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CHEMICAL COMPOSITION OF THE CELL WALLS OF *LOLIUM MULTIFLORUM* ENDOSPERM

MARGARET MERYL SMITH* and BRUCE A. STONE†
Russell Grimwade School of Biochemistry, University of Melbourne, Victoria, Australia

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Abstract—Cell walls isolated from *Lolium multiflorum* endosperm grown in liquid suspension culture contain 90% carbohydrate (as anhydro–glucose), 0·3% nitrogen, 1·9% lipid and 4·3% ash. The relative proportions of neutral sugars present in hydrolysates of the wall polysaccharides are glucose, 50%; arabinose, 19%; xylose, 26% and galactose, 5%. Extraction of the wall with 7 M urea solubilizes a polysaccharide representing 19% of the wall and composed of glucose and minor amounts of pentoses. This fraction has been examined by acid and enzymic hydrolysis and by periodate oxidation, and was shown to be a β-1,3; 1,4-glucan with approx. 79% 1,4-linkages. A specific β-glucan hydrolase has been used to determine the content of this mixed-linked glucan in isolated endosperm cell walls.

INTRODUCTION

MIXED-LINKED β-glucans, which are linear polymers of glucosyl residues joined by both 1,3- and 1,4-linkages, have been isolated from aqueous extracts of Iceland moss (*Cetraria islandica*) and from oat and barley seeds.[1] Glucans with similar properties have also been reported in aqueous extracts of the endosperm of a number of grasses[2] and in oat leaves[3,4] and maize coleoptiles.[5] The water-soluble glucans from the cereal and grass endosperm probably originate from the cell walls, although this has never been shown directly. However, barley endosperm cell walls prepared in an aqueous medium when extracted with 7 M urea yield a β-glucan which is indistinguishable from the water-soluble barley glucan.[6] An interest in the biosynthesis of mixed-linked glucans led us to examine tissue cultured endosperm from rye grass (*Lolium multiflorum*)[7] as a source of material. This paper reports on the composition of walls from *L. multiflorum* grown in suspension culture[8] and describes the characteristics of a β-glucan present in the walls. Some aspects of β-glucan synthesis by cell-free extracts of *Lolium* endosperm have been reported[9] and further studies on the biogenesis of the cell wall polysaccharides are in progress.

* Present address: Departement de Biologie, Centre Universitaire de Marseille-Luminy, Route Leon Lachamp, 13-Marseille (9e), France.
† Present address: Biochemistry Department, La Trobe University, Bundoora, Victoria, Australia.

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[9] SMITH, M. M. and STONE, B A. (1973) *Biochim. Biophys. Acta* in press.

RESULTS

Cell walls were isolated from stationary phase liquid suspension cultures of *L. multiflorum* endosperm as described in the Experimental. The wall fragments were examined microscopically and shown to be free from whole cells, cytoplasmic organelles, starch granules and protein bodies. The analytical value % by weight for carbohydrate, protein, lipid and ash contents are 90·2 (as anhydroglucose), 1·9 (N × 6·25), 1·9 and 4·3 (0·3 as P_2O_5) respectively: in addition 0·4% alkoxyl (as —OCH_3) was found.

PC of both acid and enzymic hydrolysates of the *Lolium* endosperm cell walls revealed the presence of spots co-chromatographing with xylose, arabinose, glucose and galactose. No other reducing compounds were detected in the acid hydrolysate, but the enzymic hydrolysates also contained two unidentified slower moving carbohydrate components which could be uronic acids or oligosaccharides. The relative proportions of neutral monosaccharides present in the hydrolysates were determined by GLC of the alditol acetate derivatives and are shown in Table 1. Uronic acids present in the enzymic hydrolysate, measured using uronate dehydrogenase, represented 1·3% by weight of the walls.

Fractionation of Lolium *Endosperm Cell Walls*

Walls were fractionated by gentle non-destructive procedures involving successive extractions with water, phenolacetic acid–water (PAW) and 7 M urea. The average relative weights of the fractions obtained in two separate experiments were 1·45, 1·85 and 18·85 per cent respectively and their composition and that of whole walls are summarized in Table 2. The minor components extracted with water and with PAW were not examined further.

Infrared Spectra of Cell Walls and Wall Fractions

IR spectra of the whole wall, the urea extract and the urea residue were determined and interpreted by Dr. A. J. Michell, C.S.I.R.O., Forest Products Laboratory, South Melbourne, Victoria. The spectra of the urea residue and of the whole walls strongly resembled each other and in both, the absorption bands near 1730 and 1250 cm^{-1} were indicative of

TABLE 1. NEUTRAL MONOSACCHARIDE COMPOSITION OF *Lolium* ENDOSPERM CELL WALLS

Constituent sugar	% of total sugars in hydrolysate (by wt)* HCl hydrolysate	Enzymic hydrolysate
Glucose	50	48
Galactose	5	4
Mannose		Trace
Xylose	26	27
Arabinose	19	20

* Estimated by GLC of alditol acetates.

either aliphatic ester or carboxyl groups (C=O stretching, C–O stretching and/or OH deformation vibrations respectively). A band near 1520 cm^{-1} has been reported to be associated with the presence of protein.[10] In the urea extract these three bands were absent

[10] MICHELL, A. J. and SCURFIELD, G. (1970) *Australian J. Biol. Sci.* **23**, 345.

or very weak. The lack of structure in the region 1100–1000 cm^{-1} indicates the amorphous nature of the three samples and possibly the presence of a mixture of linkages. Absorption bands were evident around 890 cm^{-1} in each sample investigated indicating the presence of β-linked residues in the component polysaccharides.[11]

Characterization of the Urea-soluble Fraction

The polysaccharide extracted from endosperm cell walls with 7 M urea was composed mainly of glucose (Table 2) and was characterized without further purification. The isolated glucan did not readily dissolve in water, but a 0·5–1·0% (w/v) aqueous solution could be prepared if the glucan was first swollen in water and then heated to 70–85°. The solution was unstable at room temperature and the polymer slowly precipitated on standing. The polysaccharide had a specific optical rotation $[\alpha]_{589nm}^{21}$ of −9·5° (*c* 0·26, H_2O) and the values at other wavelengths under the same conditions were: 578 nm, −8·7°; 546 nm, −9·5°; 436 nm, −16·7° and 365 nm, −28·5°.

The products of partial acid hydrolysis of the glucan were examined by descending PC (Solvent B) and included compounds co-chromatographing with glucose and cellobiose and a small amount of a compound which co-chromatographed with laminaribiose. A series of slower moving oligosaccharides was also present. These data indicate the presence of both 1,3- and 1,4-linked glucose residues, suggesting that the urea-soluble fraction contains a mixed-linked glucan. This was verified by enzymic hydrolysis of the sample using two β-glucan hydrolases of differing substrate specificities and determination of the relative proportions of 1,3-and 1,4-linkages by periodate oxidation.

TABLE 2. COMPOSITION OF CELL WALL FRACTION

Components	Whole walls	% of Whole wall (by wt) PAW residue	Urea extract	Urea residue
Ash	4·29	3·05	0·12	1·56
Nitrogen (Kjeldahl)	0·31	0·29	0·02	0·24
Carbohydrate*	90·2 (94·3)		18·5	75·8
Component sugar*†				
Glucose	45·1 (48·8)		15·8	33·0
Arabinose	17·1 (19·6)		1·0	18·6
Xylose	23·5 (21·7)		0·7	21·0
Galactose	4·5 (4·3)		0·94	3·34
Mannose	Trace		Trace	

* Figures in parentheses are the sum of the values obtained for the urea-extract and the urea-residue.
† These values were obtained from estimation of the relative proportion of the sugars and the total carbohydrate present in each fraction.

The *Lolium* urea-soluble glucan and three known mixed-linked β-glucans, the urea-soluble glucan from barley endosperm cell walls, a water-soluble glucan from barley endosperm and lichenin from *Cetraria islandica* were incubated separately with a β-1,3; 1,4-glucan hydrolase preparation from *Bacillus subtilis* and a β-1,3-glucan hydrolase from

[11] BARKER, S. A., BOURNE, F. J. and WHIFFEN, D. H. (1956) *Methods in Biochemical Analysis*, GLICK, D., ed.), Vol. 3, p. 213, Interscience, New York.

Rhizopus arrhizus (types 2 and 3 respectively in Table IV of Barras, Moore and Stone[12]). The products were examined by paper chromatography in solvent B and their R_{Glc} values are shown in Table 3. The oligosaccharides released from the known mixed-linked glucans are identical with those released from the *Lolium* glucan. Since the *Bacillus* preparation specifically hydrolyses β-glucans which contain adjacent 1,3- and 1,4-linked glucose residues[13] these results conclusively demonstrate the mixed-linked nature of the *Lolium* glucan.

TABLE 3. COMPARISON OF PRODUCTS RELEASED FROM *Lolium* AND OTHER β-GLUCANS BY *Rhizopus arrhizus* AND *Bacillus subtilis* β-GLUCAN HYDROLASES

Substrate	R^{G}_{1c} of products from *Rhizopus arrhizus* Enzyme preparation		*Bacillus subtilis* Enzyme preparation	
Barley glucan (water soluble)	0·1 (+++), 0 84 (+),	0 5 (++) 1 0 (+++++)	0·27 (+), 0·67 (++), 1 0 (+++)	0·5 (+++) 0 84 (++)
Barley glucan (urea soluble)	0·1 (+++), 0 84 (+),	0·5 (++) 1 0 (+++++)	0 25 (+), 0·67 (++), 1 0 (+++)	0 5 (+++) 0 84 (++)
Lolium glucan	0·1 (+++), 0·84 (+),	0·5 (++) 1·0 (+++++)	0 25 (+), 0·67 (++), 1 0 (+++)	0·5 (+++) 0 84 (++)
Lichenin	0·08 (+++), 0·84 (+),	0·51 (++) 1·0 (+++++)	0·22 (+), 0·69 (++),	0·51 (+++) 0 84 (++) 1·0 (+++)

The enzyme hydrolyses and paper chromatographic analyses of products were performed as described in Experimental. Intensity of spots: tr, trace; +, very low; ++, low; +++, moderate; +++++, strong. R_{Glc} values of standard oligosaccharides: cellobiose, 0·71; cellotriose, 0 37; cellotetraose, 0·14; cellopentaose, 0 08; laminaribiose, 0·85; laminaritriose, 0 66; laminaritetraose, 0·52, laminaripentaose, 0 39.

Because the *Bacillus* preparation is highly specific, it could also be used to selectively hydrolyse and estimate the mixed-linked glucans in whole cell walls from barley and *Lolium* endosperm. The oligosaccharides released from the wall polymers by the *Bacillus* enzyme were measured as the carbohydrate soluble in 80% aqueous ethanol (Table 4). Incubations of urea-soluble glucans from *Lolium* and barley cell walls with the enzyme were performed under the same conditions to test for the completeness of enzymic digestion. After 144 hr incubation, hydrolysis of the isolated *Lolium* and barley glucans was complete and 24 and 58% respectively of the *Lolium* and barley cell wall were solubilized. No further hydrolysis of the cell walls was detected after a further 96 hr incubation. The *Bacillus* enzyme released a slightly greater amount of wall polysaccharide than could be extracted from the cell walls with 7 M urea.

Estimation of 1,3- *and* 1,4-*Linkages by Periodate Oxidation*

Oxidation of the urea-soluble glucans from *Lolium* and barley endosperm was complete after 570 hr and 0·79 and 0·73 mol of periodate respectively were consumed per mol of

[12] BARRAS, D. R., MOORE, A. E. and STONE, B. A. (1969) *Cellulases and their Applications, Advances in Chemistry Series* (GOULD, R. F., ed.), No 95, p. 105. American Chemical Society Publications, Washington, D.C.

[13] REESE, E. T. and PERLIN, A. S. (1963) *Biochem. Biophys. Res. Commun.* **12,** 194.

anhydroglucose equivalents present, indicating that approximately 79% and 73% of the glucose residues were 1,4-linked.

TABLE 4. ESTIMATION OF MIXED-LINKED GLUCAN PRESENT IN ENDOSPERM CELL WALLS BY THE β-GLUCAN HYDROLASE FROM *Bacillus subtilis*

Sample	% hydrolysis* time of incubation (48 hr)	(96 hr)	(144 hr)
Lolium glucan	29·2	97·4	105·5
Barley glucan	58·5	75·6	94·7
Lolium walls	20·3	23·8	23·8
Barley walls	39·4	57·7	57·7

* The weight of non-precipitable carbohydrate released (as glucose equivalents) is expressed as a percentage of the total weight of cell wall or β-glucan. The *Bacillus* enzyme (4 ml containing 1 mg protein/ml) was incubated at 40° with 1 mg of glucan, 5 mg *Lolium* walls or 2 mg barley endosperm walls[6] suspended in 1 ml of 0·05 M Tris-acetate buffer, pH 6·5. Triplicate samples were removed after 0, 48, 96 and 144 hr. Further enzyme (1·0 ml) was added to the remaining incubations after 48 and 96 hr. A sample (1 ml) of the incubation mixture was evaporated to dryness and extracted with 5 ml of 80% aq. EtOH. Carbohydrate was estimated by the phenol–sulphuric acid method using 1 ml of extract.

The oxidized polymers were reduced with borohydride, hydrolysed under mild acid conditions and the products separated and examined by paper chromatography (solvent C). Reducing compounds were detected which chromatographed with glycolaldehyde, erythritol, glucosyl erythritol and laminaribiosyl erythritol and other slower moving compounds were tentatively identified from their R_{Glc} values[14] as laminaritriosyl erythritol and laminaritetraosyl erythritol and possibly laminaripentaosyl erythritol. These results suggest that blocks of 1,3-linkages of variable length (from 1 to 4, or perhaps 5 linkages in sequence) are present in both the *Lolium* and barley urea-soluble glucans.

DISCUSSION

Polysaccharides are the major components of *Lolium multiflorum* endosperm cell walls prepared from stationary phase cells in culture. Quantitatively proteins and lipids are minor components, although at other stages of growth these non-polysaccharide constituents may be present in different proportions.

The predominant monosaccharides in the wall polysaccharides are glucose, xylose and arabinose, together with small amounts of galactose. The low uronic acid content and the absence of rhamnose in the hydrolysates suggest that pectic polysaccharides are absent.

Concentrated urea solutions (7·5 M) are known to selectively remove a glucose-rich fraction (85% glucose) from amylase-treated barley seeds[15] and Costello and Stone[6] demonstrated that 7 M urea solutions selectively remove a mixed-linked β-glucan from isolated barley endosperm cell walls. Urea solutions similarly extract a mixed-linked β-glucan from *Lolium* endosperm cell walls, but more of the mixed-linked β-glucan is present in barley (50% by weight of wall) than in *Lolium* cell walls (16% by weight of wall) and the

[14] GOLDSTEIN, I. J., HAY, G. W., LEWIS, B. A. and SMITH, F. (1965) *Methods in Carbohydrate Chemistry* (WHISTLER, R. L., ed.), Vol. 5, p. 361, Academic Press, New York.
[15] VAN SUMERE, C., MASSART, L. and VAN DER STRAETEN, A. (1955) *Naturwissenschaften* **42**, 463.

barley glucan contains slightly more 1,3 linkages. The solubilization of the mixed-linked glucan by concentrated urea solutions probably results from prevention of aggregation and coiling by the elimination of inter- and intra-molecular hydrogen bonding forces.[16,17]

Glucose is a major monosaccharide in the urea-residue and the presence of microfibrillar material in electron micrographs of the isolated wall[18] suggests that some of the glucose may be cellulosic. The nature of the remaining urea-insoluble polysaccharides which contain glucose, xylose and arabinose is under investigation.

EXPERIMENTAL

Enzyme preparations. Cellulosin AP from *Trichoderma viride* was the gift of the Ueda Kagaku Kogyo Co Ltd, Neyagawashi, Osaka-Fu, Japan. The powder (6 g) was dissolved in 60 ml of 0·1 M acetate buffer (pH 5·0) and centrifuged to remove a sticky white sediment which was anthrone positive. The supernatant was dialysed to remove reducing substances and concentrated by membrane filtration in a Diaflo apparatus (Amicon Corporation, Lexington, Mass., U.S.A.) fitted with a UM 2 ultrafilter and freeze-dried.

Cultures of *Sclerotium rolfsii* (the gift of Dr. D. F. Bateman, Department of Plant Pathology, Cornell University, Ithaca, U.S.A.) were grown[19] for 7–10 days on a rotary shaker at 25–29°. Endosperm cell walls (see Preparation of Cell Wall Fraction) (1%, w/v) from *Lolium multiflorum* were added as the carbon source. Polysaccharide hydrolases were obtained by filtering the culture through sintered glass to remove mycelia, adjusting the filtrate pH to 4·8 and concentrating to 0·05 of the original vol by rotary evaporation (40°). The concentrate was dialysed against H_2O (4°), centrifuged, and the supernatant membrane-filtered (UM 2 filter) and freeze-dried. A β-1,3-glucan hydrolase from *Rhizopus arrhizus* was isolated and purified by the method of Moore and Stone.[20] A crude β-1,3; 1,4-glucan hydrolase preparation from *Bacillus subtilis* was isolated from Bacterial Amylase Novo obtained from Novo Industri A/S, Fugelbakkevej 115, Copenhagen by the method of Dunkley and Stone (unpublished).

Chemicals. L-Arabinose, D-glucose, D-mannose and D-xylose (British Drug Houses, Poole, Dorset, U.K.) were recrystallized before use. The β-1,4- and β-1,3-oligosaccharides were obtained as previously described.[20] The glucosyl erythritol and laminaribiosyl erythritol samples were the gift of Prof. A. S. Perlin, McGill University, Montreal. Lichenin from *Cetraria islandica* was from Koch-Light Laboratories, Colnbrook, Bucks., U.K. and was dialysed against distilled water before use. The water-soluble oat and barley glucans and the urea-soluble barley glucan isolated from endosperm walls were the preparations previously described.[21,6]

Other reagents. These were obtained from the following sources: dimethyl-chlorosilane (May & Baker Ltd., Dagenham, U.K.); glucose oxidase, type II (Sigma Chemical Company, St. Louis, Missouri, U.S.A.); mixed bed resin (Bio-Rad AG 501-X8, Bio-Rad Laboratories, Richmond, California, U.S.A.) and nylon bolting silk—heavy quality, linear pore size 75 μ (Henry Simon, 161 Fitzroy Street, St. Kilda, Victoria). Other chemicals were commercial analytical grade reagents.

Preparation of cell wall fraction. Stationary phase cells from sucrose-grown suspension cultures of *Lolium* endosperm[9] were collected on nylon bolting silk, washed free of culture medium and suspended in H_2O (1:1, w/v). Cells were ruptured in a French pressure cell (Aminco Bowman, Silver Spring, Maryland, U.S.A.) at 10 tons in^{-2} and the homogenate examined microscopically to ascertain whether cell breakage was complete. Wall fragments were collected by centrifugation (1-h at 1200 *g*) and washed several times with H_2O to remove free proteins, starch granules and other cytoplasmic contaminants. Adhering particles and cytoplasm were loosened from the wall ultrasonically and separated from the walls by filtration and washing on a bed of glass beads.[22] Yields of cell wall material were between 1·3 and 2 g dry wt of walls per 100 g fresh wt of cells.

Fractionation of Cell Walls

The endosperm cell walls were fractionated by successive solvent treatment.[13] Although H_2O-soluble polymers would have been extracted during the isolation procedure, the walls were further extracted with H_2O at 40° (3 hr × 3) and the H_2O-insoluble residue was then extracted with phenol–HOAc–H_2O (PAW) (2:1:1, w/v/v)[23] at 25° (17 hr × 3) since PAW mixtures have been shown to be solvents for endosperm

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[23] JENNINGS, A. C. and WATT, W. B. (1967) *J. Sci. Food Agr.* **18,** 527.

proteins.[24] The solvent was buffered to pH 4·5 with pyridine to minimize acid-catalysed depolymerization of wall polysaccharides. Both residue and extract were dialysed against 40% HOAc in the cold to remove phenol and then against H_2O. The fraction insoluble in buffered PAW was further extracted with 7 M urea at 25° (17 hr × 3) and both extract and residue were dialysed against H_2O. A polysaccharide component was precipitated from the urea-soluble fraction by addition of 4 vol. EtOH and was collected by centrifugation. This material and the urea-insoluble residue were freeze-dried.

Analyses of cell walls and cell wall fractions: *HCl hydrolysis.* Samples (5 mg) were prechilled to −77° (acetone–CO_2) and 1·0 ml HCl, saturated with HCl gas at −77° was added.[25] Hydrolysis was in sealed tubes at 35° for 1 hr. The tubes were re-frozen, opened and the contents neutralized with 1 M NaOH. A sample was assayed for reducing sugars.

Enzymic hydrolysis. Cell wall hydrolyses (triplicate) contained 8 mg of *Lolium* endosperm cell walls, 2 mg of each of the freeze-dried preparations from Cellulosin AP and *S. rolfsii* culture filtrates, 0·1 M acetate buffer (pH 5 0) and a drop of toluene to prevent bacterial growth (total vol. 4·0 ml). Samples were removed after 50 and 160 hr and the reaction terminated by boiling (3 min). A sample (0·1 ml) from each hydrolysate was used for estimation of total reducing sugars.

Partial acid hydrolysis. Glucan samples (5 mg) were hydrolysed with 0·33 M HCl in sealed tubes for 1 hr at 100°. HCl was removed by evaporation *in vacuo* over NaOH.

Analyses. Total reducing sugars were estimated as glucose.[26,27] *Total carbohydrate* was estimated as anhydro-glucose by the phenol-H_2SO_4 method.[28,29] *Uronic acids* were estimated as glucuronic acid by a procedure involving uronic acid dehydrogenase.[30,31] *Lipid.* Cell walls were refluxed with $CHCl_3$–MeOH (2:1, v/v) for 6 hr (Soxhlet). *Other analyses* of wall components were performed by the Australian Microanalytical Service, Division of Applied Chemistry, C.S.I.R.O., University of Melbourne. Samples were dried under vacuum at 100°.

Periodate oxidation. The urea-soluble glucans (100 mg) from endosperm cell walls of barley[6] and *Lolium* were oxidized with $NaIO_4$.[14] When oxidation was complete (570 hr), unreduced IO_4^- was converted to IO_3^- by the addition of 10:1 excess ethylene glycol. The oxidized glucan was dialysed free of inorganic salts and reduced with 0·5 g $NaBH_4$ (10 hr at room temp.). HCl was added (final concn. 0·2 M) and the reduced polymer hydrolysed at pH 1·0 for 8 hr at room temp. The mixture was deionized with a mixed-bed resin (Bio-Rad AG 501-X8, H^+/HCO_3^- form) and the products separated by PC.

PC. Samples for chromatography were de-ionized with mixed-bed resin and chromatograms were run on water-washed Whatman No. 3 paper by downward development. Solvent A, EtOAc–n–PrOH–H_2O (4:1:2 by vol.) upper phase, was used to separate monosaccharides. Solvent B, *n*–PrOH–EtOAc–H_2O (6:1:3 by vol.) was used to separate oligosaccharides and the products of periodate oxidation were separated in solvent C, EtOAc–pyridine–H_2O (5:2:7 by vol.). Reducing sugars were detected with alkaline $AgNO_3$.[32,33]

GLC. GLC estimation of monosaccharides in wall hydrolysates was made using an alditol acetate procedure.[34]

Optical rotation. Specific optical rotations were measured in a Perkin–Elmer 141 Polarimeter.

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