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STRUCTURAL ANALYSIS AND ENZYMIC SOLUBILIZATION OF BARLEY ENDOSPERM CELL-WALLS*

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ABSTRACT

Barley endosperm cell-walls were prepared and analysed. The carbohydrate portion, which constituted most of the wall material, consisted of 10% of L-arabinose, 13% of D-xylose, 74% of D-glucose and 2.5% of D-mannose. Mixed-linkage β-D-glucan represented 70–72% of this material; the remaining 2–4% of D-glucose may be present as cellulose and glucomannan. Water and alkali-extracted β-D-glucans contained similar ratios of (1 → 3)- to (1 → 4)-linkages, namely 3 to 7. The walls, which had a protein content of approximately 5%, contained unidentified, alkali-labile linkages. An endo-(1 → 3)-β-D-glucanase from malted barley, and a fungal endo-(1 → 4)-β-D-glucanase, caused extensive solubilization of the wall polysaccharides.

INTRODUCTION

Barley endosperm cell-walls, their constituent polysaccharides, and the enzymic degradation of these materials have been of interest for a considerable time. Brown and Morris[1] pointed out the importance of the cell wall in barley, when they observed that the cell contents in germinating barley remained undegraded as long as the cell wall was intact. Early studies on the cell-wall polysaccharides involved analysis of a viscous water-soluble "gum", and the β-D-glucan component of this gum has received considerable attention[2]. However, not all of the wall β-D-glucan is extractable with water and more-drastic extraction conditions are required to solubilize the rest of this component. The insolubility of some of the β-D-glucan may arise from the fact that it may be derived from a larger carbohydrate polymer, or from a complex of polysaccharide with protein or other material.

The present experiments involved the preparation of endosperm cell-walls and an analysis of their composition. The cell walls were then extracted at temperatures important in the brewing industry, in order to estimate the amount of wall material

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that may be solubilized by hot water alone. Furthermore, possible structural differences in the water-soluble and water-insoluble β-D-glucan components were sought. Although hot water solubilizes a significant proportion of the wall material, the presence of β-D-glucanases in a malt extract might be expected to cause further solubilization. The ability of two β-D-glucanases to solubilize wall material from isolated, endosperm cell-walls was therefore also examined.

EXPERIMENTAL

Analytical methods

Estimation of protein. — The protein content of the wall preparation was estimated directly by the Miller modification[3] of the procedure of Lowry *et al.*[4]. The walls were homogenized in the presence of the alkaline-copper reagent before the Folin–Ciocalteu reagent was added. The samples were centrifuged to remove suspended walls before measurement. Protein was also estimated by micro-Kjeldahl digestion of the walls, using 6.25 as the nitrogen-to-protein conversion-factor.

Estimation of starch. — The starch remaining bound to the walls was estimated by digestion with alpha amylase and amyloglucosidase, followed by estimation of the D-glucose released by a D-glucose-oxidase reagent[5]. The wall preparation was also heated to 100° for several min to gelatinise the starch before incubation with amyloglucosidase, again followed by the addition of D-glucose oxidase. The results from both methods were in close agreement.

Estimation of monosaccharides and methylated sugars. — For analysis by gas–liquid chromatography (g.l.c.), the monosaccharides or their derivatives were converted into their corresponding alditol acetates as described by Lindberg[6]. G.l.c. analysis was then carried out with a Pye-Series 104 Chromatograph equipped with a flame-ionization detector. A glass column (1.5 m) was packed with Silicone 0V-225 (3% stationary phase on Gas-Chrom Q, 100–200 mesh) and was maintained at 200°. Nitrogen was used as the carrier gas at flow rates of 20 ml/min and 60 ml/min for methylated and unmethylated samples respectively. The eluted peaks were identified by comparison with known standards and their identity was confirmed by m.s. with an AEI MS-30 double-beam mass spectrometer connected directly to the gas chromatograph.

Methylation and hydrolysis. — All methylations were conducted by the Hakomori method[7], with sodium dimethylsulphinyl carbanion prepared by a procedure similar to that outlined by Conrad[8]. Each sample was methylated 3 times to ensure complete methylation. The methylated polysaccharides were hydrolysed in 90% formic acid for 2 h at 100°, after which time the formic acid was evaporated off and the samples were hydrolysed for a further 3 h in M sulphuric acid at 100°. The hydrolysates were neutralized with barium carbonate and barium sulphate was removed by centrifugation.

Microscopy. — For light microscopy, the wall samples were stained for protein

with Ponceau S stain (0.9 g of Ponceau S, 1.34 g of trichloroacetic acid, and 1.34 g of salicyclic acid in 100 ml of water), and for starch, with iodine solution (0.2% iodine in 20% potassium iodide). Scanning electron microscopy was performed on samples mounted on metal stubs that were then coated with gold to a thickness of approximately 30 nm. The samples were examined with a Cambridge Stereoscan 2A electron microscope, using an accelerating voltage of 30 kV. For transmission electron microscopy, the wall materials were dried, suspended in abs. ethanol, and then redried onto copper grids. The samples were shadowed with platinum at an angle of 15° from the horizontal and examined with an AEI Model 802 Transmission electron microscope, operating with an accelerating voltage of 60 kV.

Enzymic hydrolysis of the endosperm cell-walls. — Wall samples (3 mg) were weighed into centrifuge tubes with 0.2M sodium acetate buffer, pH 5.0 (3.0 ml). The walls were maintained in suspension by using a magnetic stirrer, and the tubes were incubated at 37°. Into the tubes, water (as a control) or purified enzyme (50 μl) was added. At intervals of 24 h, the digest tubes were centrifuged and analysed for soluble carbohydrate by the phenol–sulphuric acid reagent[9]. The sedimented walls were resuspended in fresh buffer, and more enzyme was added. This procedure was continued for 5 days. The amount of solubilized carbohydrate released each day is expressed as D-glucose equivalents.

Preparative methods

Preparation of endosperm cell-walls. — Barley flour prepared from pearled barley (var. Julia) was used as the starting material and was kindly provided by Dr. G. H. Palmer of the Brewing Institute Research Foundation, Nutfield, Surrey. The flour (100–200 g lots) was slurried in 70% aqueous ethanol and wet-sieved on a 100-mesh, stainless-steel sieve (150 linear pore-size). The slurry was maintained by multiple additions of 70% ethanol while the flour was gently worked over the sieve. This procedure removed most of the starch and protein. The material retained by the sieve was then gently worked in a mortar and transferred to a nylon cloth (75-μm linear pore-size). Several treatments with a Potter–Elvejhem homogenizer equipped with a Teflon plunger were found to be very effective in loosening adhering starch and protein from the walls. Contamination by furrow tissue was removed by repeated sedimentations, as the clean walls sediment more slowly than the brownish furrow tissue. The clean wall-preparation was stored in 70% ethanol at 3°. The procedure is based on that of Mares and Stone[10], with modifications developed by Palmer[11]. An important difference is that the walls are not dried, but are kept in 70% ethanol. Although this procedure prevents quantitation of the yields of wall components, it maintains the walls in a better state for subsequent work, and avoids the dehydration that may cause undesirable changes in the physical state of the polysaccharides.

Extraction of walls. — The wall preparation (about 1 g) was centrifuged to remove as much of the 70% ethanol as possible before being suspended in 10mM sodium acetate buffer, pH 5.0 (100 ml) containing amyloglucosidase (0.1 ml) and

salivary alpha amylase (0.1 ml). The suspension was homogenized with a Potter–Elvejham homogenizer and then stirred for 1 h at 45° or 65°; one sample was extracted at 45°, while two separate samples were treated at 65°. The viscous extract was centrifuged and the pelleted walls were re-extracted three more times for 2-h periods under the same conditions. The combined supernatant solutions from each extract were treated with 3 vols. of ethanol and cooled to 3° to cause precipitation of the water-soluble polysaccharides.

The remaining water-extracted walls were then re-extracted with M potassium hydroxide containing 0.5% of potassium borohydride (100 ml). An initial extraction at room temperature resulted in a viscous solution. Three subsequent extractions were performed as for the water extraction, but at 55°. The combined extracts were then neutralized with acetic acid, and 3 vols of ethanol were added at 3° to cause precipitation. The alkali-insoluble material, referred to as the residue, was washed with distilled water and then with increasing concentrations of ethanol. The water and alkali-extracted fractions were similarly washed several times with ethanol and then all of the samples were dried in a vacuum oven at 45°.

Isolation of β-D-glucan fractions I and II. — The water-extracted polysaccharide material was solubilized by heating at 65° in water. The solution was centrifuged to remove any insoluble material and made 20% (w/v) with ammonium sulphate[2] and cooled to 3°. The recovered precipitate was β-D-glucan that contained only a trace of arabinoxylan, as shown by paper chromatography after acid hydrolysis. These contaminants were removed by a second precipitation in the same manner. The purified glucan is referred to as glucan I.

The alkali-extracted polysaccharide material could not be fractionated with ammonium sulphate as just described. Instead, ethanol to 18% (v/v) was added to the material that had been resolubilized in water and dialyzed free of ammonium sulphate, and the solution was cooled to 3°. The precipitate recovered was reprecipitated at 18% ethanol, to yield a fraction that was low in arabinose and xylose. These contaminants were subsequently removed by digestion of this fraction with an endo-(1 → 4)-β-D-xylanase which had been purified from a fungal culture filtrate. The β-D-glucan was finally recovered by ethanol precipitation at 50% (v/v) and is referred to as glucan II.

Enzyme preparations. — *A. Amyloglucosidase.* This enzyme, from *Aspergillus niger*, was purchased from BDH Chemicals Ltd., (Poole, Gt. Britain) and freed from a β-D-glucosidase impurity by chromatography on a column of DEAE-cellulose, which was eluted with a linear gradient of sodium chloride (0–0.3M) in 20mM Tris–hydrochloric acid buffer, pH 8.5. The purified preparation had no action on laminarin, barley β-D-glucan, or wheat-flour arabinoxylan.

B. Alpha amylase. Bacterial alpha amylase was purchased from the Wallerstein Laboratories (New York, U.S.A.) and used at a concentration of 2 mg/ml. Salivary alpha amylase was a laboratory preparation.

C. Endo-(1 → 4)-β-D-xylanase. This was prepared from a culture filtrate of the thermophilic fungus *Thermoascus crustaceus*, grown on 0.1% barley-husk

arabinoxylan as described by Flannigan and Sellars[12]. The enzyme was purified by column chromatography, first on DEAE-Sephadex with a linear gradient of sodium chloride (0–0.25M) in 20mM sodium phosphate buffer (pH 6.8) and second, on pachyman. The final preparation did not release arabinose from wheat-flour and barley-husk arabinoxylans, but rapidly released xylose and higher oligosaccharides, which on paper chromatography corresponded to some of the products released by partial acid hydrolysis of these substrates. It had no action on laminarin or barley β-D-glucan.

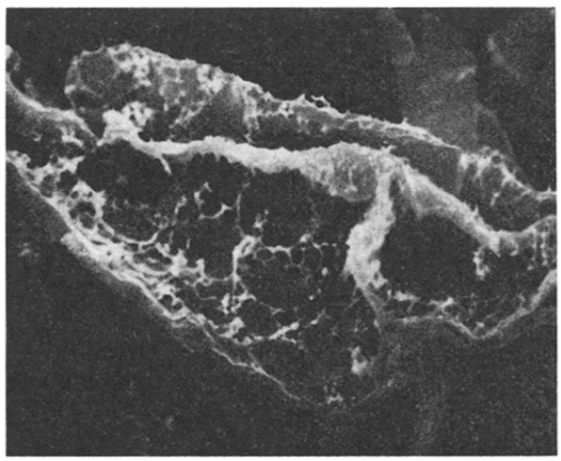
D. Endo-(1 → 4)-β-D-glucanase. This enzyme was prepared[13] from a commercial enzyme preparation, Glucanase GV, by a combination of chromatography on DEAE-cellulose and Sephadex G-150. A final purification step involved column chromatography on hydroxylapatite. With all three types of column, the elution of the activity towards lichenin always corresponded to a peak in the activity towards *O*-(carboxymethyl)cellulose. This suggests that the two activities are due to the same enzyme. Details of the purification of this enzyme will be given elsewhere. The purified enzyme had no action on laminarin or arabinoxylan, but hydrolysed *O*-(carboxymethyl)cellulose and barley β-D-glucan. The latter substrate gave the trisaccharide 4-*O*-β-laminaribiosyl-D-glucose and the related tetrasaccharide as the major products. This enzyme is therefore an endo-(1 → 4)-β-D-glucanase (EC 3.2.1.4) rather than an endo-(1 → 3(4))-β-D- glucanase (EC 3.2.1.6), which yields the trisaccharide 3-*O*-β-cellobiosyl-D-glucose from the mixed-linkage β-D-glucans[14].

RESULTS

Analysis of endosperm cell-wall preparations. — The cell walls were prepared as described in the Experimental section. Their purification was monitored by light-microscopy by using an iodine solution to stain starch granules, and Ponceau S stain for the detection of protein. In the final wall-preparation, both of these components were still present in small proportions, and occurred together on the wall. Generally, the wall surfaces were starch-free and did not stain noticeably with Ponceau S. The amount of starch that was present in the wall preparation was estimated enzymically, and accounted for 2.5% by weight of the wall. Protein was determined in the wall preparation by a colorimetric method[3] and by Kjeldahl estimation, and amounted to 4.8 and 4.9% by weight, respectively.

The major components of the walls were carbohydrate in nature. The relative monosaccharide composition of the polysaccharide constituents was estimated by acid hydrolysis and subsequent quantitative g.l.c. After correction for the glucose that was present as starch, the carbohydrate composition of the wall was arabinose, 10%; xylose, 13%; mannose, 2.5%, and glucose, 74%. On the basis of previous work in the literature, it is assumed that all the monosaccharides, except arabinose, have the D configuration.

Appearance of walls. The final preparations of endosperm cell-walls were examined by scanning electron microscopy. As observed previously by Fincher[15],



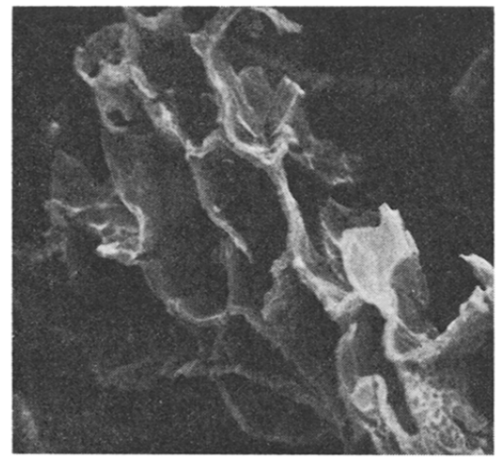


Fig. 1. Scanning electron micrograph of final wall-preparation, showing a clean wall-fragment with indentations left by starch granules of various sizes (× 1000).

Fig. 2. Scanning electron micrograph of final wall-preparation, showing groups of clean cell-walls still attached to one another (× 1000).

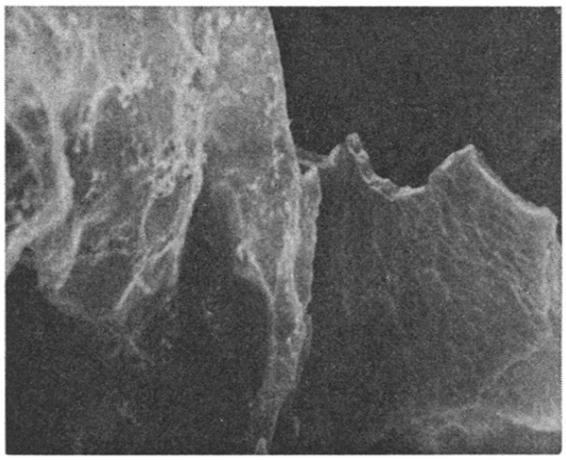


Fig. 3. Scanning electron micrograph of final wall-preparation, showing an apparently scaly cell-wall surface (× 1000).

the inner wall surface appears deeply pitted with indentations made by starch granules (Fig. 1). However, this does not always appear to be the case, as may be seen in Fig. 2. Here a group of interconnected cells appear to have both smooth and pitted inner surfaces. In Fig. 3 what is assumed to be an outer cell surface appears smooth, but scaly by comparison to the pitted inner surfaces.

Extraction of walls. — Three separate samples of the wall preparation were extracted as described in the Experimental section. In the first case, the walls were extracted with water at 45°, whereas in the second and third cases, the walls were

TABLE I

MONOSACCHARIDE COMPOSITION OF EXTRACTED WALL FRACTIONS, ESTIMATED AS THEIR ALDITOL ACETATES

Extraction temperature	*Fraction*	*% of total walls (w/w)*	*%(w/w)*				
			Ara	*Xyl*	*Man*	*Glc*	*Ara/Xyl*
45°	H_2O soluble	9.1	11	14	2	71	0.79
55°	KOH soluble	82.1	13	15	0	72	0.87
	Residue	8.8	9	11	21	58	0.82
65°	H_2O soluble	32.6	4	6	1	89	0.67
55°	KOH soluble	63.1	11	18	0	70	0.61
	Residue	4.3	11	13	37	39	0.84
65°	H_2O soluble	34.5	5	7	1	88	0.71
55°	KOH soluble	62.0	12	17	2	69	0.70
	Residue	3.6	10	11	46	33	0.91

TABLE II

RELATIVE COMPOSITION OF METHYLATED β-D-GLUCANS

Sample	*Methyl ether*	*Mole (%)*	
		1	*2*
Glucan I	2,3,4,6-Me_4-Glc	0	0
	2,4,6-Me_3-Glc	34	32
	2,3,6-Me_3-Glc	65	68
Glucan II	2,3,4,6-Me_4-Glc	0	0
	2,4,6-Me_3-Glc	29	30
	2,3,6-Me_3-Glc	68	68
	3,6 or 4,6-Me_2-Glc	1.5	1.8

TABLE III

DEGREE OF POLYMERIZATION OF β-GLUCAN FRACTIONS

Sample	*D.p.*
Glucan I	405
Glucan I (xylanase treated)	390
Glucan II	258
Barley β-D-glucan[a]	254

[a]Prepared from barley flour.

extracted at 65°. The amount of wall material extracted with water and alkali and the relative composition of the extracted materials are given in Table I. About one-third of the wall is solubilized by water at the temperature normally used in mashing (about 65°), and ~90% of the soluble material is glucan. Water- and alkali-extracted glucans (referred to as glucans I and II respectively) were also prepared.

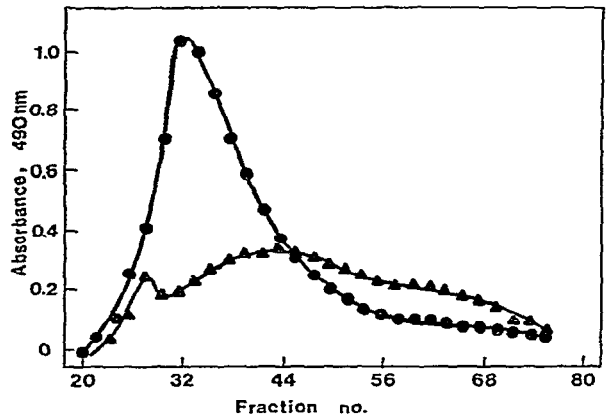


Fig. 4. Polydispersity of water- (●—●) and alkali- (▲—▲) extracted β-D-glucans as shown by their separation profiles on Sepharose 4B. Samples were assayed for total carbohydrate by the phenol–sulphuric acid method[9].

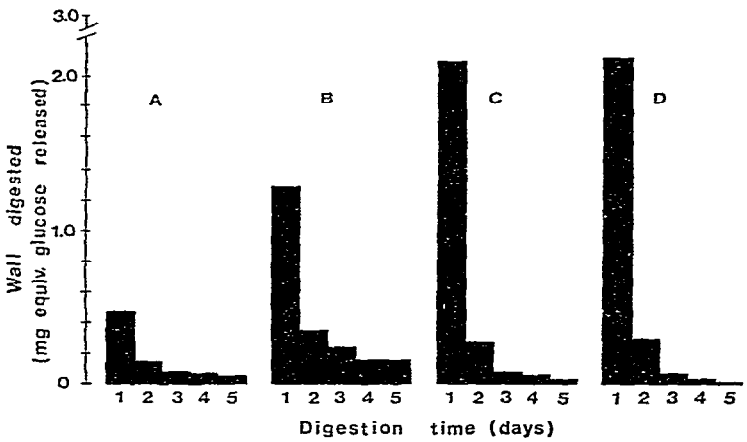


Fig. 5. Digestion of barley endosperm cell-walls by barley endo-(1 → 3)-β-D-glucanase and fungal endo-(1 → 4)-β-D-glucanase. A, control experiment indicating leaching of wall carbohydrate by buffer alone. B, carbohydrate released by endo-(1 → 3)-β-D-glucanase. C, carbohydrate released by endo-(1 → 4)-β-D-glucanase. D, solubilization of wall polysaccharide by the two enzymes acting together.

Methylation analysis of glucan fractions. — Methylation analysis of glucan I and II was carried out. Glycogen β-limit dextrin and laminarin were used as standards to identify the methylated derivatives of glucose on the basis of relative retention-times by g.l.c., the identity of the derivatives being confirmed by m.s. The results are given in Table II. As the 2,3,4,6-tetra-*O*-methyl-D-glucose peak was small (less than 1 %), it was not included in the calculation of the percentage composition of the types of linkages.

Degree of polymerization of the glucan fractions. — The D-glucitol dehydrogenase method was used[16] to estimate the degree of polymerization of the glucan

fractions I and II. To ensure that the xylanase used to purify fraction II did not cause any change in the degree of polymerization, fraction I was treated under identical conditions. The results of these determinations are given in Table III, and show that the xylanase preparation was free of β-D-glucanase activity.

Polydispersity. — The range of the molecular size of the components present in each of the β-D-glucan fractions was examined by molecular-sieve chromatography on a column of Sepharose 4B. The results are shown in Fig. 4, and indicate a significant difference in the separation profiles.

Enzymic hydrolysis of the endosperm cell-walls. — A histogram showing the amount of carbohydrate solubilized by barley endo-(1 → 3)-β-D-glucanase[17] and the fungal endo-(1 → 4)-β-D-glucanase, acting separately and together, is presented in Fig. 5. For this enzymolysis, the walls were dried from the 70% ethanol suspension by washing several times with 95% ethanol, followed by drying in a vacuum oven.

DISCUSSION

The relative carbohydrate composition of the barley endosperm cell-walls (Table I) is in good agreement with the values reported by Fincher[15]. The 5% of protein present in the wall preparation is considered to be at least partially due to cytoplasmic protein, as walls stained with Ponceau S showed localized areas of red stain. These areas also often contained small starch granules. However, starch granules were not observed by scanning electron microscopy, which suggested that those granules remaining in the purified preparation of walls may occur in protected areas, such as in partially broken cells (Fig. 2). Cross-sections of the walls showed no discernible layers. This is not unexpected as the walls are likely to be considerably compacted by desiccation. This contrasts with *Bromus* endosperm cell-walls where, after first fixing and embedding the walls, several discernible layers have been observed by transmission electron microscopy[18].

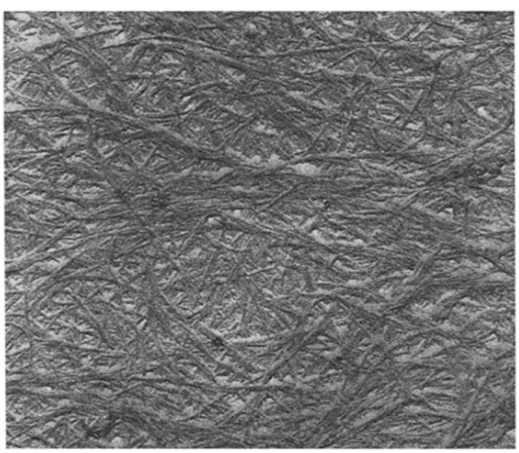


Fig. 6. Transmission electron micrographs of walls after alkali extraction, showing a multi-net arrangement of microfibrils (× 53,000).

Nature of mannose in the wall. — Mannose present in the wall amounted to 2.5% by weight of the total carbohydrate material. Although the origin of the mannose, largely present in the alkali-insoluble residue, has not been determined, it should be noted that a glucomannan has also been isolated from the alkali-insoluble fraction of wheat endosperm cell-walls[10].

Cellulose. — Brown and Morris[1] originally assumed that the major endosperm cell-wall material was cellulose. However, when the cellulose content of the barley endosperm was estimated by MacLeod and Napier[19], a very low value was found, and it was suggested that this small amount of cellulose could be accounted for by furrow contamination alone. Tentative evidence for the presence of cellulose is provided by alkaline extraction of the endosperm cell-walls; a glucose-rich material is found that shows a microfibrillar structure by transmission electron microscopy (Fig. 6). Similar microfibrillar structures have been observed in the endosperm cell-walls prepared from barley, by Fincher[15], and from rye, by Mares and Stone[20]. If the microfibrillar material is, in fact, cellulose, the present data indicate that its concentration in the endosperm cell wall is 1% or less.

β-D-Glucan. — Approximately 70% of the endosperm cell-walls of barley is composed of mixed-linkage β-D-glucan. The ratio of (1 → 3)- to (1 → 4)-linkages estimated by methylation was essentially 3 to 7 for both fractions I and II (Table II). Thus, the present methylation results confirm the linkage ratio of soluble cereal β-D-glucan that had previously been determined by other methods[21–23], and extends it to show that the alkali-extracted β-D-glucan is not significantly different. The relatively small proportion (1.5–1.8%) of a di-*O*-methyl D-glucose derivative is not regarded as being structurally significant, particularly since a corresponding amount of tetra-*O*-methyl D-glucose could not be detected.

One possible reason for the difference in extractability of the two glucan fractions is that the less-soluble one could have been of higher molecular weight. For this reason, the molecular size of the two β-D-glucan fractions was examined (Table III and Fig. 4). The results indicate that the less-soluble fraction actually had a lower degree of polymerisation. Alkaline degradation[24] should have been minimised during the extraction, as potassium borohydride was present at all times.

One explanation for the difference in the β-D-glucan components could be that the less extractable β-D-glucan is bound physically, ionically, or covalently into the wall by other components. Possible covalent linkages are seryl- or threonyl-carbohydrate linkages, or ester linkages[25] between the numerous free hydroxyl groups of the β-D-glucan and carboxyl groups, such as are found in aspartic and glutamic acids of the protein material, and to a lesser extent, in the arabinoxylan fraction. Ferulic acid and other phenolic materials have also been suggested as possible cross-linking components within plant cell-walls[25], and the presence of ferulic acid–carbohydrate complexes in barley endosperm cell-walls has recently been established[26].

During the extraction of the walls, when potassium borohydride was added to the water-extracted walls after re-suspension in water, there was an immediate

further release of wall material. This suggested the breakage of labile linkages, with subsequent solubilization of polymeric material. Thus, the present results would suggest that a more-detailed examination of the wall will be necessary before possible cross-linkages or carbohydrate–protein linkages can be positively identified. It should be noted that there is now convincing evidence that certain glucans (such as starch, glycogen[27], and paramylon[28]) are synthesized on polypeptide or protein backbones, and it is possible that the mixed-linkage β-D-glucan could be formed by a similar type of mechanism.

A preliminary examination of the arabinoxylan component was also made[29]. After considerable fractionation and enzymic digestion with endo-β-D-glucanases (free of arabinoxylanase activity), a small fraction of purified arabinoxylan was obtained. Methylation analysis by the procedure described indicated an arabinose: xylose ratio of ~2:3, and that approximately 15% of the molecule was composed of quadruply-substituted xylose residues, which were linked at O-1 and O-4 to other xylose residues, and at O-2 and O-3 to single arabinofuranose residues. Whether the fraction prepared was representative of the total arabinoxylan material in the endosperm cell-walls is not yet known. Other workers have also reported[30] the occurrence of the quadruply-substituted xylose residues, in an arabinoxylan from barley aleurone cells. Work is continuing to clarify the structure of the endosperm cell-wall arabinoxylan.

The possible function of β-D-glucanases in germinating barley has not been extensively examined from the point of view of wall solubilization. In the present study, a fungal endo-(1 → 4)-β-D-glucanase was a most effective solubilizing enzyme and was used as a possible comparator to the endo-barley-β-D-glucanase (namely, the mixed-linkage β-D-glucanase, E.C. 3.2.1.73) from malted barley[31]. The latter enzyme is considered to be of major importance in the degradation of barley β-D-glucan, but its potential to attack the insoluble wall directly has not yet been examined. The present results suggest that the fungal enzyme is very effective as a wall-solubilizing enzyme. The barley endo-(1 → 3)-β-D-glucanase[17] also appears to be able to release a substantial amount of wall material, but required a longer incubation time. Because of the different specificities of the fungal and barley enzymes, a direct comparison of their enzyme activities is not very meaningful. Together, these two enzymes rapidly released approximately the same amount of soluble material as either enzyme would eventually release by itself. A possible role for the endo-(1 → 3)-β-D-glucanase is that of an initial wall-solubilizing enzyme. A more detailed study with the endo-barley-β-D-glucanase, rather than the fungal enzyme, might therefore help to clarify the role of these enzymes in the initial solubilization of the endosperm cell-wall.

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