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Further Studies of the Ability of Xyloglucan Oligosaccharides to Inhibit Auxin-Stimulated Growth[1]

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ABSTRACT

The structural features required for xyloglucan oligosaccharides to inhibit 2,4-dichlorophenoxyacetic acid-stimulated elongation of pea stem segments have been investigated. A nonasaccharide (XG9) containing one fucosyl-galactosyl side chain and an undecasaccharide (XG11) containing two fucosyl-galactosyl side chains were purified from endo-β-1,4-glucanase-treated xyloglucan, which had been isolated from soluble extracellular polysaccharides of suspension-cultured sycamore (*Acer pseudoplatanus*) cells and tested in the pea stem bioassay. A novel octasaccharide (XG8′) was prepared by treatment of XG9 with a xyloglucan oligosaccharide-specific α-xylosidase from pea seedlings. XG8′ was characterized and tested for its ability to inhibit auxin-induced growth. All three oligosaccharides, at a concentration of 0.1 microgram per milliliter, inhibited 2,4-dichlorophenoxyacetic acid-stimulated growth of pea stem segments. XG11 inhibited the growth to a greater extent than did XG9. Chemically synthesized nona- and pentasaccharides (XG9, XG5) inhibited 2,4-dichlorophenoxyacetic acid-stimulated elongation of pea stems to the same extent as the same oligosaccharides isolated from xyloglucan. A chemically synthesized structurally related heptasaccharide that lacked a fucosyl-galactosyl side chain did not, unlike the identical heptasaccharide isolated from xyloglucan, significantly inhibit 2,4-dichlorophenoxyacetic acid-stimulated growth.

Xyloglucans are hemicellulosic polysaccharides present in the primary cell walls of all types of higher plants (2, 6, 8, 10, 11, 23). They consist of a β-1,4-D-glucan backbone with α-D-xylosyl residues glycosidically attached, in a regular pattern, to the 6-position of approximately 75% of the glucosyl residues of the backbone (3, 15, 19). Some of the α-D-xylosyl residues are themselves substituted at the 2-position with β-galactosyl or α-fucosyl-β-1,6-galactosyl moieties.

Treatment of xyloglucan with a fungal endo-β-1,4-glucanase results in the cleavage of unbranched 4-linked glucosyl residues in the glucan backbone, generating oligosaccharide subunits of the polymer (2, 21, 22). The quantitatively most prevalent subunits are XG7[3] and XG9. XG9 has been shown to inhibit auxin (2,4-D) induced elongation of pea stem segments, whereas XG7 did not significantly inhibit the elongation (5, 12, 13, 21).

XG5, purified from rose xyloglucan, and a trisaccharide fucosyl lactose, both related in structure to XG9, have been reported to inhibit, albeit less effectively than XG9, auxin-stimulated elongation in the pea stem bioassay (13). XG9, XG5, and fucosyl lactose each contain an α-fucosyl residue attached to the 2-position of the galactosyl residue; however, XG8, which is identical with XG9 except for the absence of the terminal α-fucosyl residue, is unable to inhibit auxin-induced growth, providing evidence that the terminal α-fucosyl residue of XG9 is essential for its growth-inhibiting activity. In this paper, we examine the abilities of three other fucose-containing oligosaccharides to inhibit 2,4-D-stimulated elongation of stem segments excised from etiolated pea seedlings.

The effect on the pea stem bioassay activity of adding a second fucosyl-galactosyl side chain to XG9 was determined by isolating and testing XG11. We also purified, characterized, and tested novel XG8′, prepared by treatment of XG9 with an endogenous pea enzyme, a xyloglucan oligosaccharide-specific α-xylosidase that removes the xylosyl residue farthest from the reducing end of XG9. Finally, we present the results of determining, in the pea stem bioassay, the activity of chemically synthesized XG9, XG7, and XG5.

MATERIALS AND METHODS

Purification of a Xyloglucan Oligosaccharide-Specific α-Xylosidase from Pea Stems

XG8′ (Fig. 1) was prepared by digestion of XG9 with a selective α-xylosidase purified from auxin-treated pea (*Pisum sativum*) stems as described previously (16), except that the following procedures were performed after cation-exchange chromatography on S-Sepharose (Pharmacia). Fractions from the S-Sepharose chromatography that contained the peak of

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[3] Abbreviations: XG5, XG7, XG8′, and XG9, xyloglucan-derived penta-, hepta-, octa-, and nonasaccharides illustrated in Figure 1; XG9r and XG11r, the reduced xyloglucan-derived nona- and undecasaccharides illustrated in Figure 1; HPAE, high-performance anion-exchange; FAB, fast atom bombardment.

α-xylosidase activity were pooled and dialyzed against 50 mM Tris-HCl, pH 8.0, in preparation for copper-chelating chromatography. A 1- × 7-cm column containing chelating Sepharose (Pharmacia) was prepared as described before (17). The sample was loaded onto the column that was then washed with 60 mL of 50 mM Tris-HCl, pH 8.0, followed by 35 mL of 100 mM sodium phosphate, pH 6.5, containing 0.8 M NaCl. The column was subsequently washed with 35 mL of 100 mM sodium acetate, pH 4.5, containing 0.8 M NaCl. The flow rate was 1 mL/min. The proteins eluted by the phosphate and acetate buffers had no xylosidase activity. The α-xylosidase was eluted with 50 mM of EDTA, pH 7.6, containing 0.5 M NaCl. The EDTA fraction (35 mL) that contained most of the xylosidase activity was dialyzed against 100 mM sodium acetate, pH 4.5, in preparation for XG9 digestion.

Colorimetric Assays

Pentoses were quantitated by the orcinol method (4) and hexoses by the anthrone method (21).

Preparation of XG9, XG7, and XG11

Xyloglucan was purified from the extracellular polysaccharides of suspension-cultured sycamore cells as described previously (22). *Trichoderma viride* endo-β-1,4-glucanase was purified and used to generate xyloglucan oligosaccharides as described before (2, 21, 22). Xyloglucan oligosaccharides (25 mg of total sugar) were desalted on a 1.5- × 20-cm column of Sephadex G-10 (Pharmacia) equilibrated in water. Anthrone-positive (22) material that eluted free of salts was pooled and lyophilized. The resulting dry carbohydrate was dissolved in 0.5 mL of water and applied to a 1.6- × 196-cm column of Bio-Gel P-2, minus 40 mesh, and eluted at 10 mL/h with water. The nonasaccharide- and heptasaccharide-rich fractions (16) were collected and lyophilized to dryness. XG11 was purified as described previously (9).

Reduction of XG9 and XG11

XG9 was converted into its corresponding octaglycosyl alditol (XG9r) by reduction with $NaBH_4$ of the aldehyde moiety of the glucose residue located at the reducing end of the oligosaccharide (20). The reduction was achieved by dissolving XG9 (1 mg) in 0.5 mL of 1 M NH_4OH containing 5 mg of $NaBH_4$. After 2 h at room temperature, 100 μL of glacial acetic acid was added to the reaction mixture to convert the remaining borohydride to borate. The reaction mixture was then dried under a stream of filtered air, and borate was removed as trimethyl borate by evaporation with methanol (1). The reduced nonasaccharide was passed through a column (1 × 4 cm) of Amberlite MB-3 (Sigma) to remove sodium ions. The eluate was lyophilized. XG11 was reduced to XG11r as described before (9).

Formation and Purification of XG8′

XG9 (1.2 mg) was incubated (30°C for 20 h) in 250 μL of 100 mM sodium acetate, pH 4.5, containing 0.02% sodium azide and 40 units of α-xylosidase. (One unit of α-xylosidase is defined as the amount of enzyme required to produce 1 nmol of reducing xylose per hour, using reduced xyloglucan heptasaccharide as substrate.) The resulting digest was passed through a column (1 × 4 cm) of Amberlite MB-3 (Sigma) to remove sodium ions and then lyophilized to remove acetic acid. XG8′ was purified from the residue by HPAE chromatography on a CarboPac PA-1 column using a Dionex metal-free BioLc liquid chromatograph interfaced to an AutoIon Series 450 data system (Dionex Corp., Sunnyvale, CA).

Unaltered XG9, XG8′, and free α-D-xylose were detected in the column eluant using a Dionex pulsed amperometric detector equipped with a gold working electrode. The amperometric detector was operated in the integrated amperometry mode at 1000 nA sensitivity. The following pulse potentials ($E_1 - E_3$) and durations ($t_1 - t_3$) were used for detection of the oligosaccharides: $E_1 = 0.05$ V ($t_1 = 500$ ms); $E_2 = 0.6$ V ($t_2 = 80$ ms); $E_3 = -0.6$ V ($t_3 = 50$ ms). The column was eluted with 100 mM NaOH (0–5 min) followed by a linear gradient of sodium acetate (0–60 mM) in 100 mM NaOH (5.1–40 min). The column was then eluted isocratically with 60 mM sodium acetate in 100 mM NaOH (40.1–50 min) and reequilibrated for 10 min in 100 mM NaOH before the next injection. The flow rate was 1 mL/min. The eluants were prepared by suitable dilution of aqueous 50% NaOH with ultrapure water and sodium acetate trihydrate (biochemical grade, Fluka). Eluants were filtered (0.2-μm Nylon 66 membranes) and degassed with helium using an eluant degas module (Dionex). XG8′ was separated from XG9 and free α-D-xylose on a 9- × 250-mm CarboPac PA-1 column (Dionex). Fractions were collected every 30 s. The flow rate was 5 mL/min. Orcinol-positive peaks were pooled for further analysis.

FAB-MS of XG8′

Positive-ion FAB-mass spectra of purified XG8′ (Fig. 3) were obtained using a VG Analytical ZAB SE mass spectrometer operated at low resolution (1:1000), with an accelerating voltage of 8 kV. Underivatized XG8′ (10 μg) in 1 μL of water was applied with 2 μL of glycerol matrix to the stainless steel target of the spectrometer. Spectra were recorded at a scan rate of 30 scans/decade, covering a range of 2000 to 200 atomic mass units. Ions were labeled with their nominal masses.

Pea Stem Growth Bioassay

The oligosaccharides were tested for their ability to inhibit 2,4-D-stimulated elongation of pea stem segments. Each oligosaccharide was dissolved in 2 mL of freshly prepared incubation medium consisting of 1% sucrose, 0.02% benzylpenicillin (potassium salt; Sigma), and 5 mM potassium phosphate (pH 6.1). Stem segments (6 mm long starting 3 mm from the maximum curvature of the apical hook) of Early Alaska peas were cut from the third internode of 7-d-old, etiolated pea seedlings. The segments were washed with the incubation medium for 30 min and individually floated (10 per treatment) in a small glass vial (2.5 cm diameter; 5 cm height) containing 2 mL of the same medium plus oligosaccharides to be tested. The vials were placed on a gyratory shaker (70 rpm at 22°C) for 30 min. A synthetic auxin, 2,4-D, was then added to a final concentration of 5 μM, the lowest

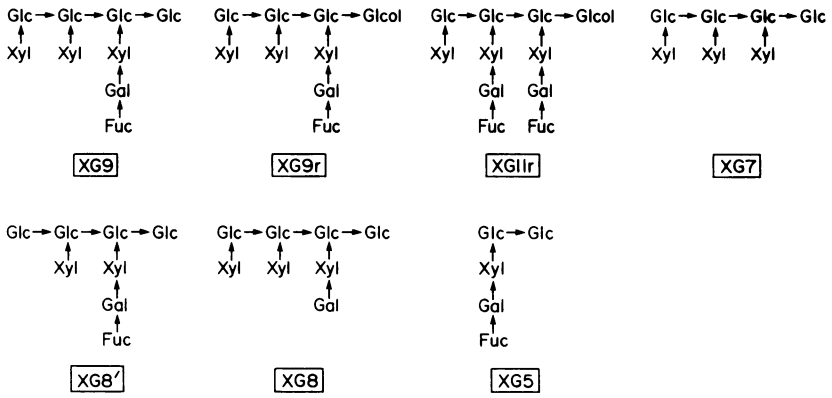

Figure 1. Structures of xyloglucan oligosaccharides. XG8′ is the octasaccharide generated by xylosidase digestion of XG9 (Note: The octasaccharide XG8 is XG9 minus the fucose [9].) XG9r and XG11r are the oligoglycosyl alditols prepared by converting the reducing glucose residue of XG9 and XG11, respectively, to glucitol.

concentration that resulted in the maximum rate of elongation of the stem segments. The segments were incubated with shaking for 17 h. The final length of each segment was measured using a profile projector. Data are presented as the percentage of inhibition of 2,4-D-stimulated growth, calculated as

$$\% \text{ inhibition} = \frac{L_{(2,4\text{-D})} - L_{(2,4\text{-D+test})}}{L_{(2,4\text{-D})} - L_{(\text{con})}} \times 100\%,$$

where $L_{(2,4\text{-D})}$ is the mean final length of segments treated with 2,4-D, $L_{(\text{con})}$ is the mean final length of segments incubated without 2,4-D, and $L_{(2,4\text{-D+test})}$ is the mean final length of segments treated with 2,4-D plus the oligosaccharide.

Preparation of Synthetic Oligosaccharides

Samples of chemically synthesized XG9, XG7, and XG5 were prepared as described previously (18).

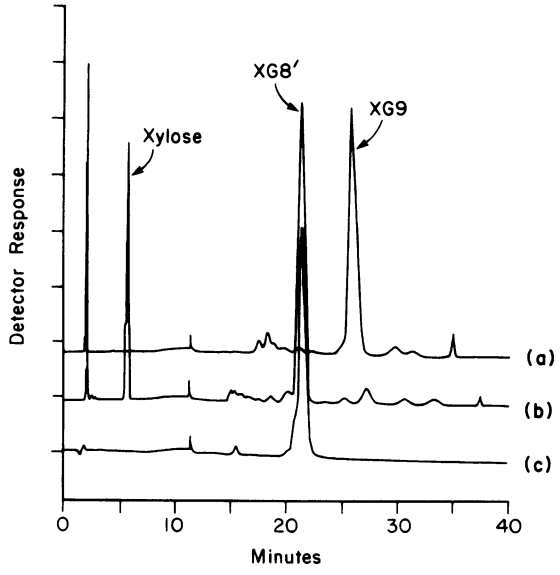

Figure 2. Separation of xyloglucan oligosaccharides using HPAE. The chromatography was on a Dionex CarboPac PA-1 column (4 × 250 mm) with pulsed amperometric detection. a, Chromatography of ≈2 μg of XG9; b, chromatography of products generated by α-xylosidase digestion of XG9; c, rechromatography of purified XG8 ′.

RESULTS AND DISCUSSION

Purification of XG8′

One of our goals is to determine which glycosyl residues of XG9 are required for its growth-inhibiting activity. A highly specific α-xylosidase is present in pea stem tissue that converts XG9 into XG8′ and free xylose (16; Fig. 1). XG8′ was generated from XG9, purified, and bioassayed to determine whether the loss of the xylosyl residue altered the biological activity of XG9. XG8′ was purified by HPAE (Fig. 2) as described in "Materials and Methods." The HPAE peak eluting at 5 min (Fig. 2b) was identified as xylose by GC of its alditol acetate derivative (data not shown). The peak eluting at 21 min was pooled and, to remove sodium ions, was passed through a Dionex Onguard-H, strong cation-exchange column in the hydrogen form. The eluate was lyophilized, and an aliquot was rechromatographed on the Dionex CarboPac PA-1 column (Fig. 2c). A single peak with a retention time of 21 min was obtained. The mol wt of this oligosaccharide product was determined by FAB-MS (Fig. 3). The observed pseudomolecular ion [M + H] at m/z 1239 corresponded in mass to XG8′ (Fig. 1). The peaks at 1261 and 1277 were equivalent to the [M + Na^+] and [M + K^+] ions, respectively, of XG8′. Thus, XG8′ was generated, purified, and available for bioassay.

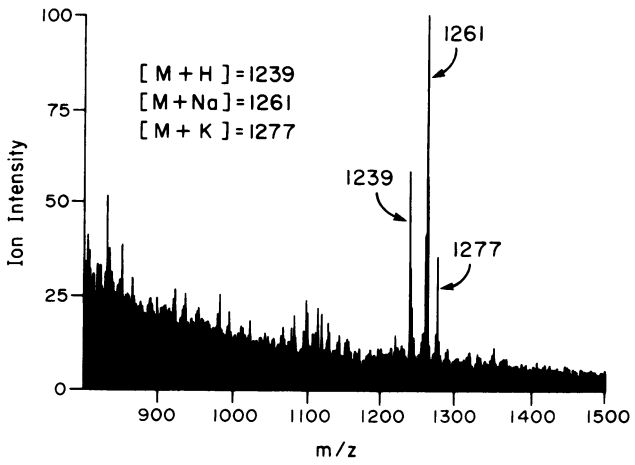

Figure 3. Positive-ion FAB-mass spectrum of XG8′.

	Expt 1	Expt 2	Expt 3	Expt 4
	Mean final length of segments (mm)			
−2,4-D	7.8 ±0.1	7.0 ±0.1	7.0 ±0.1	8.0 ±0.1
+2,4-D	10.9 ±0.2	9.6 ±0.1	9.7 ±0.2	11.0 ±0.1

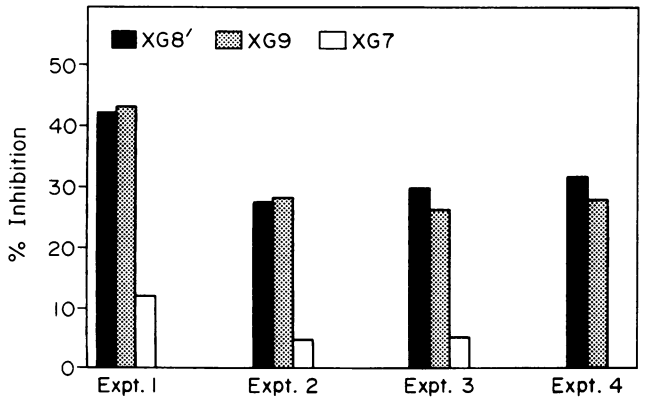

Figure 4. Effect of XG8′, XG9, and XG7 on 2,4-D-stimulated elongation of pea stem segments. Experiments were conducted on different days. The mean final length of 10 or 20 segments ± SE, grown in the presence or absence of 5 μM 2,4-D and in the absence of oligosaccharides, is given for each of the four experiments. The effect of the oligosaccharides on the percentage of inhibition of 2,4-D-stimulated growth is given below (as defined in "Materials and Methods"). Each value was calculated from measurements of 20 segments. Two replicate vials (10 segments each) were used for each treatment. The effect of XG7 was not tested in experiment 4.

	Expt 1	Expt 2	Expt 3
	Mean final length of segments (mm)		
−2,4-D	8.0 ±0.1	7.0 ±0.1	7.1 ±0.1
+2,4-D	11.0 ±0.1	9.7 ±0.1	10.3 ±0.1

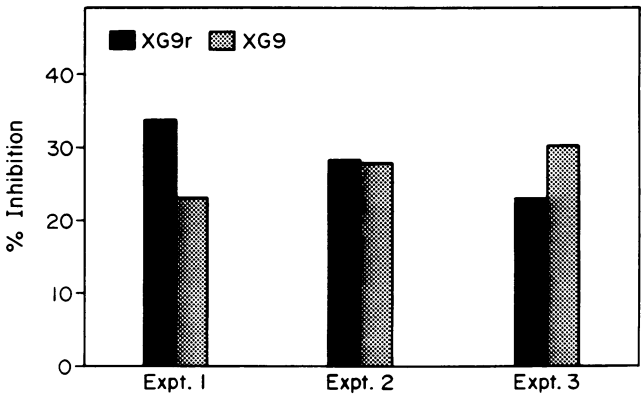

Figure 5. Effect of XG9r and XG9 on 2,4-D-stimulated elongation of pea stem segments. Details are the same as given in the legend to Figure 4.

Inhibition by Xyloglucan Oligosaccharides (XG8′, XG9, XG9r, XG11r) of Auxin-Induced Growth of Pea Stem Segments

The ability of xyloglucan oligosaccharides, at 0.1 μg/mL, to inhibit 5 μM 2,4-D-induced growth of pea stem segments was determined (Figs. 4–6). The 2,4-D concentration (5 μM) was the lowest concentration resulting in maximum elongation. The oligosaccharides were assayed at 0.1 μg/mL (between 10^{-8} and 10^{-7} M), because the growth-inhibiting activity of XG9 exhibited a well-defined optimum at that concentration. In addition, the effect of xyloglucan oligosaccharides on the endogenous growth of pea stem segments (*i.e.* in the absence of 2,4-D) was evaluated (Table I). At the concentrations tested, XG9 and XG7 did not significantly inhibit the endogenous growth of pea stem segments.

Biological Activity of XG8′

The fucosyl-galactosyl side chain of XG9 is essential but not sufficient for its biological activity. We were interested, therefore, to investigate to what extent the remainder of the XG9 molecule is essential for inhibiting activity. XG9 was treated with a specific α-fucosidase yielding XG8′, which was purified and tested in the pea stem bioassay. The effect of the

	Expt 1	Expt 2	Expt 3	Expt 4
	Mean final length of segments (mm)			
−2,4-D	6.9 ±0.1	7.3 ±0.1	6.9 ±0.1	6.5 ±0.1
+2,4-D	9.4 ±0.2	9.5 ±0.1	9.0 ±0.1	9.1 ±0.1

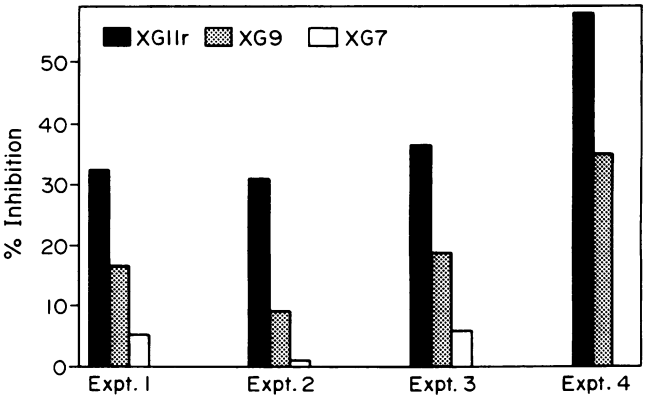

Figure 6. Effect of XG11r, XG9, and XG7 on 2,4-D-stimulated elongation of pea stem segments. Details are the same as given in the legend to Figure 4.

Table I. *Effect of Xyloglucan Oligosaccharides on Endogenous Growth of Pea Stem Segments*

Additives		Oligosaccharide Concentration (μg/mL)					
5 μM 2,4-D	Oligosaccharide	10	1	10^{-1}	10^{-2}	10^{-3}	0
		mean final length of segments (mm)					
−	None						6.57 ± 0.10
+	None						9.05 ± 0.16
+	XG9	nd[a]	nd	8.46 ± 0.13	nd	nd	
−	XG9	6.60 ± 0.06	6.56 ± 0.05	6.47 ± 0.06	6.55 ± 0.08	6.59 ± 0.08	
−	XG7	6.54 ± 0.06	6.58 ± 0.06	6.53 ± 0.05	6.52 ± 0.07	6.56 ± 0.07	

[a] nd, Not done.

concentration of XG8′ on its bioactivity was shown to be the same (optimum approximately 10^{-8} M) as for synthetic XG9 (Fig. 7), the activity of which is also indistinguishable from that of natural XG9. In four independent experiments, XG8′ was as effective as XG9 in inhibiting 2,4-D-stimulated elongation of pea stem segments (Fig. 4). Thus, the terminal xylosyl residue of XG9 is not essential for its biological activity.

Biological Activity of XG11r and XG9r

XG11 is a recently isolated (9) xyloglucan undecasaccharide fragment that contains two fucosyl-galactosyl side chains. It was of interest, therefore, to determine the effect of a second fucosyl residue on the ability of the oligosaccharide to inhibit pea stem growth. XG11 was purified from the endo-β-1,4-glucanase digest of the xyloglucan that had been purified from the extracellular polysaccharides of suspension-cultured sycamore cells (9). XG11 was reduced to its corresponding oligoglycosyl alditol derivative, XG11r, to facilitate its characterization. The unreduced undecasaccharide was not available for testing in the pea stem bioassay. Therefore, XG9 was converted to XG9r (the glucose residue at the reducing end was converted to glucitol) to determine whether this modification would interfere with its growth-inhibiting activity. In three different experiments, XG9r was found to be as effective as XG9 in inhibiting 2,4-D-stimulated elongation of pea stem segments (Fig. 5).

XG9r differs from XG11r in that the former lacks a second fucosyl-galactosyl side chain (Fig. 1). XG11r was, in four independent experiments, more effective than XG9 in inhibiting 2,4-D-stimulated elongation of pea stem segments (Fig. 6). Because XG9 and its corresponding oligoglycosyl alditol derivative, XG9r, show no detectable difference in growth-inhibiting activity (Fig. 5), the increased inhibition provided by XG11r relative to XG9 can be attributed to the presence of the second fucosyl-galactosyl side chain. We have identified, therefore, a new biologically active oligosaccharide that inhibited the growth of pea stem segments to a greater extent than did XG9.

XG7 did not significantly inhibit auxin-induced elongation of pea stem segments, as shown in earlier reports (12–14, 21).

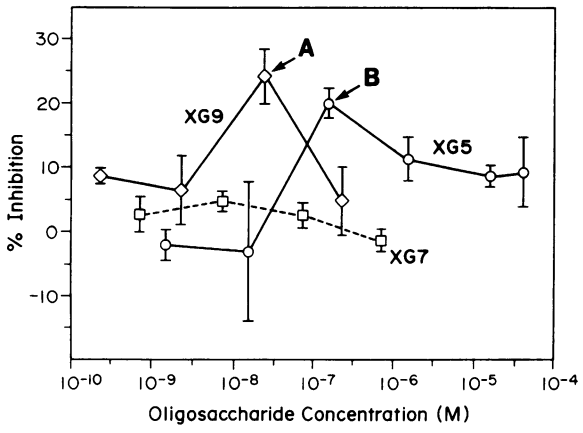

Figure 7. Effect of synthetic xyloglucan oligosaccharides on 2,4-D-stimulated elongation of pea stem segments. ◇, Synthetic XG9; □, synthetic XG7; ○, synthetic XG5. A, Value for XG9 isolated from sycamore extracellular polysaccharide xyloglucan; B, value for XG5 isolated from sycamore extracellular polysaccharide xyloglucan. Values are percentages of inhibition as defined in "Materials and Methods." Each value is the average of four treatments ± SD. The value of each treatment was calculated from measurements of 20 segments (two replicate vials of 10 segments each).

Biological Activity of Synthetic Oligosaccharides

The structure of XG9 has now been confirmed by chemical synthesis (18). The synthetic nonasaccharide was tested in the pea stem bioassay to rule out the possibility that a contaminant, which may have copurified along with XG9 of natural origin, was responsible for the ability to inhibit pea stem growth. Therefore, we tested synthetic XG9 as well as synthetic XG7 and synthetic XG5 in the pea stem bioassay. The ability of synthetic XG9 to inhibit auxin-induced growth of pea stems was, at the concentrations tested (10^{-6}–10^{-10} M), identical with that of XG9 of natural origin (Fig. 7). The concentration optimum for inhibition was approximately 10^{-8} M. Higher concentrations of synthetic XG9 did not inhibit 2,4-D-stimulated growth. The same result was obtained with natural XG9. Like natural XG7, synthetic XG7 did not result in significant inhibition of growth (Fig. 7). XG5 inhibited 2,4-D-stimulated growth with a concentration optimum for inhibition of approximately 10^{-7} M (Fig. 7). The inhibition by XG5 did not show a clear-cut diminution in growth-inhibiting activity at concentrations $>10^{-7}$ M, as shown by XG9; the diminution in growth-inhibiting activity was also not evident with XG5 of natural origin (13). Thus, XG9 and XG5, and not an undetected contaminant, were

responsible for the inhibition of 2,4-D-stimulated growth of pea stems.

CONCLUSIONS

XG9, XG11, and XG5 are oligosaccharins (oligosaccharides with regulatory properties) that inhibit 2,4-D-stimulated elongation of pea stem segments (5, 12, 14, 21). Chemically synthesized XG9 and XG5 confirmed that these oligosaccharides and not an unidentified contaminant are responsible for the observed inhibition of 2,4-D-stimulated growth of pea stem segments.

McDougall and Fry (14) reported that a decasaccharide (XG10) similar to XG11 but lacking the fucosyl residue farthest from the reducing end showed no biological activity despite the presence of the required fucosyl-galactosyl side chain. Apparently, the terminal β-galactosyl residue in XG10 interferes with binding to the putative receptor. Substitution at the 2-position of this β-galactosyl residue with a terminal α-fucosyl residue restored and even elevated the growth-inhibiting activity (and, therefore, binding to its putative receptor). The addition of the second fucosyl residue is either required by the oligosaccharide to adopt the conformation that binds with high affinity to its physiological receptor or is required to cause a more effective conformation change in the receptor by more completely filling the active site.

The present paper provides further evidence for the major role of terminal α-L-fucosyl residues in the ability of xyloglucan oligosaccharides to inhibit 2,4-D-stimulated growth of pea stem segments. The results strengthen the proposed feedback control loop hypothesis (21) in which high concentrations of auxin promote the formation of bioactive oligosaccharides that play an essential role in regulating the development of the growing plant.

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