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STIMULATION OF PEA 1,4-β-GLUCANASE ACTIVITY BY OLIGOSACCHARIDES DERIVED FROM XYLOGLUCAN

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ABSTRACT

Oligosaccharide fragments prepared by enzymic digestion of pea xyloglucan at micromolar concentrations exhibited marked stimulatory effects on pea endo-1,4-β-glucanase activity during viscometric assays with xyloglucan as the substrate. The nonasaccharide repeating subunit ($Glc_4Xyl_3GalFuc$) at 200μM evoked a 7–10-fold stimulation of β-glucanase activity, and the heptasaccharide subunit (Glc_4Xyl_3) at 200μM evoked a 4–5-fold stimulation. The stimulatory effect was substrate- and enzyme-specific; *e.g.*, it could not be detected when *O*-(carboxymethyl)cellulose was used as the substrate or when endo-1,4-β-glucanase from *Trichoderma* was tested with xyloglucan as the substrate.

INTRODUCTION

Oligosaccharide fragments derived from various plant and microbial polysaccharides, the so-called[1] "oligosaccharins", may fulfil diverse biological functions in plants. They can act as elicitors of plant response towards various microbial infections[2–4], and they may also display a hormone-like function[5]. For example, it was shown by York *et al.*[6] that the repeating nonasaccharide subunit (Glc_4Xyl_3-GalFuc), isolated from endo-1,4-β-glucanase hydrolyzates of sycamore xyloglucan, and supplied to pea-stem segments at nanomolar concentration, effectively inhibited auxin-induced growth. The heptasaccharide subunit of xyloglucan (Glc_4Xyl_3), which is also formed in hydrolyzates, had no effect on growth. The specific anti-auxin effect of the xyloglucan nonasaccharide has recently been confirmed by independent studies[7]. One of the most marked responses of pea stems to auxin treatment is[8] the induction and accumulation (up to 200-fold) of pea endo-1,4-β-glucanase. When secreted, this β-glucanase causes degradation of xyloglucan to repeating-subunit oligosaccharides, including nona- and hepta-saccharides[9–11], which may be the basis for an auxin-evoked wall loosening and growth[12].

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As part of a program designed to permit us to understand the mode of action of oligosaccharins, we have investigated the possibility that fragments derived from xyloglucan interact directly with pea endo-1,4-β-glucanase, in which event they could be feedback modulators of this key enzyme which is required in order to produce the xyloglucan oligosaccharins in the first place. It is known that cellodextrins can act as competitive inhibitors of pea cellulase acting *vs.* xyloglucan[12] or *O*-(carboxymethyl)cellulose[13]. Thus, we thought a study of the effects of xyloglucan fragments on pea endo-1,4-β-glucanase activity might reveal the mechanism of the effects of the fragments on growth.

For present purposes, we have elaborated a method for the preparation of xyloglucan subunits that is based on hydrolysis of xyloglucan by partially purified endo-1,4-β-D-glucanase, and subsequent separation of fragments by preparative thin-layer chromatography on silica gel 60. The results unexpectedly indicated that pea xyloglucan subunit oligosaccharides at micromolar levels actually *stimulate* pea endo-1,4-β-glucanase activity. The nonasaccharide subunit is the most effective, and the effect is only manifested when xyloglucan itself is the substrate.

RESULTS AND DISCUSSION

Methods described in the literature for the preparation of oligosaccharide subunits derived from xyloglucan usually involve gel-permeation chromatography[6,14] or paper chromatography[11], for the separation step. Preparative thin-layer chromatography (t.l.c.) on silica gel plates, using the newly devised solvent system described herein, is faster and gives good separation when smaller amounts of the material are processed. It is necessary to remove soluble and insoluble impurities [*e.g.*, poly(acrylic) and poly(methacrylic) acid esters] that may be extracted from the silica gel together with separated oligosaccharides. For this purpose, we deionize the extracts, and then employ gel chromatography. The oligosaccharides isolated by t.l.c. were chromatographically distinct bands (see Fig. 1), although it cannot be excluded that they represent a mixture of isomers of identical molecular weight. Typical yields from 200 mg of pea xyloglucan after digestion with *Trichoderma* β-glucanase were 2 mg of nonasaccharide, 6 mg of heptasaccharide, and 5 mg of pentasaccharide.

When incubated with purified pea endo-1,4-β-glucanase acting *vs.* purified tamarind xyloglucan, xyloglucan oligosaccharide subunits caused an immediate increase in the rate of decomposition of the xyloglucan, as assayed viscometrically. Tamarind xyloglucan was used routinely because it possesses a viscosity higher than that of pea xyloglucan, but the oligosaccharide subunits also stimulate the depolymerization of pea xyloglucan by pea β-glucanase. The stimulatory effects of the oligosaccharides increase with the concentration of the fragments up to at least 200μM, which approaches saturation (see Figs. 2 and 3). Pentasaccharide, heptasaccharide, and nonasaccharide were all found to be stimulatory at micromolar levels, with the longer chain lengths the most effective (see Fig. 3). Cello-oligo-

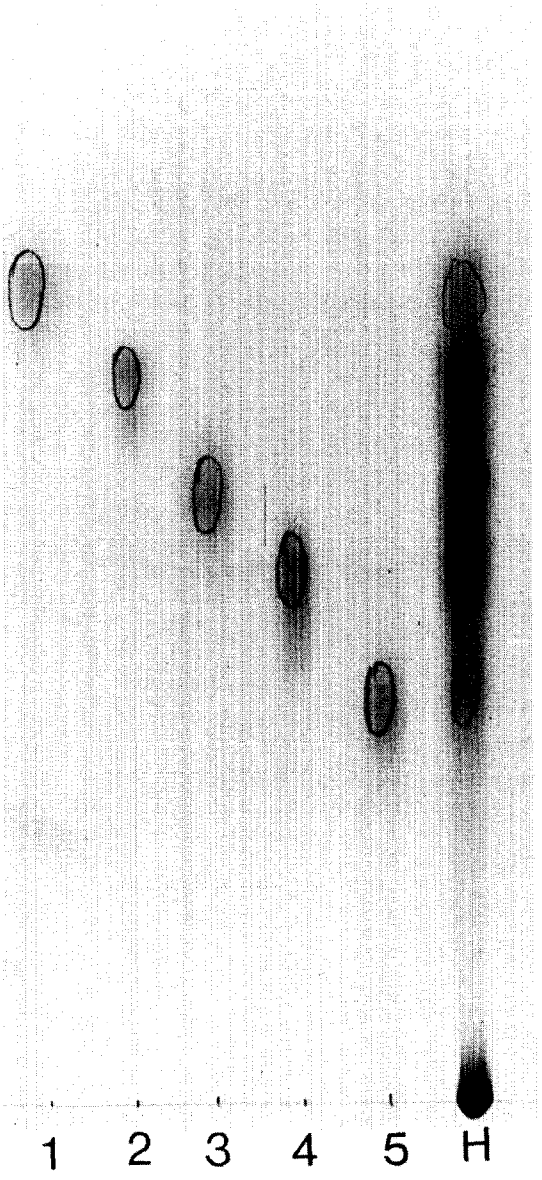

Fig. 1. Analytical thin-layer chromatogram on plate of Silica Gel 60 (0.2 mm thick) showing individual isolated xyloglucan oligosaccharides (glucose, 1; cellobiose, 2; pentasaccharide, 3; heptasaccharide, 4; and nonasaccharide, 5). H is the crude xyloglucan hydrolyzate.

saccharides (G_2–G_6) at similar concentrations are inhibitory, as observed previously[12], but no inhibition by xyloglucan fragments was detected at any concentration or any size. The L-fucose terminal residue in the nonasaccharide is not essential for stimulation (see Fig. 3), and oligosaccharides derived from tamarind xyloglucan (up to octasaccharide, all non-L-fucosated) also stimulate pea 1,4-β-glucanase activity (not shown).

Pea β-glucanase also effectively[13] hydrolyzes barley mixed (1→3)-/(1→4)-linked β-glucan, but xyloglucan fragments evoke only a modest stimulation of this reaction as assayed viscometrically (~20% of the rate of hydrolysis of xyloglucan). No effect whatsoever could be detected by xyloglucan oligosaccharides, at any size

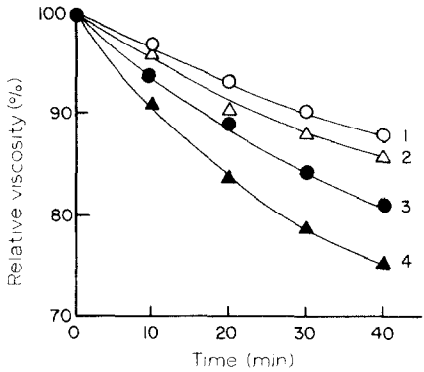

Fig. 2. Effect of various concentrations (0–50μM) of heptasaccharide from pea xyloglucan on the decrease of relative viscosity of tamarind xyloglucan solutions in the presence of pea endo-1,4-β-glucanase. 1, control; 2, 10μM; 3, 20μM; 4, 50μM. 0.3 unit of the enzyme was used in the assay, performed under conditions described in Materials and Methods.

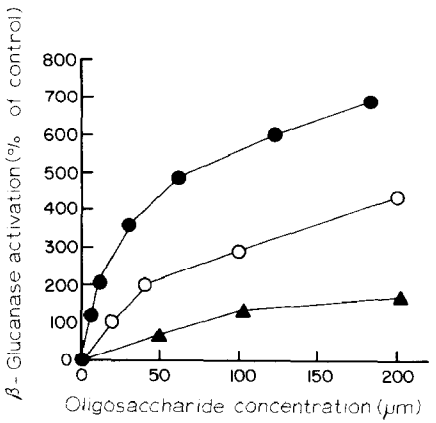

Fig. 3. Concentration dependence of the activating effect of different pea xyloglucan oligosaccharides on pea endo-1,4-β-glucanase. Viscometric assays were performed with 0.1 unit of β-glucanase and 30-min incubation. Key: ●, nonasaccharide; ○, heptasaccharide; and ▲, pentasaccharide.

or any concentration, on the rate of hydrolysis of *O*-(carboxymethyl)cellulose by pea β-glucanase.

No stimulation of xyloglucan hydrolysis by xyloglucan oligosaccharides was observed when an endo-1,4-β-D-glucanase preparation from *Trichoderma viride* was used instead of pea β-glucanase. The *Trichoderma* β-glucanases appear to have specificities of action *vs.* xyloglucan different from those of pea endo-1,4-β-glucanase, because only the former generate fragments smaller than heptasaccharide from pea xyloglucan (*cf.* ref. 12).

It is clear from the results of these experiments that the structure of the oligosaccharides used is important for obtaining stimulation of pea β-glucanase, as only those fragments containing elements of the structure of pea xyloglucan subunits were found effective. The unsubstituted cellotetraose backbone was inhibitory, but the more substituted it was, the more it was converted into an activator. Substrate structure was also found to contribute to the specificity of the reaction, because stimulation by these fragments was virtually confined to the hydrolysis of xyloglucan, from which they are derived. Finally, the stimulatory response was enzyme-specific because pea, but not *Trichoderma*, endo-1,4-β-glucanase was susceptible to activation.

Although unusual, 1,4-β-glucanase activation by oligosaccharides is not an unprecedented observation. In early studies, Reese *et al.*[15] noted that cellobiose at low concentrations is capable of stimulating the rate of hydrolysis of *O*-(carboxymethyl)cellulose by β-glucanase preparations from certain fungi. They explained this phenomenon as due to the protective (stabilizing) effect of cellobiose on the enzyme, although there was no stimulation when unsubstituted cellulose was used as the substrate.

An alternative explanation of this phenomenon could be that there is positive cooperativity between the enzyme and substrate (xyloglucan). The xyloglucan subunits up to nonasaccharide are not substrates for pea β-glucanase, but are end products[12] that clearly do not act as competitive inhibitors of the reaction that produces them. The active site is known[13] to bind to at least 3 cellobiose units (cellohexaose), whereas the xyloglucan subunits have only a cellotetraose backbone. Thus, they may bind at a site other than the catalytic site, in such a way that an allosteric activation results.

It is impossible at this point to decide whether the observed stimulation of pea endo-1,4-β-glucanase activity by xyloglucan fragments has any physiological meaning. York *et al.*[6] observed that the anti-auxin effect of nonasaccharide was maximal at nanomolar concentration, and disappeared at higher concentrations, as if a secondary set of reactions was introduced at high oligosaccharide levels that annulled the antiauxin effect. The effective concentration of xyloglucan oligosaccharides as β-glucanase activators was found higher, by at least by three orders of magnitude, than the concentration optimal for the antiauxin effect. If local concentrations ever reach such values *in vivo*, this could only serve to activate wall β-glucanase to generate more fragments of xyloglucan, and thus to reverse their antiauxin effect.

EXPERIMENTAL

General. — *O*-(Carboxymethyl)celluloses (Na salts), Types 7 HSP and 7LP, were obtained from Hercules. Tamarind-seed xyloglucan was prepared by a procedure modified from that of Hayashi *et al.*[16]. Xyloglucan from etiolated pea seedlings was prepared according to Hayashi and Maclachlan[14].

Preparation of xyloglucan oligosaccharides. — Xyloglucan (200 mg) was dissolved in 20 mL of 25mM acetate buffer, pH 5, containing partially purified endo-1,4-β-D-glucanase (0.5 mg/mL) from *Trichoderma viride*, a mixture of β-glucanases prepared from Cellulysin (Calbiochem) by adsorption on columns of microcellulose powder at pH 4.5 and elution at pH 7.5. The fungal β-glucanases and xyloglucan were incubated under toluene for 12–16 h at 40°. Precipitated undigested xyloglucan was removed by centrifugation, and the supernatant liquor was evaporated to dryness in a rotary vacuum evaporator. The residue was extracted with methanol (3 × 10 mL), and the undissolved portion was removed by centrifugation. The solution, containing xyloglucan hydrolyzate, was concentrated to a small volume by evaporation, and applied to 10–12 Silica Gel 60 (Merck) preparative thin-layer plates (20 × 20 cm, 0.5 mm thick). The plates were developed in 2:1:1 (v/v/v) 1-propanol–methanol–water for 5–6 h. After thorough drying in a stream of air, the margins of the plates were sprayed with 1% orcinol in 10% H_2SO_4 dissolved in ethanol, and then heated for 10 min at 100°.

Zones corresponding to carbohydrate, detected at t.l.c. margins, were scraped from the plates and eluted with 5% (v/v) acetic acid–methanol. After evaporation of the eluates to dryness, the residues were mixed with methanol, and the suspensions filtered through Gelman Acropor filters (pore size 0.45 μm). The filtrates were evaporated to dryness, the residues dissolved in water (2 mL), and the solutions de-ionized by Biorad AG 501-S(8)D mixed-bed resin. The solution of oligosaccharide was concentrated to ~1 mL in a stream of air, and the concentrate was applied to a column (1 × 60 cm) of Biogel P2 and eluted with water. Fractions containing peaks of carbohydrate, as detected by the method of Dubois *et al.*[17], were concentrated to a small volume, and their carbohydrate content determined. Individual fractions were checked for purity by analytical thin-layer chromatography on plates (0.2 mm thick) of Silica Gel 60, using the same system as just described, and that described by Holmes and O'Brien[18]. Detailed structures for the xyloglucan nona- and hepta-saccharide subunits have been published[6,14,16].

Preparation and assay of pea β-glucanase. — Endo-1,4-β-glucanase [1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase; EC 3.2.1.4] was prepared from auxin-treated apices of pea seedlings according to the procedure of Byrne *et al.*[19], except that the gel and ion-exchange chromatographies were omitted. The isozyme (mol. wt. 70,000) which was insoluble in buffer but soluble in M NaCl was used in the present experiments, as it is this form that is secreted, and found concentrated on the inner surface of primary cell-walls.

The assays were performed in 1-mL, Cannon–Manning semimicroviscometers

(capillary size 200) at 37°. Enzyme (0.2–0.5 unit, 0.1 mL) was mixed with purified oligosaccharide (0.1 mL), and after a few minutes reaction was initiated by adding a solution of tamarind-seed xyloglucan (0.8 mL, 0.6% w/v) in 20mM phosphate buffer, pH 6.2. Readings were taken immediately after mixing the enzyme with the substrate (efflux time t_o) and then after different time-intervals (efflux time t_i). Relative viscosity was expressed as the ratio t_i/t_o. As controls, enzyme inactivated by boiling for 5 min at 100°, or oligosaccharides decomposed by hydrolysis in M trifluoroacetic acid for 4 h at 100°, or both, were used. One unit of β-glucanase activity is expressed as the amount of enzyme required to cause 1% loss in initial viscosity during 1 min under the given conditions.

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REFERENCES

1 P. ALBERSHEIM AND A. G. DARVILL, *Sci. Am.*, 253 (1985) 58–64.
2 R. BISHOP, D. J. MAKUS, G. PEARCE, AND C. A. RYAN, *Proc. Natl. Acad. Sci. U.S.A.*, 78 (1981) 3536–3540.
3 A. G. DARVILL AND P. ALBERSHEIM, *Annu. Rev. Plant Physiol.*, 35 (1981) 243–275.
4 J. K. SHARP, B. VALENT, AND P. ALBERSHEIM, *J. Biol. Chem.*, 259 (1984) 11,312–11,320.
5 K. T. T. VAN, P. TOUBART, A. COUSSON, A. G. DARVILL, D. J. GOLLIN, P. CHELF, AND P. ALBERSHEIM, *Nature (London)*, 314 (1985) 615–617.
6 W. S. YORK, A. G. DARVILL, AND P. ALBERSHEIM, *Plant Physiol.*, 75 (1984) 295–297.
7 G. J. MCDOUGALL AND S. C. FRY, *Abstr. Pap. Int. Workshop Plant Polysacch. Struct. Funct., 2nd*, July 8–10, 1987, Grenoble, France, p. 34.
8 D. P. S. VERMA, G. A. MACLACHLAN, H. BYRNE, AND D. EWINGS, *J. Biol. Chem.*, 250 (1975) 1019–1028.
9 J. M. LABAVITCH AND P. M. RAY, *Plant Physiol.*, 54 (1974) 499–502.
10 M. E. TERRY, R. L. JONES, AND B. A. BONNER, *Plant Physiol.*, 68 (1981) 531–537.
11 S. C. FRY, *Planta*, 169 (1986) 443–453.
12 T. HAYASHI, Y.-S. WONG, AND G. A. MACLACHLAN, *Plant Physiol.*, 75 (1984) 605–610.
13 Y.-S. WONG, G. B. FINCHER, AND G. A. MACLACHLAN, *J. Biol. Chem.*, 252 (1977) 1402–1407.
14 T. HAYASHI AND G. A. MACLACHLAN, *Plant Physiol.*, 75 (1984) 596–604.
15 E. T. REESE, W. GILLIGAN, AND B. NORKRANS, *Physiol. Plant.*, 5 (1952) 379–390.
16 T. HAYASHI, Y. KATO, AND K. MATSUDA, *Plant Cell Physiol.*, 21 (1980) 1405–1418.
17 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
18 E. W. HOLMES AND J. S. O'BRIEN, *Anal. Biochem.*, 93 (1979) 167–170.
19 H. BYRNE, N. V. CHRISTOU, D. P. S. VERMA, AND G. MACLACHLAN, *J. Biol. Chem.*, 250 (1975) 1012–1018.