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Project Summary

We are investigating the biosynthesis of mixed-linkage β-D-glucan and glucuronoarabinoxylans which make up the hemicellulosic matrix of the primary cell walls of maize and other cereal grasses. The Golgi apparatus was enriched from plasma membrane and other organelles by flotation density gradient centrifugation. Glucan synthase I and II, which are established markers for Golgi and plasma membrane, respectively, displayed considerable overlap in conventional separations with sucrose density gradients. Flotation gradients improved separation of the membranes substantially, but the different synthases themselves also incorporated radioactivity from either 10 μM or 1 mM UDP-[^{14}C]-glucose into polymer. Relative incorporation of radioactivity into polymers from UDP-[^{14}C]-xylose by the various membrane fractions was nearly identical to relative IDPase activities, indicating that combined xylosyl transferase-xylan synthase represents a new, unequivocal marker for the Golgi apparatus. We also have developed techniques of gas-liquid chromatography and radiogas proportional counting to achieve capillary quality separation of partially methylated alditol acetates with simultaneous determination of radioactivity in the derivatives. We determined that both terminal and 4-linked xylosyl units from UDP-[^{14}C]-xylose and 3- and 4-linked glucosyl units from UDP-[^{14}C]-glucose were formed with Golgi membranes, whereas the plasma membranes made mostly 3-linked glucosyl units. We now are using digestion of polymeric products by specific endo-glycanohydrolases to diagnostic oligosaccharides that reveal specific kinds of polysaccharides synthesized by the Golgi membranes. We will then verify the linkage structure of these diagnostic oligomers by separation of partially methylated alditol acetates and detection of radioactivity by radiogas proportional counting. Efforts in the coming year are directed toward determining the factors required for branching of the xylan with arabinose and formation of the cellobiosyl- and cellotriosyl-(1→3)β-D-glucose units of the *bona fide* mixed-linkage β-D-glucan.

DISCLAIMER

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Our efforts since May 1, 1988 have been directed toward 1) improvement of the isolation of the Golgi apparatus from plasma membrane and other internal membranes of developing maize seedlings, 2) identification of radioactive reaction products by methylation analysis, radiogas proportional counting and specific enzyme digestion, and 3) pulse-labeling of intact tissues *in vivo* with radioactive arabinose and identification of sugars and linkages by methylation analysis and radiogas proportional counting. We also initiated investigations of the protein constituents of maize cell walls during development and collaborated with Dr. Peter Kaufman, Department of Biological Sciences, University of Michigan, on studies of the alterations of the polysaccharides in cells of gibberellin-induced oat internodes and gravistimulated pulvinus tissues.

Radioactive Gas Proportional Counting

To determine radioactivity incorporated into specific sugars and sugar linkages, we devoted considerable effort to engineer efficient recovery of derivatives of the cell wall sugars separated by gas-liquid chromatography (GLC) for continuous monitoring of the chromatograph effluent by radiogas proportional counting. We are delighted to report that use of wide-bore capillary columns and a two-stage make-up gas pre- and post-effluent stream-splitting permitted maintenance of near capillary quality separation of derivatives and detection of radioactivity in derivatives to about 200 dpm. We were easily able to separate the principal partially methylated alditol acetate derviatives of the GAXs of maize and the radioactivity incorporated into each of the derivatives (see Carpita and Gibeaut 1988).

Biosynthesis of Glucuronoarabinoxylans <u>In Vivo</u>

[^{14}C]-L-Arabinose is an excellent marker for synthesis of GAX in cereal grasses. Arabinose and xylose of the polysaccharide are normally formed through nucleotide sugar interconversion pathways beginning with UDP-glucose, but arabinose is incorporated through a salvage pathway catalyzed by arabinokinase to form arabinose-1-phosphate and a pyrophosphorylase to form UDP-arabinose. A C-4 epimerase converts a portion of this pool to UDP-xylose. In both millet cells in liquid culture and maize seedlings, arabinose is absorbed rapidly and incorporated into both buffer-soluble and cell wall polymers. With millet cells, over 70% of 250 μM arabinose is absorbed from the medium by 0.5 g fresh wt of cells in 2 h, and chase is accomplished conveniently by addition of 25 mM arabinose. Upon chase, radioactivity decreases rapidly in both free arabinose and buffer-soluble polymers with concomitant increases in radioactivity incorporated into GAX of the

cell wall. Similar pulses are effective if small amounts of [^{14}C]-L-arabinose are fed to the millet cells and radioactivity is chased by continuous flow of glucose into the nucleotide sugar pools. We observed that that a majority of the buffer-soluble polysaccharides turns over to the cell wall. If a highly-substituted form of GAX (HS-GAX) that we discovered in growing grass cells is precursor for the bulk cell wall GAX, then substantial amounts of terminal α-L-arabinose hydrolyzed from the xylan during this interconversion should be reabsorbed by the cells and used to form new HS-GAX. With this pattern of metabolism, arabinose cycles between cytoplasm and wall. True "chase" is attenuated by continued formation of the precursor, but with each cycle of arabinose from wall to cytoplasm, additional UDP-[^{14}C]-xylose is formed by the C-4 epimerase, and the proportion of arabinose to xylose should decrease relative to the rate and extent of the arabinose recycling. We found that, in fact, very little change in the ratio in the GAX of proso millet cells in which the GAX maintains a relatively high degree of substitution, but a marked decrease in the labeled arabinose to xylose ratio in GAXs of developing maize leaves (see Carpita and Gibeaut 1988).

Biosynthesis of Cell Wall Polysaccharides In Vitro

We have improved considerably methods for separation of the Golgi apparatus from plasma membrane and other cellular organelles by a density gradient flotation technique. The Golgi complex comprising the stacked membranes, forming secretory vesicles, and associated endoplasmic reticulum and transition vesicles were visualized by TEM, and negative-staining techniques revealed that the cisternae complex was preserved after density gradient centrifugation. Although enrichment of the Golgi system is satisfactory with simple density gradients, investigation of polysaccharide synthesis *in vitro* using nucelotide-[^{14}C]-sugar substrates revealed problems inherent in separation of the Golgi from plasma membrane. "Glucan synthase I" for Golgi membranes and "glucan synthase II" for plasma membrane are established markers. For grasses, however, we found considerable overlap in activities resulting both from overlap of the two membranes in the gradient as well as overlap in the actual activities of the different glucan synthases themselves. At low UDP-[^{14}C]-glucose, synthesis of hemicellulosic glucan is favored, whereas glucan synthase II, or the (1→3)β-D-glucan "callose" synthase, is favored at high substrate concentrations. In maize preparations, however, substantial amounts of radioactivity in glucan synthase I reactions are incorporated into polysaccharide in fractions containing mostly plasma membrane. We found that rigorous removal of Ca^{2+} was necessary to reduce this activity. Separation of Golgi and plasma membrane was improved substantially with flotation after preliminary sedimentation in a density gradient. We then compared activities of xylan synthase and xylosyl transferase by incorporation of UDP-[^{14}C]-xylose, glucan synthase I with 10 μM UDP-

[^{14}C]-glucose, and glucan synthase II with 1 mM UDP-[^{14}C]-glucose. The relative incorporation of xylose in polymer from UDP-xylose by the different membrane fractions were nearly identical to the IDPase marker for Golgi apparatus, whereas considerable amounts of radioactivity in glucan synthase I reactions were incorporated in polysaccharides with the plasma membrane fractions. Surprisingly, when Ca^{2+} was rigorously scrubbed from the membranes, considerable amounts of hemicellulosic glucan were made in the Golgi-enriched fractions at high concentrations of UDP-glucose. We clearly needed improved procedures for product characterization rather than just incorporation of radioactivity into polymeric material.

Linkage analysis of the radioactive polymers formed by the floated membranes, using radiogas proportional counting of partially methylated alditol acetates, was then exploited. With Golgi membranes, both terminal- and 4-linked xylosyl units were formed with UDP-[^{14}C]-xylose, and 3- and 4-linked glucosyl units in a ratio expected for the mixed-linkage β-D-glucans were formed with UDP-[^{14}C]-glucose. As expected, substantial amounts of 3-linked glucosyl units constituted the reaction products from plasma membrane (see Carpita and Gibeaut 1988).

Alteration of Cell Wall Structure in Gibberellin-induced Oat Internodes and Gravistimulated Oat Leaf Sheath Pulvinus

In collaboration with Dr. Peter Kaufman, University of Michigan, we initiated studies of the alteration in chemical structure of the cell walls of oat in response to growth stimulation. These studies were of interest to us because they provided new cell development systems to which we could apply our special techniques of polysaccharide analysis. Experiments were performed by Kaufman at the University of Michigan, and lyophilized tissues were sent to us for analysis. Excised internodes exhibited a marked elongation induced by gibberellin, and the response was enhanced when sucrose is added to the incubation medium. Sucrose, supplied to the tissues, was the primary carbon source for cell growth. Gibberellin enhanced the degradation of endogenous starch, but the contribution of glucose from starch to increase the mass of the cell walls was small compared to exogenous sucrose. The leaf-sheath pulvinus is the graviresponsive tissue. When excised pulvini are oriented horizontally, growth is enhanced in the lower portion and bends the pulvinus upward. In the intact seedling, this response produces an upright plant. Starch was a much higher proportion of the mass of the dry weight in the pulvinus compared to the internode, and gravistimulation resulted in concomitant loss of starch in the lower portions of the pulvinus. There was a net increase of all cell wall constituents of both internode and pulvinus and a proportional increase in mixed linkage β-D-glucan when internodes were incubated in sucrose. When elongation of internodes was enhanced with gibberellin, the proportion of β-D-glucan decreased. However, when pulvini were gravistimulated, the proportion of β-D-glucan increased

in the more rapidly growing lower portions. These data suggest that a delicate balance of synthesis and degradation of β-D-glucan during growth appears to be related to cell wall synthesis supported by sucrose and gibberellin-enhanced cell elongation.

PUBLICATIONS:

Journal articles:

1. Gibeaut, D. M., P. Dayanandan, T. G. Brock, N. S. Ghosheh, P. B. Kaufman, N. C. Carpita. Cell wall and starch metabolism of *Avena sativa*: Gibberellin-enhanced growth of the internode and gravistimulated growth of the leaf-sheath pulvinus. In preparation for *Plant Physiology*.

2. Gibeaut, D. M., N. C. Carpita. Synthesis of cell wall xylans and glucans by Golgi membranes enriched by flotation density gradient centrifugation. In preparation for *Protoplasma.*

Invited review articles:

1. Carpita, N. C., D. M. Gibeaut. 1988. Biosynthesis and secretion of plant cell wall polysaccharides. *In* Current Topics in Plant Biochemistry and Physiology, Volume 7, D. D. Randall, et al., eds., University of Missouri-Columbia, pp. 112-133.

2. Carpita, N. C. 1989. Chemical structure of the cell walls of higher plants. *In* Proceedings of the Third Vahouny Conference on Dietary Fiber, D. Kritchevsky, ed., Washington, D. C., *in press*

RENEWAL REQUEST FOR DOE GRANT DE-FG02-88ER13903

We propose to continue our studies of the biosynthesis of β-D-glucans and glucurono-arabinoxylans in developing maize seedlings. Radioactivity from UDP-[^{14}C]-xylose was incorporated into both terminal and 4-linked xylosyl units of polymers. Although these kinds of linkage data are valuable, we are presently developing enzymic techniques that produce diagnostic oligosaccharides that reveal which the sugars are linked together. A *Trichoderma* cellulase cleaves (1→4)β-D-glucosyl units of xyloglucan made at the Golgi apparatus with terminal xylosyl units remaining attached. This oligomer, an α-D-xylosyl-(1→6)-D-glucose (isoprimeverose), is resolved by TLC from cellobiose and glucose. The TLC plates with radioactive oligomer are exposed to X-ray film and developed, and the isoprimeverose spot quantified by densitometry. This quantitation will give a reliable estimation of the proportion of terminal xylosyl units associated with xyloglucan or as termini of the hemicellulosic xylans. Although 4-xylosyl units demonstrated that xylan was synthesized, a proportion of the UDP[^{14}C]-xylose is converted to UDP[^{14}C]-arabinose catalyzed by a C-4 epimerase associated with the Golgi membranes (data not shown) and ostensibly should be used for synthesis of arabinoxylan. We don't know why arabinose was not incorporated to indirectly give radioactive 3,4-xylosyl units and terminal arabinosyl units (see Carpita and Gibeaut 1988). We propose to examine the interaction of UDP-xylose and UDP-arabinose in formation of the appropriate branched arabinoxylan. We are considering two possibilities: 1) synthesis of these branch points requires factors not provided in the incubation medium, and 2) that UDP-GlcA is the actual precursor *in vivo* that is transported into the lumen of the Golgi apparatus and forms the UDP-ara needed for transfer to the growing chain. This paradigm is valid for xyloglucan synthesis in dicots where UDP-Glc is a cytoplasmic donor of glucose for the growing chain, whereas UDP-xylose is from UDP-GlcA absorbed by the Golgi apparatus, decarboxylated in the lumen, and the xylosyl units added to the glucan chain secreted into the lumen (Hayashi et al., 1988, Plant Physiol 87: 341).

The ratio of 3- and 4-linked glucosyl units formed in Golgi preparations can vary depending on reaction conditions (divalent cations, pH, etc.), but whether or not these linkages are in the same polymer is the critical question--a small amount of callose formed by contamination of the preparation with plasma membrane could contribute the 3-linked glucosyl units, and the 4-linked glucan could have arisen from the glucan synthase that normally would make xyloglucan. To establish a fundamental criterion for synthesis of the mixed linkage β-D-glucan, we have used a specific restriction endoglycosidase of *Bacillus subtilis* to yield oligomers representative of the β-D-glucan. This enzymes cleaves a (1→4)β-D-glucosyl units but only if preceded by a (1→3)β-D-glucose. Digestion of purified β-D-glucan with this enzyme yields about equal amounts of cellobiosyl-(1→3)β-D-glucose and cellotriosyl-(1→3)β-D-glucose, indicating that the

polysaccharide comprises repeating units of cellotriosyl and cellotetraosyl units connected by a single (1→3)β-D-glucosyl unit. We are able to resolve the tri- and tetrasaccharide by TLC. The products from *Trichoderma* cellulase digestions of the glucans synthesized *in vitro* give a wide range of oligomeric products, but the *B. subtilis* digestions give larger amounts of the tri- and tetrasaccharide. Surprisingly, the trisaccharide is made in great excess to the tetrasaccharide. We propose to continue studies of the synthesis of β-D-glucan *in vitro* and analyze of the reaction products by a combination of enzymic digestion and then methylation analysis and radiogas proportional counting. We are hampered somewhat by relatively poor resolution of the tri- and tetrasaccharides, diagnostic of the β-D-glucan, from cellodextrins and 3-linked glucose oligosaccharides. Ideally, we want to recover the radioactive tri- and tetrasaccharides and verify their composition by methylation analysis and radioactive gas proportional counting. We are developing alternative methods for separation of the radioactive oligomers for convenient preparation of derivatives.