteins. The cell walls of *Arabidopsis* leaves contain each of these components and no others that we could detect. The cell walls of *Arabidopsis* leaves are remarkable in that they are particularly rich in water-soluble polysaccharides (34% of the wall). The pectic polysaccharides consist of RG-I (11%), RG-II (8%), and homogalacturonan (23%). Xyloglucan accounts for 20% of the wall and the oligosaccharide fragments generated from xyloglucan by endoglucanase consist of the typical subunits of other higher plant xyloglucan. Glucuronoarabinoxylan (4%), cellulose (14%) and protein (14%) account for the remainder of the wall. Except for the water-soluble pectic polysaccharides, the polysaccharides of *Arabidopsis* leaf cell walls occur in proportions similar to those of other plants. The structures of the *Arabidopsis* cell wall polysaccharides are typical of those of other plants.

14. An, J., M.A. O'Neill, R. Guillen, A.G. Darvill, and P. Albersheim. 1993. Isolation and partial characterization of rhamnogalacturonan I from the stem, leaf, and root of light-grown and etiolated peas. In preparation.

Rhamnogalacturonan I (RG-I), a pectic polysaccharide, has been solubilized from stem, root, and leaf cell walls of light- and dark-grown pea plants by treatment with a fungal *endo*-polygalacturonase. The glycosyl-residue and glycosyl-linkage compositions of RG-I from each type of tissue have been determined. The oligoglycosyl alditols released by lithium fragmentation of each RG-I preparation were compared using a method based on high-performance anion-exchange chromatography with pulsed amperometric detection. The combined results of these studies have shown: (1) RG-I isolated from light-grown pea stems and leaves contains more arabinosyl residues and less galactosyl residues than RG-I isolated from etiolated pea tissue, (2) no major structural differences were detected in RG-I isolated from the roots of both light- and dark-grown plants, (3) the backbone of RG-I isolated from light-grown tissue contains fewer branched rhamnosyl residues than the backbone of RG-I isolated from etiolated pea tissue, (4) RG-I isolated from etiolated pea tissue contains shorter arabinosyl side chains and longer galactosyl side chains than light-grown RG-I, and (5) RG-I isolated from etiolated pea stem cell walls and root cell walls are structurally similar. These results provide strong evidence that light plays an important role in regulating the structure of a cell wall pectic polysaccharide.