Use of Per-C-Deuterated *myo*-Inositol for Study of Cell Wall Synthesis in Germinating Beans[1]

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ABSTRACT

Cell wall polysaccharides of the hypocotyl and roots in germinating beans (*Phaseolus vulgaris* L.) were selectively labeled in arabinosyl, xylosyl, and galacturonosyl residues by per-C-deuterated *myo*-inositol, which was introduced through 72 hours of imbibition. Glucuronate residues remained unlabeled. Selected ion gas chromatography-mass spectrometry analysis revealed that deuterium was not redistributed in these three sugar residues or into other carbohydrate residues during this conversion, suggesting that the labeled residues are formed exclusively via the *myo*-inositol oxidation pathway and that no glucogenesis from *myo*-inositol takes place during this conversion. The presence of a significant level of deuterated arabinose, xylose, and galacturonate after just 72 hours of imbibitional uptake of per-C-deuterated *myo*-inositol indicated that the *myo*-inositol oxidation pathway has a predominant role in the biosynthesis of new cell walls.

myo-Inositol is an important precursor of plant cell wall polysaccharides. During seed development and germination, *myo*-inositol that has been stored in seeds in various forms is transported to the growing seedling parts and then converted to uronic acid and pentose residues for the synthesis of new cell wall polysaccharides (16–18). In germinating beans, *myo*-inositol is formed from reserve sugars at a very early stage that precedes phytic acid hydrolysis (19). Along with stored reserves of *myo*-inositol, this newly synthesized *myo*-inositol is available for cell wall synthesis (19). Thus, studies of *myo*-inositol metabolism will enhance our understanding of cell wall formation and eventually reveal the role of *myo*-inositol in this process. Until now, most studies have utilized radiolabeled *myo*-inositol in this regard. Use of per-C-deuterated *myo*-inositol would provide an attractive, powerful technique that has advantages not available through tracer methodology. (a) Stable isotopes are not radioactive and are, therefore, easy to handle. (b) Labeled, nonlabeled, and total sugar compositions are readily analyzed simultaneously by GC-MS using selected ion and total ion-monitoring systems. (c) Selected ion monitoring easily and quickly provides important information on the redistribution of deuterium atoms into sugar residues other than those produced exclusively by the *myo*-inositol oxidation pathway (10–12). (d) Specific labeling with a stable isotope permits one to use ^{2}H- or ^{13}C-NMR for nondestructive investigation of the structures involved. Recently, we developed a method for preparation and purification of large quantities of per-C-deuterated *myo*-inositol (14). Here we report on the use of this material to label cell walls of germinating bean hypocotyls and roots and perform GC-MS analysis on hydrolyzed products from these cell walls.

MATERIALS AND METHODS

Preparation of Per-C-Deuterated *myo*-Inositol-Labeled Bean Seedlings

The preparation of per-C-deuterated *myo*-inositol by Raney nickel-catalyzed exchange and its separation from other isomers of inositol and their degradation products have been described (14). For the present study, per-C-deuterated *myo*-inositol was mixed with a trace amount of *myo*-[2-^{3}H]inositol (to provide a radiolabeled marker), purified by ion exchange chromatography, and recrystallized (14).

Labeled *myo*-inositol (0.2 M in per-C-deuterated *myo*-inositol in distilled water) was sterilized by autoclaving and was supplied in 1 mL amounts to each of five surface-sterilized bean (*Phaseolus vulgaris* L.) seeds by imbibition as described previously (18, 19). There was no adverse effect of *myo*-inositol on germination and growth of beans even at 0.5 M (18). After 72 h at 23°C, the labeled germinating seedlings (hypocotyl length, 1 cm) were harvested and divided into cotyledons, hypocotyls, and roots.

Fractionation of Labeled Seedling Parts

Parts from five seedlings were ground in 80% ethanol with mortar and pestle, extracted twice with hot ethanol (70–75°C), and rinsed twice with 80% ethanol and then with 95% ethanol. Extracts and rinses were combined as the ethanol-soluble fraction. Residues were then extracted overnight with methanol:chloroform (1:1, v/v) at 23°C and rinsed with 95% ethanol (methanol-chloroform fraction). These residues were washed twice with water followed by potassium phosphate buffer (0.01 M, pH 6.9), and all aqueous extracts were combined (water-soluble fraction). The water-insoluble residues were treated with α-amylase (Sigma Type I-A from porcine pancreas) (0.01 M potassium phosphate containing 0.02% sodium azide [pH 6.9], 30°C, 24 h) and rinsed twice with water (amylase-soluble fraction). Finally, the residues were

lyophilized, resuspended, and hydrolyzed, first in 2 N trifluoroacetic acid and then in 72% sulfuric acid (TFA- and H_2SO_4-soluble fraction, respectively) (18).

Sugar Analysis by GC-MS

The TFA-soluble fraction was separated into neutral and acidic components by ion-exchange chromatography on a column of Dowex 1 (formate) resin (19). Neutral sugar residues were converted to their alditols (1). Acidic components were lactonized (9), treated with $NaBH_4$, and then passed through a column of Rexyn 101 (H^+ form). The process of reduction and cation-exchange chromatography was repeated three times to completely convert lactones to the corresponding alditols (20).

Alditols were acetylated and then analyzed by GC-MS on a Finnigan-MAT model 800 spectrometer equipped with a fused silica capillary column coated with SP 2330 (15 m × 0.25 mm) (Supelco, Bellefonte, PA). Products were separated with a programmed temperature gradient (initial 220°C for 4 min, gradient 2°C/min, final 240°C). Total sugar composition was determined from the total ion chromatogram. Since the retention time of a deuterated alditol acetate was slightly less than that of its nondeuterated form, an alditol acetate derived from a deuterated neutral sugar could be determined from its selected ion chromatogram of m/z 191, that derived from a deuterated sugar acid by use of combined m/z 190 and 191, and that of nondeuterated sugars, m/z 187 (4).

RESULTS

Distribution of Tritium from *myo*-[2-^{3}H]Inositol in Germinating Beans

During 72 h of imbibition the bean seeds took up approximately 9.5% of the available tritium. Assuming no discrimination between nondeuterated (but tritiated) and per-C-deuterated *myo*-inositol, the amount of tritium taken up by a seedling corresponded to approximately 3.5 mg of per-C-deuterated *myo*-inositol.

Of this, 71 to 74% was in ethanol-soluble fractions from hypocotyl and root, and 91% in the same fraction from cotyledons (Table I). Another 8 to 9% was present in each seedling part in the water-soluble fraction. Virtually no tritium appeared in the methanol-chloroform fractions and very little in the amylase fractions. None of these fractions were further analyzed. Acid hydrolysis released 15 to 19% of the tritium present in hypocotyl and root. Most of this was present in the TFA-soluble fractions (70–73% as neutral sugars and 27–30% as sugar acids). None was found in the corresponding fractions from cotyledons.

Table I. *Distribution of ^{3}H from myo-[2-^{3}H]Inositol in Germinating Beans*

Fraction	Hypocotyl	Root	Cotyledons
		% ^{3}H (by fraction)	
Ethanol	73.9	71.0	91.1
Water	9.0	8.1	7.8
Amylase	2.0	2.0	0.4
TFA	13.8	16.5	0.7
H_2SO_4	1.3	2.4	
		% ^{3}H (by seedling part)	
Total ^{3}H	11.9	6.7	81.4

Analysis of Total and Deuterated Sugar Compositions in TFA-Soluble Fractions as Determined by GC-MS

Total ion and selected ion chromatogram analyses of the alditol acetates from TFA-soluble fractions of hypocotyl and root supplied total, deuterated, and nonlabeled sugar and sugar acid compositions. The compositions are summarized in Table II. Of the seven common cell wall neutral sugar residues released by TFA hydrolysis, only arabinose and xylose were deuterated. Other neutral sugar residues were devoid of the fragment ion, m/z 191. Of the two common cell wall sugar acid residues released by TFA hydrolysis, only galacturonate was deuterated. These observations applied to both hypocotyl and root portions of the seedling. Although glucuronosyl residues accounted for about 40% of the total sugar acids in the cell walls (Table II), selected ion analysis revealed no fragment ions with m/z 190 or 191 which would have indicated deuteration (Fig. 1).

Comparison of total ion to selected ion data provides information on the amount of per-C-deuterated *myo*-inositol utilized for synthesis of arabinosyl, xylosyl, and galacturonosyl residues during the 72 h period of imbibition (Table II). Over 20% of xylosyl and galacturonosyl residues in hypocotyl and roots were deuterated, while another 10% of the arabinosyl residues in hypocotyl and 18% of that in roots were also deuterated. Neither neutral sugar nor sugar acid residues from the TFA-soluble fraction of cotyledons had greater than natural abundance of deuterium, although arabinose was the major sugar component of the hydrolyzate (data not shown).

Investigation of Redistribution of Deuterium into Cell Wall Components Other Than Products of the *myo*-Inositol Oxidation Pathway

Selected ion chromatograms which compare the abundance of fragment ions with m/z 189, 190, and 191 as produced by

Table II. *TFA-Soluble Sugar Compositions of Bean Cell Walls as Determined from GC-MS Chromatograms of Alditol Acetate Derivatives*

Sugar	Hypocotyl		Root	
	Total	Deuterated	Total	Deuterated
		mol %		
Rhamnose	5.1		4.5	
Fucose	0.8		4.4	
Arabinose	23.5	2.3 (9.8)[a]	22.1	4.0 (18.1)
Xylose	9.6	2.2 (22.9)	9.9	2.7 (27.3)
Mannose	4.2		3.3	
Galactose	19.1		27.0	
Glucose	24.2		12.3	
Gal.UA	8.4	1.7 (20.2)	9.6	2.8 (29.2)
Glc.UA	5.2		6.8	

[a] Numbers in parentheses are percentages of ^{2}H-labeled sugars in the total amount of each individual sugar residue.

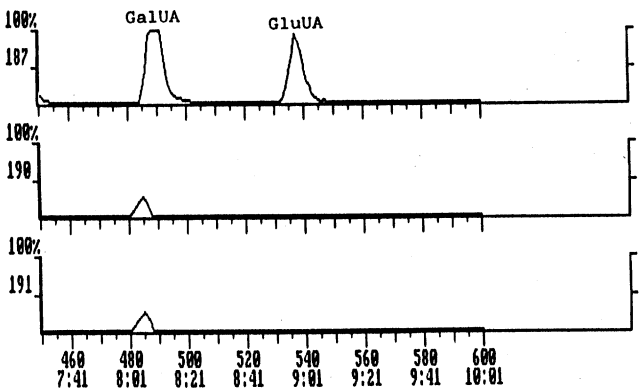

Figure 1. Selected ion chromatograms of uronic acid residues from hypocotyl cell walls. The numbers on the ordinates (187, 190, 191) show fragment ions m/z 187, 190 and 191, respectively. The numbers on the abscissas are scan numbers (upper) and retention times (lower). GalUA: galactitol hexaacetate from galacturonic acid, GluUA: glucitol hexaacetate from glucuronic acid.

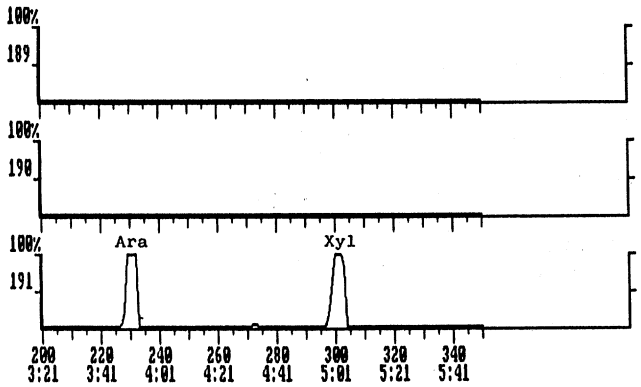

Figure 2. Selected ion chromatograms of arabinose and xylose residues from hypocotyl cell walls. The numbers on the ordinates (189, 190, 191) are fragment ions m/z 189, 190 and 191, respectively. The numbers on the abscissas are scan numbers (upper) and retention times (lower). Ara: arabinitol pentaacetate, Xyl: xylitol pentaacetate.

arabinosyl and xylosyl residues of hypocotyl cell walls are seen in Figure 2. Neither the deuterated arabinosyl nor the deuterated xylosyl residue produced fragment ions with m/z 189 or 190. None of these fragment ions were produced from other neutral sugar residues (data not shown). Similar results were obtained from neutral sugars of root cell walls. Moreover, deuterated galacturonosyl residues produced equal amounts of fragment ions with m/z 190 and 191 (Fig. 1), but no ions with m/z 189 (data not shown). This indicates that all carbon-linked deuteriums of *myo*-inositol (except carbon 1-linked deuterium, which was lost during oxidation of *myo*-inositol to glucuronic acid) were retained when per-C-deuterated *myo*-inositol was converted to arabinose, xylose, and galacturonic acid residues.

DISCUSSION

GC-MS analyses of bean seedlings which imbibed per-C-deuterated *myo*-inositol clearly demonstrate the fact that arabinosyl and xylosyl residues are the only deuterated neutral sugar components of cell wall polysaccharides from hypocotyl and root tissue. This observation resembles results obtained with *myo*-[2-^{3}H]inositol-treated bean seeds at still later stages of germination (18). From the present study emerges the new fact that only galacturonosyl residues, but not glucuronosyl residues, are deuterated. This interesting finding suggests that, during early stages of germination, intermediary UDP-glucuronate in the *myo*-inositol oxidation pathway is utilized exclusively for the biosynthesis of galacturonosyl and pentosyl groups. Although 40% of the uronosyl groups in the TFA-soluble fractions from hypocotyl and root was glucuronate, it was devoid of deuterium.

This study also indicates that an endogenous source of *myo*-inositol is available as substrate for oxidation to cell wall constituents. It can arise from at least two metabolic processes, one involving preformed sources of *myo*-inositol and another utilizing *de novo* synthesis of *myo*-inositol from sugar reserves in the seed. The former may be free *myo*-inositol or a bound form such as galactinol or phytic acid. In wheat caryopses, *myo*-inositol is stored in all three forms, but only free *myo*-inositol and galactinol are utilized rapidly for cell wall biosynthesis, while phytic acid is depleted much more slowly (16, 17). In beans, phytic acid hydrolysis is evident 3 d after germination (2, 3) and the *myo*-inositol produced therein is transported to and utilized at actively elongating regions for new cell wall biosynthesis and at nonelongating regions for consolidation of cell wall structure following the completion of elongation (18). A significant amount of *myo*-inositol is synthesized *de novo* during very early stages of germination, certainly within the 72 h period of imbibition (19). Exogenously supplied per-C-deuterated *myo*-inositol probably combines with the two sources endogenous to the imbibing seed and enters the biosynthetic path to new cell wall polysaccharides via the *myo*-inositol oxidation pathway (5–8). The absence of deuterated glucuronate suggests that this uronosyl residue is not required for cell wall biosynthesis during very early stages of germination or that UDP-glucuronate within the *myo*-inositol oxidation pathway is unavailable for biosynthetic processes involving transfer of glucuronosyl groups during this period of development. Possibly, the alternative oxidation pathway through UDP-glucose (7) supplies this need if such a requirement does exist.

The absence of redistribution of deuterium into other cell wall sugar residues, notably glucosyl and galactosyl groups, is interesting. In germinating lily pollen, a portion of the UDP-xylose that is generated through the *myo*-inositol oxidation pathway undergoes hydrolysis and enters hexose phosphate through pentose phosphate metabolism, eventually finding its way into hexosyl residues of cell wall polysaccharides (10–12). If this process was present during imbibition of per-C-deuterated *myo*-inositol-treated beans, deuterated hexosyl units would have appeared. None were found. There was no redistribution of deuterium into such groups through glucogenesis. Moreover, all carbon-linked deuteriums of arabinose, xylose, and galacturonic acid residues in the cell walls were retained. This also indicates that per-C-deuterated *myo*-inositol was converted to these cell wall sugar residues exclusively via the *myo*-inositol oxidation pathway.

If one considers the nondeuterated pentosyl and galacturonosyl units that were present in cell walls of the embryonic axis prior to imbibition and the stored reserves capable of endogenous synthesis or release of nondeuterated free *myo*-inositol, it is remarkable that such a significant portion of the cell wall pentosyl and galacturonosyl residues is deuterated in the brief 72-h period allowed for this experiment. This indicates that *myo*-inositol is the major source for these cell wall sugar residues in germinating beans. The facility with which *myo*-inositol is utilized for galacturonosyl unit biosynthesis within the germinating bean seed strongly suggests that this process may have a similar role in oligosaccharide signaling (13).

Use of per-C-deuterated *myo*-inositol and GC-MS analysis is a powerful technique for studies involving *myo*-inositol and cell wall metabolism and provides a convenient route to the specific labeling of selected polysaccharides that contain a large burden of deuterated atoms. Further, the method can be extended to include other isomeric forms of deuterated inositol since all eight isomers are now readily resolved by HPLC (15). Current efforts include the use of ^{2}H-NMR to explore the structure of heavily ^{2}H-labeled cell walls which were prepared by the procedures described here. A similar approach can be applied to other studies involving *myo*-inositol metabolism such as phytate biosynthesis and breakdown or phosphoinositide metabolism.

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