Biosynthesis of the Fucose-Containing Xyloglucan Nonasaccharide by Pea Microsomal Membranes

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Abstract

Pea microsomal membranes catalyze the transfer of [1-3H]fucose (Fuc) from GDP-[U-14C]fucose (Glc), UDP-xyllose (Xyl) or UDP-galactose (Gal), to an insoluble product with properties characteristic of xyloglucan. After digestion of the ethanol-insoluble pellet with Streptomyces griseus endocellulase, [14C]fucose residues occur exclusively in a fragment corresponding in size to the xyloglucan nonasaccharide, Glc2XylGalFuc2. This fragment contains a single labeled fucose residue per oligomer, α-linked in a terminal nonreducing position. By comparison, in incubations where GDP-[14C]fucose is absent and replaced by UDP-[3H]xyllose, the maximum size of labeled oligosaccharide found following cellulase digestion of products is an octasaccharide. In the presence of both GDP-[14C]fucose and UDP-[3H]xyllose, a nonasaccharide containing the two labels is produced. Fucose and xylose residues are transferred within a few minutes to acceptor molecules of molecular weight up to 300,000. Such products do not elute detectably over 60 minutes of incubation. The data support the conclusion that the nonasaccharide subunit of xyloglucan may be generated in vitro by transfucosylation to preformed acceptor chains, and that its synthesis is dependent on the inclusion of exogenous GDP-fucose.

Xyloglucan is a widely occurring polysaccharide and a major component of dicot primary cell walls. Its structural features have been elucidated in several species; for reviews, see Dey and Brinson (4) and McNeil et al. (12). The polymer from soybean, pea, and sycamore cell walls contains equal amounts of two repeating subunits which can be obtained by endocellulase digestion: a nona- and a heptasaccharide (GlcXylGalFuc and Glc2Xyl), which are arranged mostly in alternating sequence (7, 8, 10, 12). The mechanism of xyloglucan biosynthesis in vitro has been described partially, but results to date deal only with the elaboration of the glucose-xylose backbone (9, 10, 14). The product formed by soybean microsomes from UDP-Glc2 plus UDP-Xyl, with either sugar labeled, is cleaved by endocellulase to produce mainly radioactive hepta- and pentasaccharides. Nothing is known of the mechanism involved in the addition of the two terminal side chain sugars (galactose and fucose) that are required to make a complete nonasaccharide. Particular interest in the synthesis of the nonasaccharide arises from the fact that it is formed with regularity in the xyloglucan structure, although how this precision is achieved is unknown. Also intriguing are recent findings of York and co-workers (17) who report an inhibition of 2,4-D-induced elongation of pea stem segments by the nonasaccharide subunit of xyloglucan, but not by the heptasaccharide (1). Biological significance appears to be related to fucosylation and the present study therefore concentrated on the formation of this sugar to form xyloglucan nonasaccharides. This is the first report describing the in vitro formation of the fucose-containing xyloglucan nonasaccharide subunit.

Materials and Methods

Chemicals. Silica Gel 60 TLC plates were purchased from Merck; GDP-[U-14C]fucose (217 mCi/mmol) and NaB[1-3H] (500 mCi/mmol) were from Amersham; and UDP-[1-3H]xyllose (9.9 Ci/mmol) from New England Nuclear. All unlabeled sugar nucleotides were from Sigma. The endocellulase from Streptomyces griseus S199 was a generous gift from Dr. E. T. Reese, U.S. Army Laboratories, Natick, MA; a glucosidase mixture from Aspergillus oryzae as well as Tamarindus indica seed xyloglcan were kindly provided by Drs T. Hayashi and K. Matsuda, Tokoh University, Sendai; α-L-fucosidase from beef kidney and β-D-galactosidase from Aspergillus niger were purchased from Sigma; Charonia lampas β-D-galactosidase was from Miles Biochemicals. p-Nitrophenyl α-L-fucopyranoside and α-nitrophenyl β-D-galactopyranoside were from Sigma. Bio-Gel P-4 (200–400 mesh) was from Bio-Rad, Sepharose CL-6B from Pharmacia, and cellulose powder from Macherey, Nagel and Co. (Duren, Germany).

Plant Material. Seeds of Pism sativum var. Alaska were surface-sterilized with 6% NaClO2, soaked in water for 4 h and planted in vermiculite. After 1 week of growth in the dark at 22°C, 1-cm stem segments were excised from growing regions below the hook and plumule, and homogenized (400 segments) with pestle in an ice-cold mortar for 1 min in 15 ml buffer A: 0.1 M Hepes/KOH buffer (pH 7.0), 1 mm EDTA, 1 mm DTT, 0.4 M sucrose, and 0.1% BSA. The preparation was filtered through Miracloth (Calbiochem) and centrifuged at 1000 g for 10 min. The supernatant was layered over a 55% sucrose cushion and centrifuged 45 min at 100,000 g at 4°C in an IEC B-60 ultracentrifuge with a SB-283 rotor. The membrane fraction was collected from the interface.

Conditions of Incubation. Reactions were conducted with membranes resuspended in buffer B: Hepes/KOH 100 mm (pH 7.0), 10 mm MnCl2, 0.6 mm EDTA, 0.6 mm DTT, 0.25 mm sucrose, and 0.06% BSA (10). Membranes (200 μl) containing approximately 2 mg protein were incubated with the appropriate radioactive sugar nucleotide: 4.5 μM GDP-[U-14C]fucose, and/or 2 μM UDP-[1-3H]xylose. Unless specified otherwise, unlabeled sugar nucleotides were at 200 μM.

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1 Abbreviations: UDP-Glc, uridine diphosphoglucone; UDP-Xyl, uridine diphospho-xylose; UDP-Gal, uridine diphosphogalactose; NaBt, sodium borotritide; GDP-Fuc, guansidine diprophosphofucate.
nucleotides were included in all of the reaction mixtures at the following concentrations: UDP-Glc (2 mm), UDP-Xyl (20 μM), and UDP-Gal (20 μM). Reactions were started by the addition of membranes in buffer B to the labeled and unlabeled sugar nucleotide mixtures. The reactions were left to proceed at 20°C for 30 min then stopped by boiling for 5 min. Where except mentioned, 100 μg of T. indica seed xyloglucan was added as a carrier after boiling, and the mixture extracted with 1 ml 90% ethanol. The insoluble pellet was further extracted 3 times with 70% ethanol, and used for cellulase digestions, or extracted at room temperature for 30 min in 24% KOH containing 0.1% NaBH₄ to solubilize xyloglucans.

Endocellulase Digestion. Insoluble products were suspended in 0.5 ml 50 mM K-acetate buffer (pH 5.0), and 0.4 mg S. griseus S199 endocellulase was added per tube. The digestions were left to proceed under toluene for 48 h at 40°C. Reactions were stopped by boiling and, after centrifugation, the supernatant was collected and used for gel filtration analysis.

Mixed Glycosidases Digestion. The oligosaccharides from cellulase digestes were air-dried, resuspended in 1 ml 50 mM K-acetate buffer (pH 5.0), and 1 mg of A. oryzae mixed glycosidases preparation was added per tube. The digestions were left to proceed under toluene for 20 h at 40°C, stopped by boiling, and the supernatants desalted in 0.5 × 3.0 cm columns of Bio-Rad Dowex 50W-X2 (200–400 mesh) eluted with 3 column volumes of water. The eluate was used for paper or TLC.

Specific Glycosidase Digestions. Oligosaccharides from cellulase digestes were air-dried, resuspended in 0.5 ml 0.1 M sodium citrate buffer (pH 5.6), and 3 μl (10 milliunits) of beef kidney α-L-fucosidase was added per tube. The digestion was left to proceed under toluene for 20 h at 40°C, then stopped by boiling. For α-L-fucosidase digestion, oligosaccharides were resuspended as for the fucosidase digestion, 10 milliunits (5 μl) of β-galactosidase from A. niger or from C. lampa was added and the reactions left to proceed for 30 h at 40°C. All three enzymes were tested for activity on their respective p- or o-nitrophenol substrates.

NaBT₄ Labeling. Titrination of reducing terminals of the oligosaccharides was conducted according to a modification of the method of Forsee et al. (6). Oligosaccharides were air-dried and resuspended in 200 μl of 20 mM Na₂CO₃, 150 μl of freshly solubilized sodium borotritide (2 × 10⁶ dpm) in water, and the mixture was incubated at room temperature for 20 h. After acidification with 2 N acetic acid (100 μl), the solution was air-dried. The evaporation procedure was repeated 3 times with water, then 3 times with methanol resuspension. The final fraction was solubilized in water and used for gel filtration analysis.

Gel Filtration. Gel filtration chromatography was conducted in two Bio-Gel P-4 columns connected in sequence (1 × 230 cm total bed volume) and eluted with water containing 0.02% NaN₃. Fractions of 1 ml were collected for radioactivity estimation. Alkali-soluble polysaccharides were fractionated in 0.1 N NaOH on a 95 cm Sepharose CL-6B column calibrated with dextrans of known molecular size (Sigma).

Cellulose Binding Assays. The ¹⁴C-labeled nonasaccharide was solubilized in 2 ml of water, or in 60 or 80% acetone, then 100 mg of cellulose powder were added and the solutions incubated 2 h at 22°C with occasional stirring. After centrifugation, supernatants were collected and counted for radioactivity. The pellets were subsequently extracted for 30 min in 0.5 ml of 24% KOH. After centrifugation, the supernatants and pellets were neutralized and counted in Aquasol II.

General Chromatography Methods. Ascending paper chromatography for monosaccharides was conducted using Whatman 3MM paper in solvent system A: 1-propanol:ethyl acetate:water (3:2:1, v/v) for 1.5 h at 70°C in a sealed tank. For TLC, Silica Gel 60 plates were run in solvent B: 1-butanol:acetone:water (4:5:1, v/v). Unlabeled sugars were detected on paper by the silver nitrate reagent (15), and on TLC by spraying the plates with 5% H₂SO₄ in ethanol followed by heating at 100°C. Radioactive sugars were localized by cutting the paper chromatogram or TLC plate and counting the strips in Aquasol II.

Pea Cell Wall Xyloglucan Extraction Procedure. Cell wall xyloglucan was extracted from growing regions of etiolated pea stems according to Hayashi and Maclachlan (8). Carbohydrate determination was made with the phenol-sulfuric acid test (5).

RESULTS

In Vitro Nonasaccharide Synthesis from GDP-[¹⁴C]Fucose. Pea membranes were incubated with GDP-[U-¹⁴C]fucose (4.5 μM) for 30 min in the presence of the unlabeled sugar nucleotides expected to be required for nonasaccharide synthesis. The insoluble, ethanol-washed products were digested with cellulase as described. A ¹⁴C-labeled digestion product was observed to elute from Bio-Gel P-4 columns in a single peak (Fig. 1A) corresponding to the position of the nonasaccharide obtained by cellulase digestion of untreated pea xyloglucan extracted from cell walls (Fig. 1B). Approximately 85% of the ethanol-insoluble radioactivity was found in this peak. The labeled product was capable of binding to commercial cellulose in aqueous acetone solutions with percentages of retention similar to those reported by Valen and Albersheim (16) for the sycamore xyloglucan nonasaccharide. Its binding capacity increased with acetone content of the solution, from 25% in water to 37% in 80% acetone. Furthermore, up to two-thirds of the bound radioactivity was desorbed by 24% KOH, a property consistent with that of xyloglucan and its digestion products (2). Complete digestion of the ¹⁴C-labeled

![Figure 1](https://example.com/fig1.png)
nonasaccharide with a glycosidase mixture from A. niger produced a single labeled compound co-migrating on paper chromatography and TLC with authentic fucose.

A sample of \(^{14}C\)-fucosylated nonasaccharide, obtained from digested reaction mixtures where tamarind xyloglucan had not been added during extraction procedures, was labeled with NaB\(^{3H}\) as described. A peak containing both \(^{14}C\) and \(^3H\) label eluted from the P-4 column with standard wall xyloglucan nonasaccharide. This radioactive nonasaccharide was incubated with bovine kidney \(\alpha\)-fucosidase for 48 h and the digest was refractionated on Bio-Gel P-4 (Fig. 2). All \(^{14}C\) now eluted as fucose (confirmed by paper chromatography), and tritium eluted in the position of an octasaccharide. This presumably corresponds to tritiated Glc\(_2\)Xyl\(_2\)Gal. This compound was not sensitive to digestion with \(\beta\)-galactosidase from various sources (see "Materials and Methods"), but terminal galactose in xyloglucan appears to be resistant to attack by most \(\beta\)-galactosidases (13).

The Acceptor Molecule. Time-course incubations were conducted with labeled GDP-Fuc or UDP-Xyl in the presence of unlabeled UDP-Glc, UDP-Xyl, and UDP-Gal; the reactions were stopped by boiling and the ethanol-washed insoluble products were extracted with 24% KOH as described in "Materials and Methods." The solubilized products were applied to a Sepharose CL-6B column and eluted in 0.1 M NaOH. Both fucose and xylose were incorporated into a high mol wt alkali-soluble polymer which did not appear to elongate with time (Fig. 3, A and B). The elution range of this product corresponded with that of natural pea xyloglucan (8). The low mol wt peak at the right of both patterns was found in the boiled control as well and is probably substrate or its degradation products.

The addition of unlabeled UDP-Glc, UDP-Xyl, and/or UDP-Gal enhanced the synthesis of the nonasaccharide from GDP-[\(^{14}C\)]fucose, but their presence was not essential for substantial fucosyl transfer (Table 1). It is clear that synthesis of the nonasaccharide from GDP-[\(^{14}C\)]fucose can proceed in vitro to a considerable extent in the absence of other exogenous sugar nucleotides. Thirty-min incubations with labeled GDP-fucose in the absence of exogenous unlabeled sugar nucleotides produced lesser amounts of alkali-soluble polymers, but their mol wt distribution on Sepharose CL-6B was identical to the patterns obtained in the presence of exogenous unlabeled sugar nucleotides. These observations together with the results of Figure 3 imply that addition of fucose occurs onto preexisting xyloglucan chains that are present in the preparation. Similar experiments conducted with membranes incubated with UDP-[\(^3H\)]Xyl, in the presence or absence of unlabeled sugar nucleotides, confirmed earlier results (10, 14), namely that the addition of UDP-Glc greatly enhanced the incorporation of radioactive xylose into xyloglucan (in our tests, by 7-fold). The mol wt profiles (Sepharose CL-6B) of products labeled with or without added sugar nucleotides were however very similar.

It is concluded that the presence of UDP-Glc, UDP-Xyl, and UDP-Gal, while enhancing xylose transfer, is not essential to the fucosylation of xyloglucan; and that the addition of these sugar nucleotides does not lead to an elongation of the acceptor chains which is sufficient to be detected by Sepharose chromatography.

Role of GDP-Fucose. When microsomal preparations were incubated with UDP-[\(^3H\)]xylose in the presence of unlabeled UDP-Glc, UDP-Xyl, and UDP-Gal, cellulose digestion produced mainly labeled hepta, penta and trisaccharides (Fig. 4A). The pentasaccharide most likely derives from an incomplete xyloglucan backbone (11), but the trisaccharide is possibly a degradation product from xylan (3, 10). The small amount of octasaccharide present is presumably galactosylated heptasaccharide. No labeled nonasaccharide could be detected in these incubations.

Figure 4B shows the P-4 elution pattern of cellulase digestion products from an incubation containing both UDP-[\(^3H\)]xylose
Fig. 4. Fractionation on Bio-Gel P-4 of oligosaccharides obtained from *Streptomyces* endocellulase digestion of insoluble products synthesized by pea microsomes in 30-min incubations in the presence of UDP-[3H]xylose (A); or of both UDP-[3H]xylose and GDP-[14C]fucose (B). (C) shows the profile after α-fucosidase digestion of the nonasaccharide obtained in (B). 9, Nonasaccharide; 7, heptasaccharide; 5, pentasaccharide; 3, trisaccharide; F, fucose; X, xylose; (O—O) 14C; (——) 3H.

and GDP-[14C]fucose, with the standard unlabeled sugar nucleotides. Tritium label eluted in the same peaks as seen in Figure 4A, however there was no octasaccharide and a new peak appeared, corresponding to nonasaccharide. The 14C label co-eluted exclusively with the nonasaccharide and fucose.

The nonasaccharide peak from the above experiment was collected, digested with fucosidase and fractionated on a P-4 column. All of the 14C eluted with fucose, indicating complete defucosylation (Fig. 4C). Tritium eluted as an octasaccharide exclusively (apparent tritium activity co-eluting with the 14C peak is a spillover from [14C]fucose, as checked by paper chromatography and TLC). The octasaccharide was digested with mixed glycosidases from *Aspergillus* to labeled isopimarose (xylosylglucose disaccharide). It is concluded that in the presence of exogenous GDP-fucose, a newly xylosylated acceptor can be completed *in vitro* to the nonasaccharide stage.

**DISCUSSION**

These results show that pea microsomal membranes transfer [14C]fucose from radioactive GDP-fucose to an ethanol-insoluble microsomal acceptor. This polymer can be digested by endocellulase to yield one labeled oligosaccharide which co-elutes from Bio-Gel columns with pea cell wall xyloglucan nonasaccharide (Fig. 1). This fucose-labeled nonamer binds to cellulose in a manner similar to authentic xyloglucan and contains a single terminal α-linked fucose which is nonreducing (Fig. 2). The polymer from which this nonasaccharide derived is soluble in concentrated alkali and comparable in size to pea xyloglucan (Fig. 3A). In experiments where pea microsomes are supplied with labeled UDP-Xyl plus or minus unlabeled UDP-Glc, an alkali-soluble product is also formed with a similar size distribution (Fig. 3B). The fucose and xylose-labeled products show no detectable size increase with time, suggesting that they are not initiated *de novo* but represent endogenous "primers" which are acceptors for transglycosylation reactions.

Endocellulase digestion of the polymers formed from tritiated UDP-Xyl in the presence of unlabeled UDP-Glc, UDP-Xyl, and UDP-Gal produces a mixture of labeled oligosaccharides, the largest of which are hepta and octasaccharide (Fig. 4A) (10, 11, 14). If GDP-[14C]fucose is also included in the incubation mixture, the octasaccharide disappears from the digestion products and a nonasaccharide appears, in which both isotopes are present (Fig. 4, B and C). It is concluded that in these preparations, pre-made microsomal xyloglucan can incorporate both xylose and fucose from exogenous sugar nucleotides to form complete nonasaccharide subunits.

It is possible to estimate the relative fractions of fucosylated nonasaccharides formed by incorporation into preexisting chains, and those derived from newly elongated (xylose-labeled) portions of xyloglucans primers. This can be achieved by calculating the relative specific activity of [14C]fucose to [3H]xylose in the nonasaccharide formed in double-labeling experiments. Assuming that all three xylose residues on any newly elongated nonasaccharides must be labeled, the ratio of label for xyI:fuc for all of the [14C]fucose were added uniquely to these new terminals. However, the observed ratio from specific activity estimates in the peaks for xyI:fuc was found to vary from 0.1:1 to 0.2:1, depending on the experiments. This implies that approximately fifteen to thirty times as much fucose was incorporated into preexisting xyloglucan as into newly elongated regions. This is consistent with the observation that fucose incorporation was not dependent upon elongation of the chains (Table I).

The data show that *in vitro* in the presence of exogenous UDP-Glc, UDP-Xyl, and UDP-Gal, elongation of acceptor xyloglucan molecules is not sufficient to be detectable. Elongation to some extent must presumably occur, including the addition of fucose, but it is concluded that synthesis of xyloglucan nonasaccharide by microsomes proceeds primarily by transfer of fucose residues from GDP-fucose to large pre-made primer chains.

**LITERATURE CITED**

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