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Note

A (1→3)- and (1→4)-linked β-D-glucan in the endosperm cell-walls of wheat*

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Endosperm cell-walls of wheat (cv. Insignia) isolated in a medium of 70% ethanol are shown to be composed of polysaccharides containing 53.5% xylose, 18.5% arabinose, 20% glucose, 6.5% mannose, and 1.0% galactose, together with 4.7% (w/w) of protein. Both (1→3)- and (1→4)-linked glucopyranosyl residues are present, as shown by methylation studies. Treatment of the walls with the β-D-glucan endo-hydrolase of *Bacillus subtilis* liberates oligoglucosides characteristic of a (1→3)- and (1→4)-linked β-D-glucan constituting 94% of the non-cellulosic glucose in the wall. The methylation data confirm the presence of a heteroxylan having a (1→4)-linked xylopyranosyl backbone substituted by arabinofuranosyl residues.

The non-starchy polysaccharides from wheat flour have been extensively studied and comprise arabinoxylans[1,2], arabinogalactan–peptides[3], glucomannans, and cellulose[4]. Although there are reports of glucose in the hydrolysates of both water and alkali-soluble extracts of wheat flour after prolonged incubation with alpha amylase[1,5], there has been no definitive study of the nature of the polysaccharides in which glucose is present. β-D-Glucans containing both (1→3) and (1→4) linkages are important components of the non-cellulosic polysaccharides in cereal and grass endosperms and have been shown to be localised in the endosperm walls of barley[6,7], *Lolium*[8,9], and rice[10], and in the aleurone walls of barley and wheat[11].

Although earlier studies on water-soluble polysaccharides from wheat endosperm[12,13] and isolated wheat-endosperm walls[4] had failed to reveal (1→3)- and (1→4)-linked β-D-glucans, significant quantities of these glucans were recently found by a specific enzymic procedure developed to determine (1→3)- and (1→4)-linked β-D-glucans in plant materials[14]. This development led to the present reappraisal of the composition of wheat endosperm cell-walls.

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MATERIALS AND METHODS

Materials. — Wheat (*Triticum aestivum* L. cv. Insignia) was kindly supplied by Mr. K. Mander, Victorian Department of Agriculture. Alpha amylase (porcine pancreatic, Type VI-A), glucose oxidase, and *o*-dianisidine dihydrochloride were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Sodium hydride (80% suspension in mineral oil), sodium borohydride, Bromothymol Blue, and soluble starch were purchased from B.D.H., Poole, U.K. Lactoperoxidase was prepared by the method of Hogg and Jago[15] to the ammonium sulphate fractionation stage. Bio-Rad AG 501-X8, (20–50) mesh resin (Bio-Rad Laboratories, Richmond, CA, U.S.A.) was used in the H^+/HCO_3^- form. The β-D-glucan endo-hydrolase from *Bacillus subtilis* strain SP99 was purified from a concentrate obtained from Novo Industri A/S, Copenhagen, Denmark[14]. The (1→3)-β-D-glucan exo-hydrolase from *Euglena gracilis* was purified by the method of Barras and Stone[16]. Gas-chromatographic column packings, 3% of SP-2340 on 100–120 mesh Supelcoport and Gas Chrom Q, 100–120 mesh, were purchased from Supelco Inc., Supelco Park, Bellefonte, PA, U.S.A., and OV-225 from Pierce Chemical Co., Rockford, IL, U.S.A.

Isolation of endosperm walls. — Cell walls were isolated from wheat flour in 70% ethanol according to the method of Mares and Stone[4], except that the step of treatment with alpha amylase was omitted and starch granules were removed by an additional treatment in a French pressure-cell (Aminco Bowman, Silver Springs, MD, U.S.A.) and in an ultrasonic disintegrator (Rapidis 600, Ultrasonics Ltd., Shipley, Yorkshire, U.K.). The final cell-wall preparation was dried by solvent exchange and stored in a desiccator over silica gel.

Assessment of purity of the cell-wall preparation. — Light- and fluorescence-microscopy revealed contamination with non-endospermic cell walls to be less than 5%. The preparation was essentially free of small starch-granules. Digestion with porcine-pancreatic alpha amylase and determination of 80% ethanol-soluble products by the phenol–sulphuric acid method gave a starch content of 2%. Monosaccharide analyses were corrected appropriately.

Monosaccharide analyses. — Cell walls were hydrolysed with fuming hydrochloric acid and the monosaccharide composition of hydrolysates determined by gas–liquid chromatography of their alditol acetates on 3% SP-2340 on 100–200 mesh Supelcoport[4].

Methylation. — Polysaccharide fractions were methylated by the method of Hakomori[17] as modified by Björndal *et al.*[18]. Cell walls had to be treated three times to achieve complete methylation. The g.l.c. peaks were identified by their retention times relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol by using the values given by Lönngren and Pilotti[19] and by g.l.c.–m.s.[11]. G.l.c. was performed with a glass column (1.8 m × 3.2 mm o.d.) packed with 3% OV-225 on Gas Chrom Q (100–120 mesh) maintained at 170°, with nitrogen as carrier at a flow rate of 20 mL/min.

Nitrogen analysis. — Nitrogen was estimated by the micro-Kjeldahl method by the Australian Microanalytical Service, Fisherman's Bend, Melbourne.

Enzymic hydrolysis of cell walls

Hydrolysis with Bacillus subtilis *β-D-glucan endo-hydrolase* (EC 3.2.1.73). — The endo-hydrolase used specifically hydrolyses (1→4) linkages joining 3-substituted glucosyl residues in such β-D-glucans containing both (1→3) and (1→4) linkages as barley and oat β-D-glucans[20]. The purified enzyme used has no action on O-(carboxymethyl)cellulose, laminaran, or starch. Cell walls (5–10 mg) were suspended in 1.0 mL of 0.05M maleic acid–sodium maleate buffer (pH 6.5), heated in boiling water (1–2 min), cooled, and incubated with 0.2 mL of enzyme solution (8.3 units/mL) for 24 h at 40° under toluene. A further 0.2 mL of enzyme solution was added after 20 h. The reaction was terminated by boiling (5 min) and a portion was removed and deionised with Bio-Rad AG501-X8 mixed-bed resin (20–25 mesh, H^+/HCO_3^- form), overnight at 4°. The products of hydrolysis were separated on Whatman No. 3 chromatography paper by descending chromatography (28 h) in 6:1:3 (v/v) 1-propanol–ethyl acetate–water, and the chromatographic mobility of the hydrolysis products compared to the characteristic trisaccharide, 4-*O*-β-D-glucosyl-laminarabiose, and tetrasaccharide, 4-*O*-β-cellobiosyl-laminarabiose, formed on hydrolysis of barley β-D-glucan. The sugars were detected by the alkaline silver nitrate reagent[21].

Following qualitative identification of the hydrolytic products, the β-D-glucan content of the cell walls was estimated by measuring the 80% ethanol-soluble products as follows. The enzymic digests of the cell walls were precipitated with 4 vol. of ethanol, centrifuged, and the pellet was washed thoroughly with 80% ethanol. The combined washings and supernatant solutions were dried and hydrolysed with 4 mL of 0.5M nitric acid containing 0.5% urea (w/v)[22], for 4 h at 100° in sealed tubes. Following neutralisation to the Bromothymol Blue end-point, D-glucose was measured by the glucose oxidase method[23]. The pellet was hydrolysed similarly, and the β-D-glucan content calculated from the amount of glucose in the 80% ethanol-soluble products as a percentage of the total glucose in the pellet plus the 80% ethanol-soluble fraction.

Hydrolysis with Euglena gracilis *(1→3)-β-D-glucan exo-hydrolase* (EC 3.2.1.58). — The (1→3)-β-D-glucan exo-hydrolase hydrolyses (1→3) linkages from the non-reducing end of such β-D-glucans as laminaran, to release glucose. Whole walls (5–10 mg) were suspended in 1 mL of 0.1M sodium-acetate buffer (pH 5.2) and the mixture was boiled for 1–2 min, cooled, and incubated with 1.0 mL of enzyme solution (2.54 units/mL) for 96 h at 40° in the presence of toluene. Further 1.0-mL aliquots of enzyme solution were added after 24 and 48 h. Reactions were terminated by boiling for 5 min and the mixtures prepared for chromatography as described. The content of (1→3)-β-D-glucan was estimated by measuring the glucose released by using the glucose oxidase method[23], and was expressed as a percentage of the total wall.

RESULTS

The percent monosaccharide composition (w/w) of the cell-wall preparation was: arabinose, 18.5; xylose, 53.5; mannose, 6.5; galactose, 1.0; glucose, 20; and arabinose/xylose, 0.35. The content of protein (N × 6.25) was 4.7%.

Methylation-analysis data, collected in Table I, are consistent with the presence in the cell walls of a (1→4)-linked xylan in which xylopyranosyl residues are substituted by arabinofuranosyl residues at O-3 or both O-2 and O-3. The small amounts of 2-, 3-, and 5-substituted arabinofuranosyl residues suggest that some of these substituents are in the form of short chains. Complete correspondence between the molar recovery of terminal, nonreducing arabinofuranosyl residues and the molar proportion of substituted xylosyl residues was not found. Under-methylation was not a likely cause, as no significant peaks having T values >4.0 were found. However, the peak corresponding to 1,3,4,5-tetra-*O*-acetyl-2-*O*-methylxylitol was not resolved from the peak corresponding to 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylmannitol under the conditions used, and since mannose is a component of the cell walls and is probably present as a glucomannan[4], this factor may account, in part, for the discrepancy. Furthermore, it is likely that some of the terminal residues on side-branches are monosaccharides other than arabinofuranose[2].

Glucose in the cell-wall polysaccharides is in the pyranose form and is both (1→3)- and (1→4)-linked. The arrangement of these linkages in the glucan was defined by treating the walls with *B. subtilis* β-D-glucan endo-hydrolase. Two oligosaccharides (R_{Glc} 0.08 and 0.51) were released whose paper-chromatographic mobilities corresponded to the characteristic tri- and tetra-saccharides, 4-*O*-β-D-

TABLE I

COMPOSITION OF THE HYDROLYSATE OF PERMETHYLATED WHEAT-ENDOSPERM CELL-WALLS FOLLOWING REDUCTION AND ACETYLATION

Component	T[a]	*Mole %*
1,4-Di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol	0.42	17
1,2,4-Tri-*O*-acetyl-3,5-di-*O*-methylarabinitol	0.79	1
1,3,4-Tri-*O*-acetyl-2,5-di-*O*-methylarabinitol	0.86	trace
1,4,5-Tri-*O*-acetyl-2,3-di-*O*-methylarabinitol	1.05	1
1,5-Di-*O*-acetyl-2,3,4-tri-*O*-methylxylitol	0.56	2
1,4,5-Tri-*O*-acetyl-2,3-di-*O*-methylxylitol	1.18	34
1,3,4,5-Tetra-*O*-acetyl-2-*O*-methylxylitol	2.08	16
1,2,3,4,5-Penta-*O*-acetylxylitol	3.47	4
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol	1.00	trace
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol	1.83	7
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol	2.33	19

[a]See ref. 19.

glucosyl-laminarabiose (R_{Glc} 0.08) and 4-*O*-β-cellobiosyl-laminarabiose (R_{Glc} 0.52), released from barley β-D-glucan[20]. Estimation of the ethanol-soluble hydrolysis-products following exhaustive enzymic hydrolysis of the cell-wall preparation revealed that 94% of the non-cellulosic glucose was in the form of a (1→3)- and (1→4)-linked β-D-glucan. The action of the *Euglena* (1→3)-β-D-glucan exo-hydrolase showed that a small proportion of a (1→3)-β-D-glucan, corresponding to 1% of the wall preparation, was present.

DISCUSSION

The methylation data indicate that the heteroxylan component of wheat endosperm cell-walls has a general structure comparable with that already reported for water-soluble and -insoluble wheat-endosperm heteroxylans, but that side chains may be of greater complexity than previously indicated. This would be in accord with studies made on heteroxylans from other monocotyledon cell-walls[2].

The methylation data and the results of digestion with *Bacillus subtilis* β-D-glucan endo-hydrolase clearly indicate that (1→3)- and (1→4)-linked β-D-glucans are also significant components of wheat endosperm cell-walls. In a previous study[4] on a similar cell-wall preparation that contained some associated starch, the glucose content of the wall hydrolysate was corrected for α-D-glucan on the basis of glucose released by prolonged treatment with salivary alpha amylase and glucoamylase. It has now been shown that the glucoamylase preparation used to remove the starch contained enzymes that hydrolyse barley β-D-glucan to glucose. Thus the glucose content of the cell walls was over-corrected for glucose of α-D-glucan. Re-examination of the previously isolated walls[4] by using the *Bacillus* β-D-glucan endo-hydrolase showed that a (1→3)- and (1→4)-linked β-D-glucan was, in fact, present.

The reports of variable amounts of non-α-glucan glucose in both the water- and alkali-soluble fractions from wheat endosperm[1,5] suggest that, as in barley[14], the amounts of β-D-glucan are subject to varietal and probably seasonal variation. In barley endosperm cell-walls, β-D-glucan is found in both water- and alkali-soluble fractions[6,7], but in rye-grass endosperm cell-walls, although mixed-linkage β-D-glucan is present in 8M urea- and dilute alkali-soluble fractions, it is not found in water extracts[9]. The reported absence of glucose in hydrolysates of water-soluble, wheat-flour endosperm polysaccharides in many studies[1,12,13] suggests that, for some wheat varieties, as in rye-grass, the β-D-glucan is in a water-insoluble form in endosperm cell-walls.

The small amount of (1→3)-β-D-glucan detected by enzymic hydrolysis with the *Euglena* (1→3)-β-D-glucan exo-hydrolase probably arises from the microscopic deposits seen on endosperm cell-walls in sections of barley[24] and wheat[25] grain and characterised by their bright-yellow fluorescence induced by Aniline Blue.

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