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Note

Structural studies on the β-D-glucan of the *Avena* coleoptile cell-wall

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The oat coleoptile has been widely used as an experimental material in studies of cell elongation mediated by a plant hormone, indole-3-acetic acid. An *exo*-(1→3)-β-D-glucanase isolated from *Sclerotinia libertiana* induces cell elongation when it is applied to oat-coleoptile segments[1,2], partially simulating the auxin effect. This enzyme causes cell wall loosening in oat coleoptiles in a manner consistent with auxin action[3,4]. Nojirimycin (5-amino-5-deoxy-D-glucopyranose), a potent inhibitor of the glucanase, inhibits auxin induced cell elongation[5]. These observations suggest that a β-D-glucan in the oat coleoptile cell-wall is associated with cell elongation. In fact, auxin-induced cell elongation of oat-coleoptile segments is accompanied by a significant decrease in the content of the noncellulosic β-D-glucan in the cell wall[6,7].

Hemicellulosic β-D-glucans containing both (1→3)- and (1→4)-β-D-glucosidic linkages have been detected in stems and leaves of grasses, in endosperm tissue of barley and oat, and in coleoptile walls of oat[8–15]. Extensive studies have been conducted by Albersheim and co-workers on the structure of primary cell walls of di- and mono-cotyledonous plant cells[16–20]. However, this group was unable to confirm the existence of any significant (1→3)-β-D-glucosidic linkages in cell walls of suspension-cultured cells of five different monocotyledonous plants[20]. This conclusion might be attributed to the use of a *Bacillus subtilis* alpha-amylase (Sigma type II-A) to remove contaminating starch. As this alpha amylase is often contaminated with a β-D-glucanase, it is likely that the β-D-glucan was inadvertently removed. The β-D-glucanse in *Bacillus subtilis* alpha amylase causes a release of tri- and tetra-saccharides from the oat coleoptile cell wall[21]. Tentative identification of these products as 3-*O*-β-cellobiosyl-D-glucose and 3-*O*-β-cellotriosyl-D-glucose has been made[21]. Additional evidence for the structure of the tri- and tetra-saccharides is given herein. For the structural analysis of the oat coleoptile glucan, the action of *Bacillus subtilis* and *Rhizopus* glucanases has been compared.

RESULTS AND DISCUSSION

The oat coleoptile cell-wall was treated with a β-D-glucanase purified from an alpha-amylase preparation (Sigma type III-A) with a Bio-Gel P-100 column as

reported by Huber and Nevins[22] The products were separated with a column of Bio-Gel P-2 and were found to be tri- and tetra-saccharides, as shown in Fig 1A Both oligosaccharides were composed entirely of D-glucose This enzyme degraded lichenan and oat-endosperm glucan, but not laminaran, cellulose, or *p*-nitrophenyl β-D-glucoside[22] It has been suggested that the structure of D-Glc-(1→4)-D-Glc-(1→3)-D-Glc-(1→4)-D-Glc is required for recognition by the enzyme, and that the β-D-(1→4) linkage of the 3-substituted group is susceptible to the cleaving action of the enzyme[23] By considering this enzyme specificity, the tri- and tetra-saccharides are deduced to be 3-*O*-β-cellobiosyl-D-glucose and 3-*O*-β-cellotriosyl-D-glucose, respectively The tri- and tetra-saccharides accounted for 5% of the wall preparation and were present in the molar ratio of 2 3 1 (Fig 1A)

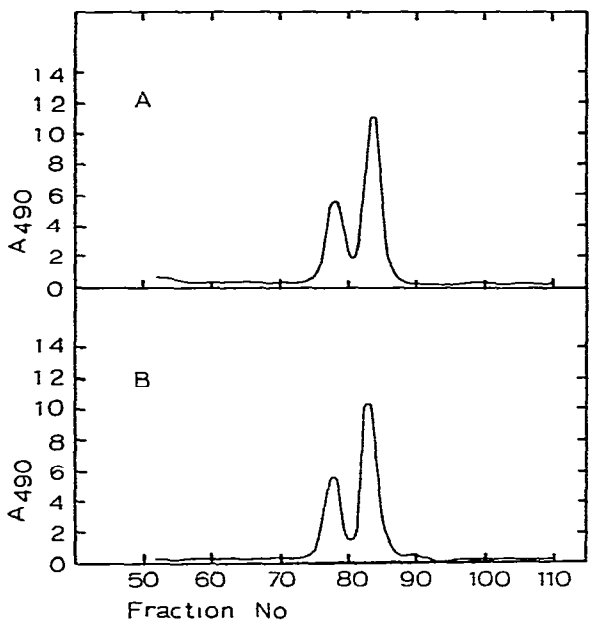

Fig 1 (A and B) Elution profiles on Bio-Gel P-2 of the hydrolysis products upon treatment of *Avena* coleoptile cell-walls (10 mg) with *Bacillus* glucanase (A) and *Rhizopus* glucanase (B)

The tri- and tetra-saccharides were methylated and subjected to formolysis and hydrolysis Hydrolyzates were acetylated and examined by g l c The molar ratios of 2,3,4,6-tetra- and 2,4,6- and 2,3,6-tri-*O*-methyl-glucitol were approximately 1 1 1 and 1 1 2 for tri- and tetra-saccharides, respectively This result agrees with the suggestion[21] that the tri- and tetra-saccharides are 3-*O*-β-cellobiosyl-D-glucose and 3-*O*-β-cellotriosyl-D-glucose

Furtner structural analyses of the tri- and tetra-saccharides were carried out on the components in partial hydrolyzates obtained by separate treatments with pyridine sulfuric acid, and β-D-glucosidase The hydrolytic products of each reaction were separated by paper chromatography Sugars detected are listed in Table I The

disaccharides derived from the trisaccharide were cellobiose upon treatment with pyridine, cellobiose and laminarabiose upon hydrolysis with sulfuric acid, and laminarabiose upon treatment with β-D-glucosidase Base (pyridine) shows preferential degradation of the constituent at the reducing end Therefore, cellobiose is produced from the trisaccharide upon removal of the 3-substituted group Laminarabiose is produced by treatment with β-D-glucosidase, which attacks from the nonreducing end This result is consistent with identification of the trisaccharide as 3-*O*-β-cellobiosyl-D-glucose Similarly, the products from the tetrasaccharide indicated it to be 3-*O*-β-cellotriosyl-D-glucose The β-D-glucan in the oat coleoptile cell-wall is a linear molecule having β-D-(1→3) linkages separated by cellotriosyl and cellotetraosyl residues The proportion of β-D-(1→3) linkages in the glucan is 30 3%

TABLE I

ANALYSIS OF TRISACCHARIDE AND TETRASACCHARIDE RELEASED FROM *Avena* WALLS BY β-D-GLUCANASE OR *endo*-(1—3)-β-D-GLUCANASE

Treatment	*Products from*	
	Trisaccharide	*Tetrasaccharide*
Partial acid hydrolysis	Cellobiose Laminarabiose Glucose	3-*O*-β-Cellobiosyl-D-glucose Cellotriose Cellobiose Laminarabiose Glucose
Base-catalyzed degradation	Cellobiose Unknown	Cellotriose Cellobiose Unknown
β-D-Glucosidase	Laminarabiose Glucose	3-*O*-β-Cellobiosyl-D-glucose Laminarabiose Glucose

Confirmation of the β-D-glucan structure was provided upon hydrolysis of the cell wall with a *Rhizopus endo*-(1→3)-β-D-glucanase This enzyme preparation degraded laminaran, lichenan, and oat-endosperm glucan, but not cellulose [*O*-(carboxymethyl)cellulose or Avicel], nigeran, pustulan, or *p*-nitrophenyl-β-D-glucoside It liberated tri- and tetra-saccharides from the oat coleoptile cell-wall, as shown in Fig 1B The molar ratio of tri- and tetra-saccharides was 2 5 1 Treatment of the tri- and tetra-saccharides with pyridine sulfuric acid, and β-D-glucosidase gave the products listed in Table I Thus, the tri- and tetra-saccharides are also identified as 3-*O*-β-cellobiosyl-D-glucose and 3-*O*-β-cellotriosyl-D-glucose The proportion of (1→3)-β-D-glucosidic linkages in the glucan is calculated to be 30 4% These values are in agreement with those derived upon application of the *Bacillus* glucanase

The enzymic hydrolysis technique as employed here appears to provide a more accurate value for the β-(1→3)/β-(1→4) ratio than methylation or Smith-degradation analysis Moreover, using these enzymes, we obtained reliable evidence

that both (1→3)- and (1→4)-β-D-glucosidic linkages exist in the same polysaccharide chain

Four components were obtained by chemical fractionation of the cell-wall preparation, and the glucan in each was examined by hydrolysis with *Bacillus* glucanase The oat coleoptile cell-wall was extracted successively with hot ammonium oxalate, and 0 5 and 4M potassium hydroxide Each fraction was treated with *Bacillus* glucanase, and the reaction mixture was applied to a column of Bio-Gel P-2 The yields of each fraction and the distribution of the tri- and tetra-saccharides are listed in Table II Most of the glucan was extracted by potassium hydroxide, indicating that the glucan is a component of the hemicellulose of oat coleoptile cell-wall A small amount of glucan remained in the α-cellulose fraction The ratios of tri- and tetra-saccharides in the different fractions are constant, suggesting that the glucan is a copolymer of 3-*O*-β-cellobiosyl-D-glucose and 3-*O*-β-cellotriosyl-D-glucose, but not a mixture of poly(3-*O*-β-cellobiosyl-D-glucose) and poly(3-*O*-β-cellotriosyl-D-glucose)

TABLE II

DISTRIBUTION OF TRISACCHARIDE AND TETRASACCHARIDE IN VARIOUS CELL-WALL FRACTIONS[a]

Cell-wall fraction	*Yield* (*mg*)	*Yield* (%)	*Trisaccharide*	*Tetrasaccharide* (μg/mg cell wall)
	281	100	33	19
Ammonium oxalate	15	5	0	0
0 5M KOH	67	24	11 5	7 9
4M KOH	105	38	17 9	11 1
α-Cellulose	92	33	3 0	1 9
Sum of fractions	279	100	32 4	20 9

[a]Each fraction was treated with *Bacillus* glucanase and the products were resolved by chromatography on Bio Gel P-2

EXPERIMENTAL

Material — Oat (*Avena sativa* L , cv Victory 1) seedlings were grown as described previously[6] Segments (20 mm) were excised from 30–35-mm coleoptiles and the apical 4-mm portions and the first leaves were removed The segments were washed with water and frozen at −20° Frozen segments were homogenized with a mortar and pestle in water and centrifuged at 20,000*g* for 20 min The pellet was suspended in water and centrifuged at 1,000*g* for 20 min This procedure was repeated three times The pellet was then washed three times with acetone and twice with 1 1 (v/v) methanol–chloroform, and dried *in vacuo* The preparation was heated in water for 10 min at 100° to inactivate β-D-glucosidase activity associated with the cell wall and then treated with 0 01 ml of hog pancreatic alpha-amylase (Sigma Type IV) in 1 ml of 25mM potassium phosphate buffer, pH 7 0, for 20 h at 30° to hydrolyze any

residual starch A drop of toluene was added to all enzyme-reaction mixtures to suppress microbial activities The residual wall-fraction was washed with water and dried

Preparation of tri- and tetra-saccharides — The cell-wall preparations (10–100 mg) were treated with 2–5 ml of *Bacillus* glucanase (6 μg protein/ml) or of *Rhizopus* glucanase (10 μg protein/ml) for 20 h at 30° A buffer solution (10mM citric acid and 20mM disodium hydrogenphosphate) at pH 6 0 for *Bacillus* glucanase or at pH 4 6 for *Rhizopus* glucanase was used as solvent for the enzyme reaction After incubation for 20 h, the insoluble wall was removed and the mixture was heated for 10 min at 100° to inactivate glucanase activity The soluble portion was applied to a column (1 5 × 90 cm) of Bio-Gel P-2 (200–400 mesh) The column was eluted with water at the rate of 6 ml/h at room temperature The elution profile was monitored with a differential refractometer Fractions (1 ml) were collected and the carbohydrate content of each fraction was measured by the phenol–sulfuric acid method[24] Trisaccharide was eluted in fractions 82–85, and tetrasaccharide in fractions 76–79 Fractions comprising each peak were combined and dried

Methylation analysis — The tri- and tetra-saccharides (~3 mg) derived from the cell wall upon hydrolysis with *Bacillus* glucanase were methylated by the method of Hakomori[25] Methylated sugars were subjected to formolysis with 90% formic acid for 1 h at 100° and hydrolyzed with 0 2M sulfuric acid[26] The hydrolyzates were neutralized (barium carbonate), and then reduced with 20 mg of sodium borohydride/ml and acetylated with 1 1 (v/v) pyridine–acetic anhydride for 1 h at 100° Borate was removed as methyl borate by vacuum rotary evaporation below 40° Methylated sugars were then examined by gas chromatography at 180° on a glass column (0 3 × 150 cm) packed with 3% of ECNSS-M on Gas Chrom Q[27]

Partial hydrolysis of the tri- and tetra-saccharides — Samples (3 × 500 μg) of the tri- and tetra-saccharides were separately treated with (*a*) 1 ml of 5% pyridine for 30–60 min at 100°, (*b*) 1 ml of 0 2M sulfuric acid for 60 min at 100° or (*c*) 0 5 ml of 1 mg/ml β-D-glucosidase (extracted from almond, Worthington Biochemical Co) in citric acid (5mM)–phosphate (10mM) buffer at pH 4 6 for 10–30 min at 30° The products were examined by descending paper chromatography on Whatman No 1 paper in 2 8 1 (v/v/v) pyridine–ethyl acetate–water for 90–135 h at 26° Components on the paper were detected with alkaline silver nitrate[28] Cellobiose, laminarabiose, gentiobiose, 3-*O*-β cellobiosyl-D-glucose, laminara-oligosaccharides, and cello-oligosaccharides were used as standard sugars 3-*O*-β-Cellobiosyl-D-glucose was prepared from oat-endosperm glucan Laminara-oligosaccharides were prepared by hydrolysis of laminaran with 0 2M sulfuric acid for 2 h at 100° Cello-oligosaccharides were prepared by acetolysis of cellulose

Fractionation of the cell wall — The cell-wall preparation (281 mg) was treated three times for 15 min each time with 0 25% ammonium oxalate (5 ml) at 100°, and the soluble portion was then dialyzed A few drops of acetic acid were added to the dialyzate Addition of ethanol gave a precipitate (the ammonium oxalate fraction) The residual cell-wall material was then treated with 5 ml of 0 5M potassium hydroxide

three times for a total of 24 h (0 5M fraction), and 5 ml of 4M potassium hydroxide in three separate 8-h treatments (4M fraction) The potassium hydroxide solutions contained 0 5 mg of sodium borohydride/ml The potassium hydroxide extracts were neutralized with acetic acid, and polysaccharide was precipitated by addition of 3 volumes of ethanol at −20° The final residue, after treatment with potassium hydroxide, was washed with water, 0 1M acetic acid, and water, and the product was precipitated with ethanol (α-cellulose fraction) The fractions (0 5M, 4M, and α-cellulose fractions) were then suspended in methanol, and dried with a stream of filtered air (procedure repeated five times) The dry weight of each fraction was determined prior to hydrolysis by *Bacillus* glucanase

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REFERENCES

1 Y Masuda and S Wada *Bot Mag*, 80 (1967) 100–102
2 Y Masuda, S Oi and Y Satomura *Plant Cell Physiol*, 11 (1970) 631–638
3 Y Masuda, *Planta* 83 (1968) 171–184
4 R Yamamoto and Y Masuda, *Physiol Plant* 25 (1971) 330–335
5 D J Nevins *Plant Cell Physiol* 16 (1975) 707–717
6 W Loescher and D J Nevins, *Plant Physiol*, 50 (1972) 556–563
7 W Loescher and D J Nevins *Plant Physiol* 52 (1973) 248–251
8 S Wada and P M Ray, *Plant Physiol*, 38 (1963) xiii
9 H M Flowers E F Neufield and W Z Hassid *Plant Physiol* 43 (1968) 1703–1709
10 A J Buchala and K C B Wilkie *Naturwissenschaften* 57 (1970) 496
11 C G Fraser and K C B Wilkie *Phytochemistry*, 10 (1971) 199–204
12 C G Fraser and K C B Wilkie, *Phytochemistry*, 10 (1971) 1539–1542
13 A J Buchala and K C B Wilkie, *Phytochemistry*, 10 (1971) 2287–2291
14 A Kivilaan, R S Bandurski, and A Schulze, *Plant Physiol* 48 (1971) 389–393
15 A J Buchala and H Meier *Carbohydr Res* 26 (1973) 421–425
16 K Talmadge K Keegstra W D Bauer and P Albersheim *Plant Physiol* 51 (1973) 158–173
17 W D Bauer K W Talmadge K Keegstra and P Albersheim *Plant Physiol* 51 (1973) 174–187
18 K Keegstra K W Talmadge W D Bauer and P Albersheim *Plant Physiol* 51 (1973) 188–196
19 B M Wilder and P Albersheim *Plant Physiol*, 51 (1973) 889–893
20 D Burke, P Kaufman, M McNeil, and P Albersheim *Plant Physiol*, 54 (1974) 109–115
21 D J Nevins D J Huber R Yamaloto and W Loescher, *Plant Physiol* 60 (1977) 617–621
22 D J Huber and D J Nevins, *Plant Physiol*, 60 (1977) 300–304
23 M A Anderson and B A Stone *FEBS Lett* 52 (1975) 202–207
24 J E Hodge and B T Hofreiter, *Methods Carbohydr Chem*, 1 (1962) 380–289
25 S Hakomori *J Biochem (Tokyo)*, 55 (1964) 205–208
26 H O Bouveng H Kiessling B Lindberg and J McKay *Acta Chem Scand* 16 (1962) 615–622
27 H Bjorndal C Hellrqvist B Lindberg and S Svensson *Angew Chem Int Ed Engl* 9 (1970) 610–619
28 W E Trevelyan D P Procter and J S Harrison *Nature* 166 (1950) 444