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Structure-Activity Relationships for Xyloglucan Oligosaccharides with Antiauxin Activity[1]

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ABSTRACT

This work was designed to investigate the structural features required for a branched xyloglucan nonasaccharide (XG9; composition: glucose$_4$xylose$_3$galactose$_1$fucose$_1$) to exhibit anti-auxin activity in the pea (*Pisum sativum* L.) stem segment straight-growth bioassay. Oligosaccharides were prepared by cellulase-catalyzed hydrolysis of *Rosa* xyloglucan, and tested for auxin antagonism. The quantitatively major hepta-, octa-, and decasaccharides (XG7, XG8, and XG10) showed no antiauxin activity at the concentrations tested and did not interfere with the antiauxin effect of 10^{-9} molar XG9 when coincubated at equimolar concentrations. The results indicate that the XG9-recognition system in pea stem segments is highly discriminating. A terminal α-L-fucose residue is essential for the antiauxin activity of XG9 and a neighboring terminal β-D-galactose residue can abolish the activity; possible reasons for the effect of the galactose residue are discussed. A sample of XG9 extensively purified by gel-permeation chromatography followed by paper chromatography in two solvent systems still exhibited antiauxin activity with a concentration optimum around 10^{-9} molar. This diminishes the likelihood that the antiauxin activity reported for previous nonsaccharide preparations was due to a compound other than XG9.

Xyloglucan contributes 20 to 25% of the dry weight of the primary walls of dicotyledons and probably plays a role in dictating wall extensibility (7, 10, 17, 20). Xyloglucan is partially degraded during auxin-induced growth. Auxins induce the nicking (11, 21), solubilisation (24), and more complete degradation of xyloglucan (9, 15). These effects are probably mediated by auxin-induced synthesis of cellulases (26) and the activation of these enzymes by H^+ ions secreted into the wall in response to auxin (8). Plant cellulases will *in vitro* hydrolyze xyloglucan into oligosaccharides including a nonsaccharide (XG9[2]) and heptasaccharide (XG7) (11).

XG9, obtained by digestion of xyloglucan by fungal cellulase, inhibited the 2,4-D-induced elongation of etiolated pea stem segments (19, 27). XG9 and other oligosaccharides of XG have been detected *in vivo* (5), indicating that XG9 may be a natural antiauxin. A feedback loop has been postulated in which excessive levels of growth-promoting auxin are countermanded by the production of the growth-inhibiting XG9 (4).

In this paper we examine the antiauxin activity of xyloglucan oligosaccharides in greater detail. We have confirmed that XG7 (which lacks the galactose and fucose residues of XG9—see Fig. 1) was neither itself an antiauxin nor did it interfere with the inhibition caused by 10^{-9} M XG9 (19). It therefore seemed logical to assume that the L-fucosyl-α-(1 → 2)-D-galactosyl-β-(1 → 2) sidechain is important in determining the biological activity of XG9. To explore further the structural requirements for antiauxin activity, we have prepared XG8 and XG10 and tested them in the pea stem bioassay. The results indicate that the fucose residue is essential for antiauxin activity.

MATERIALS AND METHODS

Maintenance of Cultures

Suspension-cultures of "Paul's Scarlet" rose (*Rosa* sp.) were maintained as described before (19).

Paper Chromatography

Descending PC was on Whatman 3MM paper in solvents BAW, BPW, EAW, and EPW. For BPW, EAW, and EPW, the paper was serrated and the solvent allowed to drip off. Monosaccharides were stained with aniline hydrogen phthalate, and oligosaccharides with $AgNO_3$/NaOH (6). For quantitative analysis, monosaccharides were stained with aniline hydrogen phthalate with heating at 105°C for 10 min and the appropriate spots eluted with 2% (w/v) $SnCl_2$ in methanol; the colored material was assayed spectrophotometrically (A_{370}) (6).

Acid Hydrolysis

Oligosaccharides were hydrolyzed in 2 M trifluoroacetic acid at 120°C for 1 h. The hydrolyzate was chromatographed in BAW followed in the same dimension by EPW (6). For analysis of fucose, BAW was used alone as this gave clearest resolution of fucose from xylose.

Gel-Permeation Chromatography

Samples (10 mL) of cellulase-hydrolysis products were mixed with 20 mg Blue Dextran + 20 mg glucose and passed

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[2] Abbreviations: XG7, XG8, XG9, XG10, the xyloglucan-derived hepta-, octa-, nona-, and decasaccharides illustrated in Figure 1; BAW, butan-1-ol/acetic acid/water (12:3:5, by volume); BPW, butan-1-ol/pyridine/water (4:3:4, by volume); EAW, ethyl acetate/acetic acid/water (10:5:6, by volume); EPW, ethyl acetate/pyridine/water (8:2:1, by volume); GPC, gel-permeation chromatography; $k_{av.}$, elution volume relative to Blue Dextran ($k_{av.}$ = 0.0) and glucose ($k_{av.}$ = 1.0); PC, paper chromatography; $R_{maltoheptaose}$, chromatographic mobility relative to maltoheptaose.

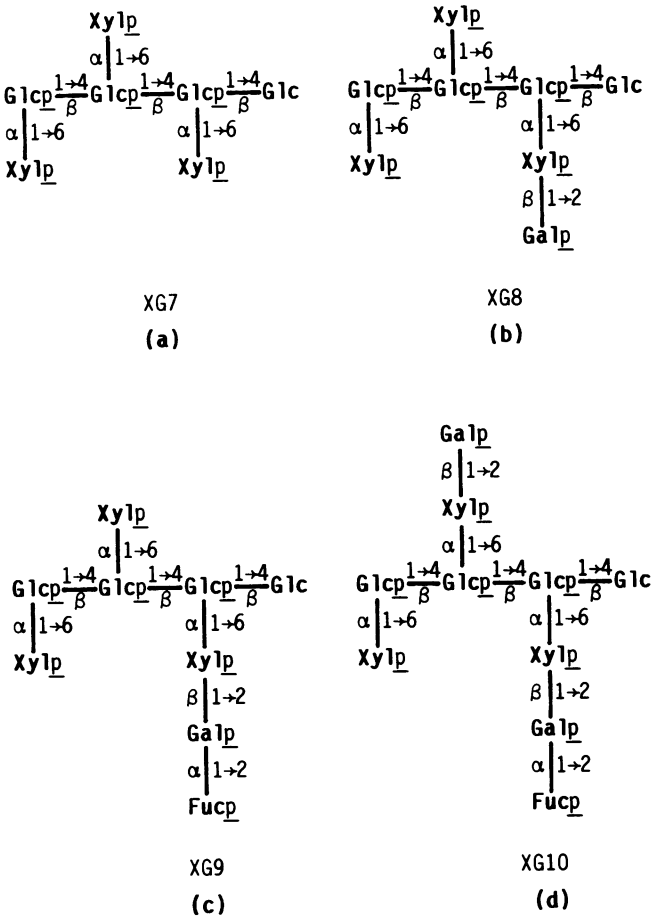

Figure 1. Probable structures of the oligosaccharides employed in the bioassays. The structures shown are those reported (2, 12, 13, 18, 25) for the quantitatively major oligosaccharides of each size-class isolated from cellulase-digests of xyloglucan (see ref. 7 for a review).

through a 280-mL bed-volume column of Bio-Gel P-2 equilibrated in water at about 20°C. Blue Dextran was detected by A_{620} and glucose by the anthrone method (1). Fractions were dried *in vacuo*.

Production of Seed Xyloglucan

A homogenate of 20 dry nasturtium seeds (*Tropaeolum majus* L.) in 10 mL H_2O was added to 90 mL 2 M NaOH/0.05% (w/v) $NaBH_4$ and heated at 100°C for 1 h (2). After centrifugation (1400*g* for 10 min) the supernatant was mixed with 200 mL of acetic acid/ethanol (1:10, v/v) and left to stand for 30 min. The crude xyloglucan which precipitated was pelleted by centrifugation and purified by the method of Rao (23). The pellet was redissolved in 80 mL water plus 10 mL Fehling's solution B (35% [w/v] potassium sodium tartarate tetrahydrate in 25% [w/v] KOH) and heated at 100°C for 30 min. After cooling, 10 mL Fehling's solution A [7.5% (w/v) $CuSO_4$] was added. The blue Cu-xyloglucan complex was collected by centrifugation and dissolved in 20% acetic acid. The decomplexed xyloglucan was reprecipitated by the addition of two volumes of ethanol and collected by centrifugation. The purified xyloglucan was resuspended in about 5% (w/v) EDTA in 50% (v/v) ethanol and washed by recentrifugation in 50% ethanol to remove any remaining complexed Cu.

Preparation of XG8 from Seed Xyloglucan

A 1% (w/v) suspension of nasturtium xyloglucan was shaken at 25°C for 20 min in 1% (w/v) *Trichoderma* cellulase (Sigma Chemical Co., product No. C 2274) containing 20 mM acetate (Na^+, pH 4.7). After centrifugation (1000*g* for 10 min), the supernatant was fractionated by GPC on Bio-Gel P-2. Fractions were assayed with anthrone (1) for hexose and the octasaccharide-enriched peak ($k_{av.}$ = 0.45–see Fig. 2a, shaded zone) was run by PC in EAW for 48 h. The major $AgNO_3$/NaOH-staining band ($R_{maltoheptaose}$ = 0.7–0.95) was eluted in water (3) and re-run in BPW for 24 h. The only $AgNO_3$/NaOH-staining zone ($R_{maltoheptaose}$ 0.80–1.10) was eluted in H_2O and stored in 25% (v/v) ethanol.

Preparation of XG7, XG9, and XG10

XG7 and XG9 were obtained from cellulase-digests of rose hemicellulose separated by GPC on Bio-Gel P-2 (19). XG10 was obtained as a shoulder on the leading edge of the XG9 peak (Fig. 1a of [19]). When re-run on Bio-Gel P-2 the material eluted as a peak with $k_{av.}$ = 0.36, characteristic of a decasaccharide, which was collected, excluding the trailing edge so as to minimize contamination with XG9. Upon acid hydrolysis the material gave the same glucose:xylose:fucose ratio as XG9 (5), but had a higher galactose:glucose ratio. It is concluded to be a decasaccharide, $Glc_4 \cdot Xyl_3 \cdot Gal_2 \cdot Fuc_1$ (XG10). This XG10 showed exact cochromatography, on HPLC on Amino-Spheri-5 (Brownlee) in 50% CH_3CN, with XG-FOS-10 (13, 18), the major decasaccharide of xyloglucan (Fig. 1d). (We are most grateful to Dr. Y. Kato [Hirosaki University, Japan] for the gift of authentic XG-FOS-10).

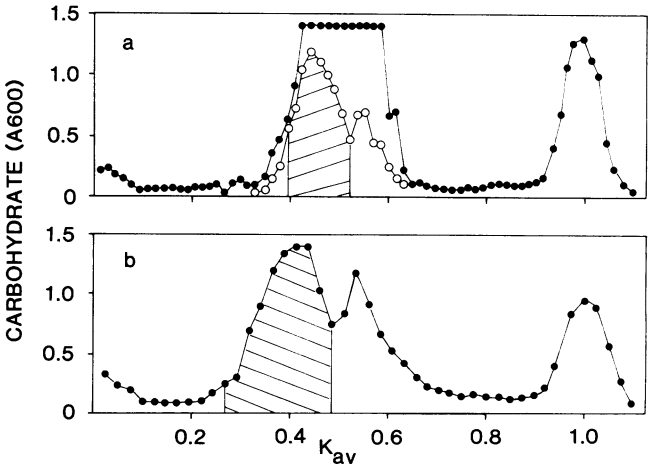

Figure 2. Elution profiles on Bio-Gel P-2 of cellulase-digests of (a) purified xyloglucan from nasturtium seeds and (b) crude hemicellulose from cultured rose cells. In (a), the two curves represent analyses of 50- (●) and 25-μL (○) aliquots from each fraction. The $k_{av.}$ values were estimated relative to the elution of internal markers of Blue Dextran ($k_{av.}$ = 0.00) and glucose ($k_{av.}$ = 1.00).

Further Purification of XG9

A batch of XG9-enriched material was prepared from rose hemicellulose as before (19) (Fig. 2b). Fractions marked by shading (XG9-rich; $k_{av.}$ about 0.4) were pooled and run by PC in EAW for 48 h. The major $AgNO_3$/NaOH-staining band ($R_{maltoheptaose}$ = 0.9–1.1) was eluted and re-run in BPW for 24 h. The only $AgNO_3$/NaOH staining band ($R_{maltoheptaose}$ = 0.8–1.1) was eluted in water and stored in 25% (v/v) ethanol. The $R_{maltoheptaose}$ values agree with those reported earlier (5).

Pea Stem Bioassay

The oligosaccharides were tested for their ability to inhibit the 2,4-D-induced elongation of pea stem segments as before (19). In brief, 7- to 8-d-old, etiolated seedlings of pea (*Pisum sativum* L. cv Alaska) were selected in which the third internode was 1 to 3 cm long, and a 6-mm segment was cut from the straight portion of this internode starting 2 mm below the hook. The excised segments were shaken gently for 30 min in water and then for 90 min in assay medium (1% [w/v] sucrose, 5 mM KH_2PO_4, and 0.02% potassium benzyl penicillin, pH 6.1). The segments were placed in 5-cm Petri dishes (8/dish) containing 5 mL assay medium + test material. The assay was started by addition of 2,4-D to a final concentration of 10^{-6} M (the lowest concentration giving maximum elongation). Controls lacking 2,4-D were run during each experiment. The Petri dishes were shaken gently in the dark at 25°C for 18 h and the segments were then measured. Two or three replicate dishes (16 or 24 segments) were used for each treatment. Data are presented as mean percentage 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 $L_{(2,4\text{-D})}$ = mean final length of segments treated with 2,4-D; $L_{(\text{con})}$ = mean final length of segments incubated without 2,4-D; $L_{(2,4\text{-D + test})}$ = mean final length of segments treated with 2,4-D plus the oligosaccharide.

RESULTS

Preparation and Purification of XG8

Nasturtium seed hemicellulose lacks fucose (2, 16) and was thus chosen as the starting material for preparation of XG8. GPC of cellulase-digestion products afforded an octasaccharide ($k_{av.}$ 0.45; Fig. 2a), which gave one major band ($R_{maltoheptaose}$ 0.83) on PC in EAW. The material from this band also ran as a single band ($R_{maltoheptaose}$ = 0.97, referred to as XG8) in BPW. On hydrolysis, XG8 gave only glucose, xylose, and galactose (molar ratio 4.0:2.8:1.2). When 250 μg XG8 was acid-hydrolyzed and the entire products were analyzed by PC in BAW, no fucose was detectable. Since the limit of detection is about 1 μg (6), this shows that the XG8 preparation was substantially free of XG9. These facts suggest that the purified XG8 sample was enriched in a major octasaccharide, probably identical with that in Figure 1b.

Further Purification of Xyloglucan Nonasaccharide

By PC in EAW and BPW, XG9 was purified further than in earlier work (19). On acid hydrolysis, the pure XG9 gave glucose, xylose, galactose, and fucose in the molar ratio 4.0:2.9:1.2:1.1, supporting its identity as XG9 (Fig. 1c), the quantitatively major repeating nonasaccharide unit of xyloglucan (12, 25).

Variability of Bioassay Data

The eight stem segments incubated in a single Petri dish showed some variability in elongation. However, no significant difference (t test; $p > 0.1$) in mean length was detected between the eight segments in one dish and the eight in a replicate dish from the same experiment. Therefore, all 16 or 24 segments subjected to a particular treatment on a given day can be viewed as a single population despite the fact that they were distributed between two or three dishes.

Biological Activity of XG7 and XG9

XG7 at 10^{-9} M showed no antiauxin activity in the pea stem bioassay, whereas Bio-Gel P-2-purified XG9 showed a consistent antiauxin effect (Table I). XG7 at 10^{-9} M did not interfere in the antiauxin activity of equimolar XG9 (Table I). Therefore, at the optimal concentration for XG9, XG7 showed no antiauxin activity. These observations agree with the literature and confirm that the following set of negative data is comparable with the earlier reports (19, 27).

Biological Activity of XG8

XG7 lacks the fucose→galactose side-chain of XG9, showing that this side-chain is essential for antiauxin activity. To determine whether the terminal fucose is required or whether the galactose residue alone will impart activity, we prepared XG8 (Fig. 1b). In three independent experiments, 10^{-9} to 10^{-7} M XG8 showed no significant antiauxin activity (Table I), indicating that the terminal fucose residue is essential. The antiauxin action of 10^{-9} M XG9 was not influenced by the simultaneous presence of 10^{-9} or 10^{-6} M XG8 (Table I).

Biological Activity of XG10

XG10 is thought to be identical with XG9 except for the presence of an additional galactose residue, probably attached to the middle xylose residue (13, 18). Since XG10 has terminal fucose, it might be expected to exhibit antiauxin activity. However, 10^{-9} M XG10 did not mimic the antiauxin activity of XG9, nor did it affect the antiauxin activity of coincubated, equimolar XG9 (Table I). Therefore, at the optimal concentration for XG9, XG10 showed no antiauxin activity. (In experiment 1 of Table I, 10^{-8} and 10^{-7} M XG10 also showed no antiauxin activity [data not shown]; lack of material prevented us repeating this.)

Biological Activity of PC-Purified Nonasaccharide

Since the results indicated a very high degree of structural specificity for antiauxin activity, the possibility was considered that the XG9 samples owed their activity to a contaminant unrelated to xyloglucan. To investigate this, PC-purified XG9 was tested at 10^{-7} to 10^{-10} M (Table II). This material gave consistent inhibition of 2,4-D-induced elongation with a concentration optimum around 10^{-9} M. The data argue strongly

Table I. *Effect of XG7, XG8, XG9, and XG10 on the Auxin-Induced Elongation of Pea Stem Segments*

The experiments were conducted on different days. In the top portion of the table, entries are the mean final length of 16 or 24 segments ± SE, defined as $16^{-1/2} \times$ SD or $24^{-1/2} \times$ SD. In the lower portion, values are % inhibition (defined as described in "Materials and Methods") due to the oligosaccharide.

Additives		Experiment							
1 μM 2,4-D	Oligosaccharide(s)	1	2	3	4	5	6	7	8
		Mean final length of segments (mm)							
−	None	7.7 ±0.1	9.1 ±0.1	8.4 ±0.2	8.0 ±0.1	8.1 ±0.1	7.8 ±0.1	9.7 ±0.1	8.5 ±0.3
+	None	9.3 ±0.3	11.1 ±0.1	11.2 ±0.2	10.1 ±0.3	10.6 ±0.3	9.1 ±0.3	12.7 ±0.3	10.9 ±0.3
		% Inhibition of 2,4-D-stimulated elongation							
+	10^{-9} M XG7	−18	3	13	7	1			
+	10^{-9} M XG8						−19	19	1
+	10^{-8} M XG8						4	5	4
+	10^{-7} M XG8						9	11	2
+	10^{-9} M XG9	42[d]	30[b]	48[b]	39[d]	32[d]	69[c]	49[b]	27[e]
+	10^{-9} M XG10	4	10	−5	5	4			
+	10^{-9} M XG7 + 10^{-9} M XG9		29[b]	39[b]	58[a]	29[d]			
+	10^{-9} M XG8 + 10^{-9} M XG9						78[b]		
+	10^{-6} M XG8 + 10^{-9} M XG9						64[c]	58[b]	
+	10^{-9} M XG10 + 10^{-9} M XG9		22[d]	42[b]	60[a]	30[d]			

[a-e] Significantly different from the 2,4-D-only control by Student's *t* test; the probability that the apparent difference was due to chance is indicated: [a] $p < 0.001$, [b] $0.001 \leq p < 0.01$, [c] $0.01 \leq p < 0.02$, [d] $0.02 \leq p < 0.05$, [e] $0.05 \leq p < 0.10$. Where no superscript is shown, $p \geq 0.1$.

Table II. *Effect of Paper Chromatographically Purified XG9 on the Auxin-Induced Elongation of Pea Stem Segments*

The experiments were conducted on different days. In the top portion of the table, entries are the mean final length of 16 or 24 segments ± SE, defined as $16^{-1/2} \times$ SD or $24^{-1/2} \times$ SD. In the lower portion, values are % inhibition (defined as described in "Materials and Methods") due to the oligosaccharide.

Additives		Experiment		
1 μM 2,4-D	Oligosaccharide(s)	9	10	11
		mean final length of segments (mm)		
−	None	9.1 ±0.2	8.1 ±0.2	8.9 ±0.1
+	None	11.3 ±0.4	10.1 ±0.2	12.9 ±0.2
		% inhibition of 2,4-D-stimulated elongation		
+	10^{-10} M XG9 (purified)	26		
+	10^{-9} M XG9 (purified)	59[d]	58[b]	37[a]
+	10^{-8} M XG9 (purified)	14	62[b]	19[a]
+	10^{-7} M XG9 (purified)	−2	−7	

[a-e] See footnotes to Table I.

against the biological activity being due to any substance (*e.g.* an oligopeptide or metal ion complex) other than a xyloglucan-derived oligosaccharide.

DISCUSSION

Purified XG9 inhibited the 2,4-D-stimulated growth of pea stem segments. In the presence of optimal (10^{-6} M) 2,4-D, the inhibition was greatest with about 10^{-9} M XG9, higher concentrations of XG9 being less inhibitory. This agrees with two earlier reports (19, 27), both of which used XG9 isolated by GPC. Our present work included studies of XG9 that had been isolated as before but then further purified by PC in two very different solvent systems. Since PC and GPC separate compounds on the basis of distinct physicochemical parameters, any contaminants of the GPC-purified XG9 that were unrelated to xyloglucan would almost certainly have been removed by PC. Our observations therefore lead us to two conclusions: (a) they strengthen the argument that it is XG9 which is responsible for antiauxin activity; (b) they indicate that the decrease in biological activity of XG9 seen at higher concentrations (*e.g.* 10^{-7} M—Table II) is a true effect of XG9 and not the result of interference by unknown contaminating compounds.

The mechanism by which XG9 exerts its antiauxin effect is unknown but the finding that XG7, XG8, and XG10 were inactive themselves and also unable to interfere with the inhibition caused by XG9 at its optimum inhibitory concentration indicates that a highly discriminating receptor is involved. Such a receptor may be biologically relevant since XG9 and a number of related oligosaccharides are present *in vivo* (5).

Since XG9 is active but XG8 is inactive, the fucose residue must be crucial to activity. However, XG10 has a fucose group but lacks antiauxin activity at concentrations where XG9 is active. One possible explanation is that the terminal fucose residue of XG10 is sterically crowded by the close proximity of the additional galactose residue (Fig. 1d). It can be speculated that the additional galactose residue prevented the essential fucose residue adopting the particular conformation necessary for binding to a putative receptor and thus for biological activity. A second possibility is that XG9 is not itself active but that it is only active in those tissues that can enzymically hydrolyze it to a smaller oligosaccharide (perhaps the pentasaccharide Fuc→Gal→Xyl→Glc→Glc, which is active in its own right [19a]); if so, the presence of the additional galactose residue in XG10 might prevent access of the necessary enzymes (α-xylosidase and β-glucosidase [14, 22]).

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