PROGRESS REPORT

STRUCTURAL STUDIES OF COMPLEX CARBOHYDRATES OF PLANT CELL WALLS

December 15, 1989 - December 14, 1990

Year 2 Continuation

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Progress Report

December 15, 1989-December 14, 1990

Introduction.

Abstracts of papers published or in press reporting work supported by this grant proposal during the past year are given here to describe the progress we have made in this research project.

Hahn, M.T., P. Bucheli, F. Cervone, S.H. Doares, R.A. O'Neill, A. Darvill, and P. Albersheim. 1989. The roles of cell wall constituents in plant-pathogen interactions. In: *Plant-Microbe Interactions*, Vol. 3 (E. Nester and T. Kosuge, eds.) McGraw-Hill, pp. 131-181.

Pathogens find themselves most commonly in the presence of plants other than their hosts. Under such circumstances, a pathogen fails in its efforts to initiate infection. What is it that renders a plant's environment inhospitable to a pathogen except in the rare instance when the plant happens to be a susceptible host? It is this question that we propose to answer by our hypothesis that, in many instances of pathogenesis by bacteria or fungi, it is an interaction between the pathogen and the carbohydrates of the host that determines the pathogen's ability to produce enzymes capable of degrading the host's cell walls.

Kiefer, L. W. York, A. Darvill, and P. Albersheim. 1989. Structure of plant cell walls XXVII. Xyloglucan isolated from suspension-cultured sycamore cell walls is O-acetylated. *Phytochem.* 28:2105-2107.

Xyloglucan oligosaccharides were enzymically released from purified cell walls isolated from suspension-cultured sycamore cells. The O-acetyl substitution pattern of these oligosaccharides was determined by ¹H-NMR spectroscopy. This analysis revealed that most of the 2-linked galactosyl residues of the decasaccharide and nonasaccharide fragments of cell wall xyloglucan are O-acetyl substituted, while the heptasaccharide fragment does not contain O-acetyl substituents. This acetylation pattern does not differ significantly from that of the same xyloglucan oligosaccharides enzymically released from the xyloglucan secreted by suspension-cultured sycamore cells.

Darvill, A.G., P. Albersheim, P. Bucheli, S. Doares, N. Doubrava, S. Eberhard, D.J. Gollin, M.G. Hahn, V. Marfà-Riera, W.S. York, and D. Mohnen. 1989. Oligosaccharins -- plant regulatory molecules In: *Signal Molecules in Plants and Plant-Microbe Interactions* (B.J.J. Lugtenberg, ed.) NATO ASI Series, Vol. H36. Springer-Verlag, Heidelberg, pp. 41-48.

The discoveries that complex carbohydrates are tissue-specific cell-surface antigens and the receptors for hormones, toxins, bacteria, and viruses have created considerable interest in complex carbohydrates. Furthermore, the complex carbohydrate portions of some glycoprotein hormones are required for their biological activity, and the complex carbohydrates of some glycoprotein enzymes keep the enzymes stable, while the carbohydrates of some glycoproteins direct the glycoproteins to their proper locations. Moreover, the immune response to glycoproteins is often directed to their carbohydrate side chains, and the carbohydrate chains have been shown to affect the activity and residence time of glycoprotein pharmaceuticals. Still another discovery that has generated considerable interest in complex carbohydrate research is that these molecules perform regulatory functions in plants and animals, often at the level of controlling gene expression.

Our laboratory has long pursued two seemingly unrelated lines of research -- one, elucidating the structure and function of the matrix polysaccharides of the walls of growing plant cells, and the other studying how plants defend themselves against disease. The combined results from these projects led to our discovery that structurally defined fragments of plant cell wall polysaccharides are able to function in plants as chemical messages with specific regulatory properties. We call oligosaccharides with regulatory activity *oligosaccharins*. We have determined that oligosaccharins not only trigger plant defenses against pathogens and other types of stress, but also are able to regulate the rate of growth and differentiation of plant tissues into roots, flowers, and vegetative buds.

The first characterized oligosaccharins were those that are commonly referred to as elicitors of defense responses in plants. The best oligogalacturonides are effective inhibitors of root formation and the oligogalacturonides can control the location on the explants where roots form. Thus, it is becoming apparent that oligogalacturonides of specific size are extremely important regulators of plant growth and development; oligogalacturonides may have as many functions as any of the classical phytohormones. Indeed, we believe that oligogalacturonides may be as important and as effective in the control of plant growth and development as auxin, cytokinin, gibberellin, abscisic acid, or ethylene. As with the phytohormones, it remains to be determined how oligogalacturonides control growth and development. Nevertheless, oligogalacturonides are very important regulatory molecules of plants.

Thomsen, J.U. and B. Meyer. 1989. Pattern recognition of the ^1H NMR spectra of sugar alditols using a neural network. *J. Magn. Res.* 84:212-217.

Artificial neural systems (ANS), or neural networks, have produced interesting examples of pattern recognition in several areas of science. To our knowledge, neural networks have not yet been applied in recognition of

NMR spectra. We have implemented a software emulation of a three-layered feed-forward neural network as described by Rumelhart et al. to explore the value of ANS in NMR spectroscopy.

O'Neill, M., P. Albersheim, and A. Darvill. 1990. The pectic polysaccharides of primary cell walls. In: *Methods in Plant Biochemistry*, Vol. 2 (D.M. Dey, ed.) London, Academic Press, pp. 415-441.

Three - and to date only three - pectic polysaccharides have been isolated from the primary cell walls of plants and characterized. These are homogalacturonan, rhamnogalacturonan I (RG-I), and a substituted galacturonan referred to as rhamnogalacturonan II (RG-II). Homogalacturonan is a chain of 1,4-linked α -D-galactosyluronic acid residues in which some (perhaps most) of the carboxyl groups are methyl esterified. RG-I is a family of closely related polysaccharides that contain a backbone of the alternating disaccharide $\rightarrow 4$)- α -D-GalpA-(1 $\rightarrow$ 2)- α -L-Rhap-(1 $\rightarrow$). About 50% of the rhamnosyl residues are substituted at C-4 with neutral, and perhaps acidic, oligosaccharides. RG-II is a polysaccharide composed of a 1,4-linked α -D-galactosyluronic acid backbone with aldehydo- and keto-sugar oligosaccharide side chains attached to C-2 and/or C-3. Some of the glycosyl residues of the pectic polysaccharides are O-acetylated, but, in general, the location of the acetyl substituents has not been determined. However, RG-I is known to be O-acetylated on C-3 of some of the backbone galactosyluronic acid residues.

Recent developments in the study of cell-wall pectic polysaccharides, in addition to elucidating new structural features, have emphasized the importance of their biological regulatory functions and physical properties. The heightened interest in structure-function relationships has led to: (1) greater awareness of the problems associated with isolating these complex macromolecules without altering their structures, (2) improved purification procedures for the preparation of homogeneous polysaccharides, and (3) refinements in established analytical procedures and development of new procedures to facilitate the structural characterization of the polysaccharides.

Kiefer, L., W.S. York, P. Albersheim, and A.G. Darvill. 1990. Structural characterization of an arabinose-containing heptadecasaccharide enzymically isolated from sycamore extracellular xyloglucan. *Carbohydr. Res.* 197:139-158.

An arabinose-containing heptadecasaccharide, generated by endoglucanase digestion of the xyloglucan secreted by suspension-cultured sycamore cells, was structurally characterized. The structure of the heptadecasaccharide was unambiguously determined through combined data generated by ^1H -n.m.r. spectroscopic, fast-atom bombardment mass spectrometric, and glycosyl-composition and glycosyl-linkage analyses of the intact heptadecasaccharide and of oligosaccharide fragments of the heptadecasaccharide. 1D and 2D ^1H -n.m.r. analyses provided data for assignment of the anomeric configurations of the glycosidic linkages, as well as information about the glycosyl and linkage compositions of the heptadecasaccharide. F.a.b.-m.s. data provided the molecular weight and supplied critical information about the glycosyl composition and glycosyl sequence of the heptadecasaccharide. The

heptadecasaccharide was found to be a combination of previously characterized nona- and hepta-saccharide components of xyloglucan. The nonasaccharide was shown to be glycosidically linked to the heptasacchride through C-4 of the β -glucosyl residue nearest to the nonreducing end of the heptasaccharide component. An arabinosyl residue was glycosidically linked at C-2 of the same β -glucosyl residue at the non-reducing end of the heptasaccharide component of the heptadecasaccharide. Although the presence of arabinosyl residues in sycamore xyloglucan has been recognized since 1973, the location of the arabinosyl residues had not been ascertained.

O'Neill, R.A., P. Albersheim, and A.G. Darvill. 1990. Purification and characterization of a xyloglucan oligosaccharide-specific xylosidase from pea seedlings. *J. Biol. Chem.* 264(34):20430-20437.

An α -xylosidase that acts on oligosaccharide fragments of xyloglucan, a plant cell wall polysaccharide, was purified from pea (*Pisum sativum*) epicotyls that had been treated with an auxin analog. The enzyme had an apparent molecular mass of 85,000 Da according to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 79,000 Da according to gel-permeation chromatography under nondenaturing conditions. The purified xylosidase consisted of a series of closely related, enzymatically active proteins with isoelectric points ranging from pH 7.35 to 7.7; the xylosidases were separated by chromatofocusing. The pH optimum of the mixed xylosidase was 4.9-5.1. The substrate specificity of the xylosidase mixture was determined by purification and structural characterization of the products of treating xyloglucan-oligosaccharide substrates with the enzyme. Characterization of the substrates and products included elution volume from a gel-permeation column, glycosyl residue and glycosyl linkage composition analyses, fast atom bombardment-mass spectrometry, and $^1\text{H-NMR}$ spectroscopy. The enzyme specifically cleaved only one of the α -xylosidic linkages of xyloglucan-oligosaccharide substrates, the one attached to a 6-linked glucosyl residue, not those attached to the 4,6-linked glucosyl residues. The enzyme was unable to cleave the xylosidic linkage of *p*-nitrophenyl- α -D-xylopyranoside or the α -xylosidic linkage to C-6 of glucose in the disaccharide isoprimeverose. The enzyme was also unable to release measurable amounts of xylose from large xyloglucan polymers.

York, W.S., H. van Halbeek, A.G. Darvill, and P. Albersheim. 1990. Structural analysis of xyloglucan oligosaccharides by $^1\text{H-n.m.r.}$ spectroscopy and fast atom bombardment mass spectrometry. *Carbohydr. Res.* 200:9-31.

A method to determine rapidly the identities and proportions of the oligosaccharide repeating-units in plant cell-wall xyloglucans by 1D $^1\text{H-n.m.r.}$ spectroscopy was developed. Six of the most commonly found xyloglucan oligosaccharide subunits (including three subunits that had not been fully characterized previously) were prepared by endo-(1 $\rightarrow$ 4)- β -D-glucanase digestion of xyloglucans from various plant species. The oligosaccharides were reduced to the corresponding oligoglycosyl-alditols, purified, and characterized by glycosyl composition and linkage analysis, $^1\text{H-n.m.r.}$ spectroscopy, and f.a.b.-mass spectroscopy. Correlations between the $^1\text{H-n.m.r.}$ spectra and the structures of the oligoglycosyl-alditols can be used to identify oligoglycosyl-alditols derived from xyloglucans of unknown structure. The

identities and relative amounts of the oligosaccharide subunits of xyloglucans isolated from tamarind seed and rapeseed hulls were determined on this basis.

Von Deyn, W., W.S. York., P. Albersheim, and A. Darvill. 1990. 1-Alkoxyamino-1-deoxy alditols, useful u.v.-absorbing derivatives of neutral and acidic oligosaccharides. *Carbohydr. Res.* 201:135-144.

The feasibility of using 1-alkoxyamino-1-deoxy alditol derivatives to facilitate the purification and structural characterization of oligosaccharides was examined. 1-Benzyloxyamino-1-deoxy, 1-deoxy-1-*p*-nitrobenzyloxyamino, and 1-deoxy-1-[4-(*p*-nitrophenyl)butoxyamino] alditol derivatives of mono- and oligosaccharides were prepared by reduction of the corresponding *O*-substituted oximes with sodium cyanoborohydride. The 1-alkoxyamino-1-deoxy alditol derivatives were separated by h.p.l.c. and characterized by ¹H-n.m.r. spectroscopy and f.a.b.-m.s. Per-*O*-methylation of the 1-benzyloxyamino-1-deoxy and 1-deoxy-1-*p*-nitrobenzyloxyamino alditol derivatives resulted in elimination of a benzaldehyde moiety and formation of a quaternary ammonium salt. The positive mode f.a.b.-mass spectra of these cationic per-*O*-methylated derivatives of oligosaccharides included very intense molecular ion signals, suggesting the possibility of a significant reduction in the amount of per-*O*-methylated oligosaccharide required for f.a.b.-m.s. analysis. Because the ionic nature of the derivatives hinders their analysis by g.c.-m.s., a new reagent, 4-(*p*-nitrophenyl)butoxyamine, was synthesized and used to prepare 1-deoxy-1-[4-(*p*-nitrophenyl)butoxyamino] alditol derivatives of several mono- and oligosaccharides. These could be separated by h.p.l.c., with detection by u.v. absorption, and they were stable under *O*-methylation conditions. The per-*O*-methylated derivatives of oligosaccharides can be analyzed by e.i.-m.s. as well as f.a.b.-m.s. *O*-Methylated 1-deoxy-1-[4-(*p*-nitrophenyl)butoxyamino] alditol derivatives of monosaccharides are amenable to processing by g.c.-m.s., allowing standard methylation analysis of derivatized oligosaccharides to be performed. Thus, the separation and structural analysis of oligosaccharides are facilitated by the physical and chemical properties (u.v. absorption, ease of separation by h.p.l.c., stability to methylation and hydrolysis conditions, and ease of analysis by g.c.-m.s. and f.a.b.-m.s.) imparted to oligosaccharides by forming the 1-deoxy-1-[4-(*p*-nitrophenyl)butoxyamino] alditol derivatives.

Hahn, M.G., A. Darvill., P. Albersheim, C. Bergmann, J.-J. Cheong, A. Koller, V.-M. L. 1990. Preparation and characterization of oligosaccharide elicitors of phytoalexin accumulation In: *Molecular Plant Pathology: A Practical Approach*, (D.J. Bowles, ed.), IRL Press. In press.

Plants utilize a variety of defense responses when confronted with invasive microorganisms. These defense responses include such diverse mechanisms as the synthesis and accumulation of low molecular weight anti-microbial compounds called phytoalexins, the production of glycosyl hydrolases capable of attacking microbial surface polymers, the synthesis of proteins that inhibit microbial degradative enzymes, and the modification of plant cell walls by deposition of callose, hydroxyproline-rich glycoproteins, and/or lignin.

Biochemical analysis of the induction of plant defense responses has been facilitated by the recognition that cell-free extracts of microbial and plant origin are capable of inducing defense responses when applied to plant tissues. The active components in the extracts are commonly referred to as "elicitors". The term "elicitor" was originally used to refer to molecules and other stimuli that induce phytoalexin synthesis in plant cells, but the term is now used to describe molecules that elicit any of the observed plant defense responses.

Crude preparations of biotic elicitors have been prepared from a variety of sources. Elicitors that have been purified to apparent homogeneity include oligosaccharide fragments of fungal and plant cell wall polysaccharides, and fungal cell surface lipids. A number of elicitor-active polypeptides of fungal and bacterial origin have also been purified and partially characterized. Several of these have been shown to be glycosyl hydrolases that release elicitor-active oligosaccharides from plant cell walls. Other elicitor-active polypeptides have not (yet) been shown to have enzymatic activities. Oligosaccharide elicitors that derive from plant and fungal cell wall polysaccharides are the best-characterized elicitors. Their purification and characterization will be described in detail in this chapter.

York, W.S., L.L. Kiefer, A.G. Darvill, and P. Albersheim. 1990. Oxidation of oligoglycosyl alditols during methylation catalyzed by sodium hydroxide and iodomethane in methyl sulfoxide. *Carbohydr. Res.* In press.

Certain artifacts in the glycosyl-linkage analysis of oligoglycosyl alditols were shown to be caused by oxidation of the alditol moieties when *O*-methylation was catalyzed by NaOH in Me₂SO. The partially methylated alditol acetates derived from 1-[²H]-cellobiitol that was *O*-methylated by this method were analyzed in detail to confirm this conclusion.

Meyer, B., T. Hansen, D. Nute, P. Albersheim, A. Darvill, W. York, and J. Sellers. 1990. Artificial neural networks identify the ¹H-NMR spectra of complex oligosaccharides. *Carbohydr. Res.* In press.

We have found that artificial neural networks are able to identify ¹H-NMR spectra of complex oligosaccharides. Feed-forward neural networks with back-propagation of errors are able to distinguish between spectra of oligosaccharides that differ by only one glycosyl residue in twenty. The artificial neural networks use features of the strongly overlapping region of the spectra (hump region) as well as features of the resolved regions of the spectra (structural reporter groups) to recognize spectra. The artificial neural networks efficiently recognize ¹H-NMR spectra even when the spectra are perturbed by minor variations in their chemical shifts. Identification of spectra by neural network-based pattern recognition techniques requires less than one second. We anticipate that artificial neural networks can be used to identify the structures of any complex carbohydrate that has been previously characterized and for which a ¹H-NMR spectrum is available.

Hisamatsu, M., G. Impallomeni, W.S. York, P. Albersheim, and A.G. Darvill. 1990. A new undecasaccharide subunit of xyloglucans with two α -fucosyl residues. Submitted.

A novel oligosaccharide subunit of xyloglucan was isolated from the β -(1 $\rightarrow$ 4)-endoglucanase-digestion products of the xyloglucan in "sycamore extracellular polysaccharides" (SEPS) and found to be an undecasaccharide having two terminal α -L-fucopyranosyl residues. The undecasaccharide was structurally characterized by ^1H -n.m.r. spectroscopy, fast atom bombardment mass spectroscopy (f.a.b.-m.s.), and glycosyl-residue and glycosyl-linkage composition analyses. The structure of the undecasaccharide was confirmed by digesting it with a hydrolase that releases isoprimeverose from the non-reducing end of xyloglucan oligosaccharides.

Doares, S.H., P. Albersheim, and A.G. Darvill. 1990. An improved method for the preparation of standards for glycosyl-linkage analysis of complex carbohydrates. Submitted.

Glycosyl-linkage analysis (*i.e.*, methylation analysis) is a widely-used procedure for determining the glycosyl-linkage composition of carbohydrates. Glycosyl linkages are deduced from the electron impact-mass spectra of partially *O*-methylated, partially *O*-acetylated alditol derivatives. While the various epimers of a given partially *O*-methylated, partially *O*-acetylated alditol (*e.g.*, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl glucitol vs 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl galactitol) give nearly identical mass spectra, they can generally be separated by g.c. Thus, complete assignment of the identities of the peaks obtained during g.c.-m.s. of partially *O*-methylated, partially *O*-acetylated alditols obtained from an unknown sample relies on the availability of appropriate g.c. standards.

Procedures have been developed for the preparation of the required standards for glycosyl-linkage analysis; these protocols involve intentional under-methylation of methyl glycosides, alditols, or aldoses. The mixture of partially methylated aldoses or methyl glycosides is then typically separated from other reactants and products by reversed-phase chromatography on C-18 cartridges or by partitioning into chloroform:methanol. The sample is then reduced with sodium borodeuteride (following acid-catalyzed hydrolysis in the case of methyl glycosides) and *O*-acetylated to form a mixture of partially methylated, partially acetylated alditols. These procedures work well for the tetra-*O*-methyl derivatives of hexoses and for the tri-*O*-methyl derivatives of deoxyhexoses and pentoses but produce poor yields of those derivatives carrying fewer *O*-methyl groups; we have found that the methyl glycosides in a mixture that are only partially *O*-methylated do not bind efficiently to a reversed-phase cartridge or efficiently extract into chloroform:methanol, due to their more polar nature. Thus, those procedures are strongly biased towards the recovery of partially *O*-methylated, partially *O*-acetylated alditols corresponding to terminal residues, with limited recoveries of the derivatives corresponding to linear glycosyl residues, and essentially no recovery of the derivatives corresponding to branched glycosyl residues.

We describe here a simple procedure that yields a mixture of all possible partially *O*-methylated, partially *O*-acetylated alditols that can be formed from a given methyl glycoside. In this procedure, a partially methylated methyl glycoside is *O*-acetylated (*i.e.*, immediately following the

under methylation step) to yield a mixture of partially O-methylated, partially O-acetylated methyl glycosides. The O-acetylation ensures that all components of the mixture are extracted into dichloromethane. The samples are extracted into dichloromethane then hydrolyzed, deuterium-reduced, and re-acetylated. The resulting mixture contains roughly equivalent amounts of each of the possible partially O-methylated, partially O-acetylated alditols theoretically obtainable from the methyl glycoside.

Sellers, J., W. York, P. Albersheim, A. Darvill, and B. Meyer. 1990. Identification of the mass spectra of partially methylated alditol acetates by artificial neural networks. Submitted.

Partially methylated alditol acetates (PMAAs) are used as key glycosyl-residue derivatives in the structural characterization of oligo- and polysaccharides. These derivatives are most readily separated by gas chromatography and identified by electron impact mass spectroscopy. Artificial neural networks were created, originally, to model biological neural networks. Continued development of the underlying mathematical processes have produced networks that can be trained to respond to specific inputs by activating the corresponding output neurons. We have used the mass spectra of the PMAAs of xylitol, arabinitol, rhamnitol and fucitol to determine whether artificial neural networks can identify mass spectra. Previous work in this laboratory has shown that artificial neural networks are capable of recognizing one-dimensional ^1H -NMR spectra of alditols and of complex carbohydrates. We now report the successful application of artificial neural networks to the recognition of the electron-impact mass spectra of PMAAs.

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