Inhibition of 2,4-Dichlorophenoxyacetic Acid-Stimulated Elongation of Pea Stem Segments by a Xyloglucan Oligosaccharide[1]

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ABSTRACT

Xyloglucan, isolated from the soluble extracellular polysaccharides of suspension-cultured sycamore (Acer pseudoplatanus) cells, was digested with an endo-β-1,4-glucanase purified from the culture fluid of Trichoderma viride. A nonasaccharide-rich Bio-Gel P-2 fraction of this digest inhibited 2,4-dichlorophenoxyacetic-acid-stimulated elongation of etiolated pea stem segments. The inhibitory activity of this oligosaccharide fraction exhibited a well-defined concentration optimum between 10^{-2} and 10^{-1} micrograms per milliliter. Another fraction of the same xyloglucan digest, rich in a structurally related heptasaccharide, did not, at similar concentrations, significantly inhibit the elongation.

Xyloglucan, a hemicellulose present in the cell walls of dicots (2), monocots (5), and gymnosperms (9), has been implicated in the process of auxin-stimulated elongation of etiolated pea stem sections. Labavitch and Ray (6) showed that incubation of excised pea stem segments with auxin increased both the rate of elongation and the amount of xyloglucan extractable with cold water from the stem segments. They suggested that auxin treatment resulted in the conversion of cell wall xyloglucan from a water-insoluble to a water-soluble form and thereby weakened the cell wall. Terry and Jones (8) obtained similar results using a technique designed to minimize contamination by cytoplasmic components. Furthermore, Verma et al. (11) have shown that auxin greatly increased the amount of xyloglucan-degrading endo-β-glucanase activity in etiolated pea stems. Induction of endo-β-glucanase activity by auxin may provide a mechanism by which the solubilization of xyloglucan occurs during auxin treatment of plant tissues. These observations are also consistent with the hypothesis that enzymically modified xyloglucan plays a regulatory role in the process of plant cell wall elongation. In this paper, we present evidence that oligosaccharides produced by the action of endo-β-glucanase on xyloglucan may, in fact, play such a role.

MATERIALS AND METHODS

Xyloglucan oligosaccharides were prepared from xyloglucan purified SEPS[2] as described (2) with the following modifications. (a) SEPS was not deesterified before anion-exchange chromatog-

raphy. (b) Anion-exchange chromatography was carried out using QAE-Q25 Sephadex (Pharmacia) by means of a batch procedure in which a SEPS solution (1 g in 450 ml of 10 mM imidazole-HCl, pH 7) was gently mixed overnight with an excess (100 ml) of ion-exchange gel equilibrated in the same buffer. The slurry was then layered on a bed of 50 ml of fresh ion-exchange gel and the neutral material was eluted with several volumes of 10 mM imidazole-HCl (pH 7). The overnight incubation of SEPS with ion-exchange gel was necessary because, without prior deesterification, the binding of the acidic components of SEPS to the gel proceeds slowly. (c) Bio-Gel P-2 chromatography was performed at ambient temperature (column dimensions 3 × 81 cm; Fig. 1).

Inhibition of 2,4-D-stimulated elongation of pea stem sections was assayed as follows. Material to be tested was dissolved in 2 ml of freshly prepared incubation medium consisting of 1% sucrose, 0.02% benzylpenicillin (potassium salt; Sigma), and 5 mM K-phosphate buffer (pH 6.1). Stem segments (6 mm long, starting 4 mm from the apical hook) were cut from the third internode (4–8 cm) of, 8- to 9-d-old etiolated pea seedlings (Alaska peas; Rocky Mountain Seed Company, Denver, CO). The stem segments were washed with distilled H_2O and floated (10/treatment) on the incubation medium contained in small glass vials (diameter, 2.5 cm; height, 5 cm). The vials were placed in a light-tight box on a gyratory shaker (110 rpm at 24°C) for 90 min. A synthetic auxin, 2,4-D, was then added to a final concentration of 0.9 μM. The vials were immediately returned to the light-tight box and incubated with shaking for an additional 16 h. Individual stem segment lengths were measured with an accuracy of ±0.1 mm using a model 6C Nikon profile projector at × 10 magnification. All experimental manipulations, except measurement of the final lengths of the stem segments, were carried out under dim red light.

Some of the initial experiments in this research, in which base-solubilized cell wall material was assayed for inhibition of auxin-stimulated elongation, were performed according to a protocol slightly different from that described above: stem segments were cut to an initial length of 10 mm; the incubation medium contained no antibiotic; and IAA (17 μM), rather than 2,4-D, was added before the stem sections were floated on the medium. Base-solubilized sycamore cell wall material (fraction B) was obtained by suspending 1 g of endopolygalacturonase-pretreated sycamore cell walls (1) in 200 ml 4 N KOH with 1 mg/ml sodium borohydride. The insoluble material was removed by centrifugation. The soluble material was simultaneously neutralized and desalted by addition of excess Dowex 50 (H^+ form), and then it was filtered through GF/C (Whatman) to remove the resin.

RESULTS

Inhibition of auxin (IAA)-stimulated growth of etiolated pea stem segments by base-solubilized material obtained from puri-

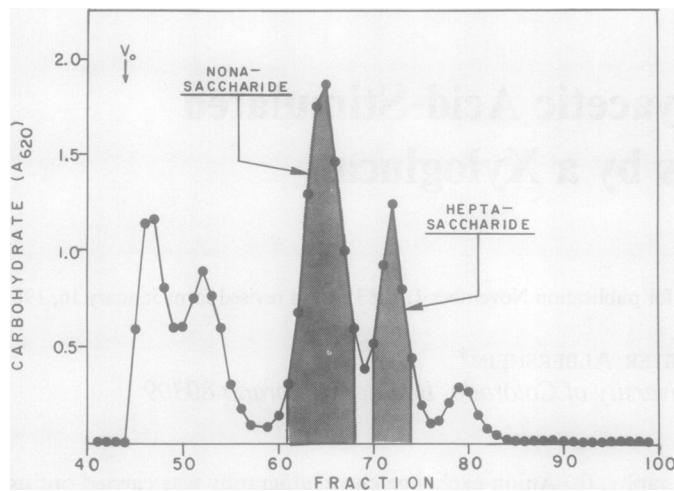[2] Abbreviation: SEPS, extracellular polysaccharides of suspension-cultured sycamore (Acer pseudoplatanus) cells.

FIG. 1. Bio-Gel P-2 elution profile of endo-β-1,4-glucanase-digested SEPS xyloglucan. The sample was loaded in 8 ml of 0.1 M sodium acetate (pH 5.2) and eluted with distilled H_2O. Fractions of 4.8 ml were collected and assayed for hexose by the anthrone method (3). The oligosaccharide-containing fractions were pooled as indicated.

Table I. *Effect of Base-Solubilized Components of Purified Sycamore Cell Walls (Fraction B) on the Auxin-Stimulated Growth of Etiolated Pea Stem Sections*

| Exp | Length after Incubation in Indicated Solution | | | Inhibition |
	Control	17 μM IAA	17 μM IAA + 1 mg/ml B	
	cm			*%*
1	1.07	1.30	1.18	52
2	1.07	1.42	1.21	60
3	1.08	1.33	1.25	32
4	1.14	1.32	1.21	61
5	1.11	1.29	1.23	35
6	1.11	1.30	1.19	58

fied sycamore cell walls (fraction B) was initially observed during experiments in which the stem segments were incubated with IAA in the absence or presence of fraction B (see Table I). The complex mixture of components in fraction B made it unrealistic to attempt to isolate and identify cell wall fragments from fraction B that possessed the inhibitory activity. In addition, the complexity of this mixture made it difficult to assess the significance of the inhibition that this material caused. Nevertheless, that this fraction was solubilized from cell walls by a strong base suggested that one of its major components was hemicellulosic xyloglucan (2). These observations led to the remaining experiments described in this paper, which were performed with purified xyloglucan that was partially depolymerized enzymically rather than chemically.

Purified xyloglucan, isolated from the soluble SEPS, was depolymerized by digestion with endo-β-1,4-glucanase isolated from *Trichoderma viride* (2). The resulting oligosaccharides were fractionated by gel-filtration chromatography on Bio-Gel P-2 (Fig. 1). The P-2 elution profile was very similar to that previously obtained for the endo-β-1,4-glucanase-digestion products of SEPS xyloglucan (2). The major endo-β-1,4-glucanase-digestion products of SEPS xyloglucan have been well characterized (10). These include the nonasaccharide and heptasaccharide illustrated in Figure 2. The nonasaccharide and heptasaccharide fractions eluting from the P-2 column were pooled as indicated in Figure 1. Previous work (10) indicated that the nonasaccharide fraction
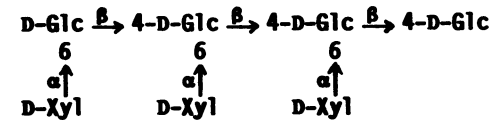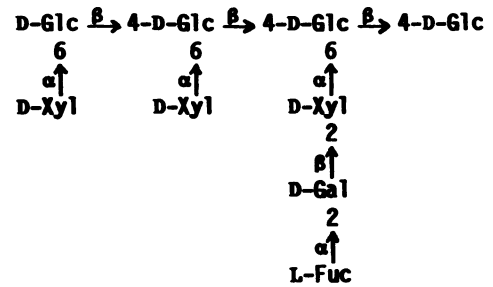

FIG. 2. Proposed structures of the major components of the (a) nonasaccharide- and (b) heptasaccharide-containing fractions of endo-β-1,4-glucanase-digested SEPS xyloglucan (9). Fuc, fucopyranosyl; Gal, galactopyranosyl; Glc, glucopyranosyl; Xyl, xylopyranosyl.
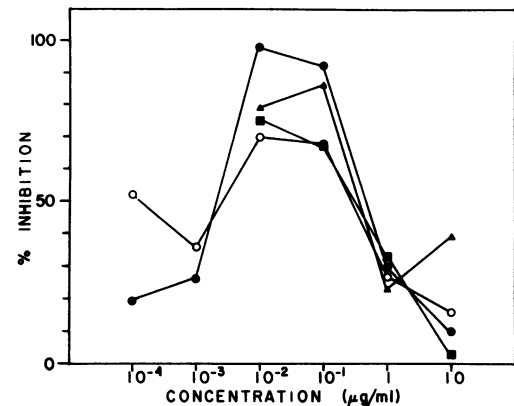

FIG. 3. Inhibition of 2,4-D-stimulated elongation of etiolated pea-stem sections by the nonasaccharide fraction. Total inhibition (100%) corresponds to an average segment length equal to that of the negative control, that is, segments to which no 2,4-D was added. Zero inhibition (0%) corresponds to the average segment length of the positive control, that is, to which 0.9 μM 2,4-D was added. The data are taken from four experiments (O, ●, ■, ▲) done on different days. In all four experiments, when either 10^{-1} or 10^{-2} μg/ml of the nonasaccharide-rich fraction was added, the inhibition was in all cases statistically significant (P < 0.001).

also contained, as minor components, oligosaccharides similar to the nonasaccharide illustrated in Figure 1. By means of ^{1}H nuclear magnetic resonance and fast-atom-bombardment MS, we have recently found that some of the oligosaccharides present in the nonasaccharide-rich fraction also have O-acetyl substituents (unpublished results of the authors and of Dr. Anne Dell, Imperial College of Science and Technology, London).

The nonasaccharide-rich fraction was further characterized as follows. The fraction contained no ninhydrin-positive material and no endo-β-1,4-glucanase activity (as determined by use of sycamore extracellular xyloglucan as the substrate). The neutral sugar composition of this fraction, as determined by GC analysis of the alditol acetate derivatives, indicated the presence of glucose, xylose, galactose, fucose, and arabinose in the ratio of 4.0:3.0:1.4:1.0:0.1. The glycosyl-linkage composition, as deter-

mined by GC and GC-MS analysis of the partially methylated alditol acetate derivatives, gave results very similar to those obtained for the nonasaccharide-rich fraction by Bauer *et al.* (2) and, thus, indicated that the major component of the fraction was the nonasaccharide depicted in Figure 2.

The nonasaccharide-rich fraction was assayed for its ability to inhibit the 2,4-D-stimulated elongation of stem segments excised from etiolated pea seedlings. The results of four experiments in which this fraction was assayed over a wide range of concentrations are summarized in Figure 3. Maximum inhibition of the elongation response was observed when the concentration of the nonasaccharide fraction was 10^{-2} μg/ml, which is approximately 10^{-8} M, if one assumes an average oligosaccharide mol wt of 10^3 D. Thus, the concentration of nonasaccharide is at least 100-fold less than the concentration of 2,4-D in the incubation medium. When the heptasaccharide fraction was assayed at similar concentrations, no significant inhibition was observed.

DISCUSSION

A highly purified xyloglucan nonasaccharide-containing fraction was shown to be an effective inhibitor of the 2,4-D-stimulated elongation of etiolated pea stem segments. The dose-response curve for this fraction indicated that the observed inhibition was not due to a toxic component of the fraction, because toxins typically cause more inhibition at higher concentrations (7). The observed inhibition was most probably not a result of direct nonasaccharide inactivation of the 2,4-D before it could induce a response, because the optimal molar concentration of nonasaccharide was approximately 100-fold less than that of the 2,4-D in the test solution.

The mechanism by which the nonasaccharide-rich fraction inhibits 2,4-D-stimulated growth is not known. The dose-response curve for the ability of the nonasaccharide-rich fraction to inhibit the growth of pea stem segments is similar in appearance to the dose-response curve for the ability of auxin to stimulate the growth of these segments: each curve exhibits a well-defined concentration optimum (4). The nonasaccharide-rich fraction does not significantly affect the growth of the stem segments in the absence of exogenously added 2,4-D. However, our method of measuring growth was not sufficiently sensitive to determine whether the nonasaccharide-rich fractions inhibited the small amount of growth that occurred in the absence of added 2,4-D.

The use of an antibiotic (penicillin) in the incubation medium is necessary in order to obtain reproducible inhibition. Attempts to perform the experiment under aseptic conditions were unsuccessful, probably due to the presence of microbes on the surface of and within the plant tissue itself. The addition of penicillin did not affect the 2,4-D-stimulated elongation of pea stem segments (data not presented) and was a convenient and effective

means of minimizing the deleterious effects of microbial contaminants.

The results presented above do not conclusively establish that the active component of the nonasaccharide fraction functions as a regulatory molecule *in vivo*. Nevertheless, it is interesting to hypothesize that xyloglucan oligosaccharides, by virtue of their ability to inhibit growth, could play an important role in the development of the plant. One possibility is that these growth-inhibiting fragments act as feedback inhibitors of auxin-stimulated growth. Another hypothesis is that, after they are released from the cell wall in the area of cell elongation by an auxin-induced endo-β-glucanase and after they are transported down the stem, the fragments subsequently inhibit the growth of axillary buds and, thus, function as regulators in the phenomenon of apical dominance.

It has not been established whether the nonasaccharide depicted in Figure 2 is the biologically active component of the nonasaccharide fraction. Future work will involve separating and characterizing the components of this fraction; the goal is to determine the identity of the biologically active constituent.

The data presented here provide another example of the regulatory properties that oligosaccharides may exhibit in plant tissues (1).

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