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The Structure of Plant Cell Walls

VII. BARLEY ALEURONE CELLS[1]

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ABSTRACT

The walls of barley (*Hordeum vulgare* var. Himalaya) aleurone cells are composed of two major polysaccharides, arabinoxylan (85%) and cellulose (8%). The cell wall preparations contain 6% protein, but this protein does not contain detectable amounts of hydroxyproline. The arabinoxylan has a linear 1,4-xylan backbone; 33% of the xylosyl residues are substituted at the 2 and/or 3 position with single arabinofuranosyl residues. The results of *in vitro* cellulose binding experiments support the hypothesis that noncovalent bonds between the arabinoxylan chains and cellulose fibers play a part in maintaining wall structure. It is suggested that bonding between the arabinoxylan chains themselves is also utilized in forming the walls.

The aleurone layer is a specialized secretory tissue situated at the periphery of the starchy endosperm in seeds of the Gramineae. Since the discovery (31, 46) that the secretory function is stimulated by gibberellic acid, aleurone layers have become a major physiological system for studying the mode of action of this hormone (21, 47). The response to gibberellic acid includes the *de novo* synthesis and secretion of α-amylase (15) and protease (20). In addition, gibberellic acid stimulates the release of a broad spectrum of other hydrolases (8) and ions (22).

Aleurone cells have thick cell walls (3–5 μM) which appear to undergo extensive degradation during the phase of gibberellic acid-stimulated enzyme release (40). It has been suggested that intact aleurone cell walls may present a barrier to the mobilization of these enzymes (44). In examining this possible wall function, attention has focused on the composition of the walls. On the basis of staining and ultraviolet fluorescence with alkaline aniline blue, considered a test for callose and related materials (11), Taiz and Jones (40) have suggested that barley aleurone cell walls contain β-1,3-glucan. Recently, Fulcher *et al.* (16) have demonstrated the nonspecificity of aniline blue analysis and have concluded that β-1,3-glucosyl linkages are not present in aleurone walls in significant amounts. In order to resolve this question, we undertook a chemical characterization of aleurone cell walls. Our results confirm the absence in aleurone cell walls of β-1,3-linked glucosyl residues and show that the walls consist of approximately 85% arabinoxylan, 8% cellulose, and 6% protein.

MATERIALS AND METHODS

Isolation of Barley Aleurone Cell Walls. About 100 aleurone layers were isolated from de-embryonated barley (*Hordeum vulgare*, L. var. Himalaya) half-seeds according to the procedure of Chrispeels and Varner (10). Isolated aleurone layers were treated with 20 ml of a 1% solution of purified bacterial α-amylase (Sigma, type IIA) in 4 mM sodium acetate, pH 4.8, containing 10 mM $CaCl_2$. Incubation of this suspension for 4 hr at 25 C on a Metabolyte waterbath shaker maintained at 150 rpm resulted in the complete removal of all adhering starch grains. Starch-free aleurone layers were collected in a sieve, washed thoroughly with distilled H_2O, and blended in a Sorvall Omnimixer for 2 min at half maximum speed to dislodge the testa-pericarps. The bulk of the testa-pericarps was discarded by careful decanting; those remaining were removed with forceps. This preparation of aleurone tissue was homogenized in 25 ml of 5 mM tris-HCl, pH 7.6, in a Ten-Broeck glass homogenizer. The homogenized tissue was dialyzed against distilled H_2O for 48 hr at 4 C. An aliquot of this suspension was analyzed and is referred to as total nondialyzable material. The remainder of the suspension was centrifuged at 3000*g* for 5 min. The resulting pellet was resuspended in 10 ml of the tris buffer containing 5 mM EDTA, 100 mg of sodium dodecyl sulfate, and 100 mg of pronase (Calbiochem) and incubated overnight at 27 C on the Metabolyte waterbath shaker. Following pronase treatment, the wall material was collected by centrifugation and washed twice with 30 ml of the tris buffer and then with 30 ml of distilled H_2O. The washed wall material was shaken overnight at 25 C in 50 ml of 50% (w/v) urea. The urea-extracted wall residue was pelleted by centrifugation and washed with distilled H_2O. The final pellet was collected on a glass fiber filter (Whatman GF/C), washed with a further 500 ml of distilled H_2O followed by 150 ml of acetone, and allowed to air dry.

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Analytical Techniques. Neutral sugar compositions were determined as described (23, 42). Cell walls were methylated by the Hakomori method (19), and analysis of the permethylated polysaccharides was performed as described (42) except that the partially methylated aldoses were reduced with sodium borodeuteride rather than sodium borohydride to aid in mass spectral analysis. The partially methylated alditol acetates were identified by gas chromatographic retention times and mass spectral analysis. Protein content was determined by the Lowry method (26). Uronic acid composition was determined by the use of the carbazole reagent (5) and by the use of metahydroxydiphenyl as described by Blumenkrantz and Absoe-Hansen (7). Cellulose was determined by the Updegraff procedure (43). Hydroxyproline was determined by the method of Kivirikko and Liesmaa (24). Total pentose was assayed by use of the orcinol reagent as described by Dische (13).

Solubilization of Aleurone Cell Wall Arabinoxylan. Cell wall arabinoxylan was solubilized in alkali by a modification of a procedure of Mares and Stone (28). Thirty-two milligrams of aleurone cell walls were treated for 5 hr at room temperature with 5 ml of 4.4 M NaOH containing 25 mM $NaBH_4$. An insoluble residue containing cellulose and some arabinoxylan was then removed by centrifugation. The supernatant solution was neutralized with glacial acetic acid and dialyzed against distilled H_2O for 3 days at 4 C. The solubilized arabinoxylan was used directly.

Cellulose Binding of Alkali-solubilized Arabinoxylan. Twenty-five milligrams of Whatman CC-41 cellulose powder were placed in each of eighteen 15-ml conical centrifuge tubes. One milliliter of a solution containing 0.38 mg of base-solubilized arabinoxylan was added to each tube, and the suspension was stirred overnight with a magnetic stirring flea. The suspension was then centrifuged, and the supernatant solution was recovered and assayed by the orcinol method for pentose. The amount of arabinoxylan bound to cellulose was calculated by subtracting the amount of pentose remaining in the supernatant solution from the amount of pentose known to be in the solution before stirring with cellulose. The remaining supernatant solution was lyophilized to obtain the arabinoxylan which does not bind to cellulose.

The cellulose pellets, containing bound arabinoxylan, were each washed with 1 ml of H_2O and stirred overnight with 1 ml of 4.4 M NaOH containing 26 mM sodium borohydride. Each tube was centrifuged, and the resolubilized arabinoxylan was decanted. This solution was neutralized with glacial acetic acid, dialyzed against distilled H_2O, and arabinoxylan was recovered by lyophilization.

RESULTS

Isolation of Aleurone Cell Walls. To be certain that significant amounts of wall carbohydrate were not discarded from the "cell wall fraction" during subsequent purification, the starch and testa-pericarps-free aleurone tissue was assayed for neutral carbohydrate. The tissue was found to contain 23% xylose, 15% arabinose, 0.7% mannose, 1.5% galactose, and 6% glucose. Comparison of the amounts of arabinose and xylose in the initial aleurone tissue and the amount of arabinose and xylose in the final cell wall fraction showed that 70% of both the arabinose and the xylose remained in the cell walls. A similar calculation revealed that 14% of the glucose, 22% of the galactose, and 10% of the mannose remained in the cell walls. The glucose, galactose, and mannose-containing polymers may or may not be part of the walls. Their structures were not further investigated.

The pronase treatment was included in an effort to free the walls of contaminating cytoplasmic proteins. It is possible that this treatment may remove some wall protein. In an effort to answer this question, the total nondialyzable fraction of crude aleurone tissue homogenates was assayed for hydroxyproline to see if the subsequent pronase treatment removes any hydroxyproline-containing protein. As with the purified cell walls, no hydroxyproline was detected, within the limits of the assay, in the total nondialyzable fraction (less than 0.1%). Thus, if the pronase treatment removes wall protein, it is not hydroxyproline-rich. Ethylenediaminetetraacetic acid was included in the pronase step to solubilize phytic acid, a major constituent of aleurone tissues.

Table I. *Composition by Weight Per Cent of Total Aleurone Cell Walls and Cell Wall Fractions*

Component	Weight Total Wall	Per Cent of Total Wall		
		Alkali-soluble Fraction of the Total Wall	Alkali-soluble Fraction Which Binds to Cellulose	Alkali-soluble Fraction Which Does Not Bind to Cellulose
Arabinose	31	29	16	14
Xylose	54	41	36	10
Mannose	0.2	0	0	0
Galactose	1	1	trace	trace
Glucose (noncellulosic)	3	5[1]		
Cellulose	8			
Protein	6			
Hydroxyproline	<0.1[2]			
Uronic acids	<1.5[2]			
Total	103.2	77	52	25
Ratio of arabinose to xylose	0.58	0.71	0.44	1.38

[1] This figure may be higher than the total wall figure due to the solubilization of some of the cell wall cellulose.

[2] Within limits of the assay used, no hydroxyproline or uronic acids were detected.

Total Cell Wall Composition. The composition of purified aleurone cell walls is presented in Table I. Neither uronic acids nor hydroxyproline was detectable in this preparation. The mole percentages of the various methylated, acetylated alditols recovered after the purified aleurone walls were methylated, hydrolyzed, reduced, and acetylated are presented in Table II. All assays in Table I and Table II were done on duplicate samples and all assays on total aleurone walls were carried out on at least two different cell wall preparations. The 2,4-linked and 3,4-linked xylosyl residues are not separable by the available gas chromatographic methods. Combination gas chromatography-mass spectrometry indicated that both derivatives are present in aleurone walls; the combined amount of these two derivatives is listed as a single entry in Table II.

Extraction of Aleurone Walls with Alkali. Aleurone walls were extracted with alkali as described in "Materials and Methods." The 4.4 M NaOH solubilized approximately 90% of the arabinose and xylose, while extraction with 0.2 M NaOH at 4 C failed to solubilize any of the wall polysaccharides. Neutral sugar analysis of the alkali-solubilized material gave a ratio of arabinose to xylose of 0.71, similar to the ratio of 0.58 obtained by analysis of the unfractionated walls. Once solubilized, the arabinoxylan remained soluble in neutral solution.

Cellulose-binding Experiments. The solubilized arabinoxylan was suspended with cellulose as described in "Materials and

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Table II. *Mole Per Cent of Total Methylated Acetylated Alditols Isolated from Methylated Aleurone Walls and from Aleurone Wall Arabinoxylan Fractionated on Cellulose*

Sugar	Determined Position of O-Methyl Groups	Deduced Glycosidic Linkage[1]	Total Aleurone Cell Walls	Arabinoxylan Which Binds to Cellulose	Arabinoxylan Which Does Not Bind to Cellulose
Arabinose	2,3,5	Term	30	24	46
	3,5	2	0.5	0	2
	2,5	3	1	0	4
	2,3	5	1	0	3
Total			32.5	24	55
Xylose	2,3,4	Term	1	trace	1
	2,3	4	39	59	15
	3 and 2	2,4 and 3,4[2]	14	10	14
		2,3,4	6	7	15
Total			60	76	45
Galactose	2,3,4,6	Term	0.3	0	0
Total			0.3	0	0
Glucose	2,3,6	4	6	0[3]	0
Total			6	0	0

[1] Glycosidic linkages were assigned as in reference 42.

[2] 2,4- and 3,4-linked xylosyl residues are both present but are not separated by the gas chromatographic conditions used. The numbers listed are for the total of 2,4- and 3,4-linked xylose.

[3] Some 4-linked glucosyl residues are present in the cellulose-bound arabinoxylan after extraction from the cellulose. This glucose was shown to originate from the cellulose and is not from the aleurone cell walls.

Methods." In several such experiments, 60 to 70% of the arabinoxylan bound to the cellulose. Less than 10% of the arabinoxylan which fails to bind to cellulose is found to bind when a second batch of fresh cellulose is added to the arabinoxylan solution. Hence, cellulose "extraction" results in two discrete arabinoxylan fractions—one that binds to cellulose and one that does not bind to cellulose. The cellulose-bound arabinoxylan was resolubilized using the alkali extraction conditions described in "Materials and Methods." Control experiments, using cellulose which had not been exposed to the solubilized arabinoxylan, showed that the base treatment solubilized small amounts of material containing glucose but no arabinose or xylose. The ratio of arabinose to xylose in the arabinoxylan which binds to cellulose is 0.44, while the ratio of arabinose to xylose in the arabinoxylan which does not bind to cellulose is 1.38. The mole percentage of various methylated acetylated alditols recovered from the bound and unbound arabinoxylan fractions is presented in Table II.

DISCUSSION

Although numerous analyses of the polysaccharides of whole barley seeds have been published (33–37), these studies are only roughly indicative of the polysaccharide composition of the walls of the various cell types of these seeds. The only direct observations on the aleurone cell wall have been cytological in nature. Our chemical study of aleurone cell walls shows that they are composed largely of only two polysaccharides, arabinoxylan and cellulose. The wall preparations contain protein, but the protein lacks hydroxyproline (25) and may not be a structural component. The walls also lack detectable amounts of uronic acids, another common constituent of plant cell walls (1).

The barley aleurone cell walls studied here are strikingly similar to the wheat endosperm cell walls studied by Mares and Stone (28, 29). The endosperm cell walls consist of 64% arabinoxylan and 11% of polysaccharides containing mannose, and galactose. The endosperm wall also contains 15% protein which, like the aleurone walls, contains no detectable hydroxyproline. The arabinoxylan of wheat endosperm cell walls has an arabinose to xylose ratio of 0.65 (28) which is similar to the ratio of these sugars, 0.58, in aleurone walls. Mares and Stone further showed that the endosperm arabinoxylan could be separated by ammonium sulfate precipitation into fractions with arabinose to xylose ratios ranging from 0.43 to 0.75 (29). Our cellulose-binding studies yielded aleurone arabinoxylan fractions with arabinose to xylose ratios ranging from 0.44 to 1.38. Thus, the arabinoxylans of the walls of both wheat endosperm and barley aleurone have a nonrandom arrangement of the arabinosyl side groups.

Aleurone and endosperm cell walls are far less complex than any of the other plant cell walls that have been studied. Aspinall (1) lists five groups of polymers generally found in plant cell walls. Suspension-cultured dicot cell walls have been shown to contain at least seven major components: rhamnogalacturonan, arabinan, arabinogalactan, galactan, xyloglucan, cellulose, and hydroxyproline-rich glycoprotein (42). The suspension-cultured cell walls of six grasses have been shown to contain arabinose, galactose, glucose, uronic acid, and hydroxyproline-containing polymers as well as cellulose and arabinoxylan (9).

Although aleurone and endosperm cell walls are unique in that arabinoxylans constitute such a large percentage of their structural polymers, a variety of other plant tissues have been shown to contain arabinoxylans (2–4, 18, 28, 32–37, 41, 45). The arabinoxylans always contain β-1,4-xylosyl backbones. Side chains of single arabinofuranosyl residues are common as are side chains of single 4-0-methyl glucuronosyl residues. Also, side chains containing galactose and nonterminal arabinose have been reported.

The aleurone cell wall arabinoxylan is a linear xylan backbone with single arabinofuranosyl residues as the predominant side chain (Tables I and II). Information on the distribution of the arabinofuranosyl side chains along the xylan backbone is provided by the results of the cellulose-binding experiments. Only 22% of the 1,4-xylosyl residues in the cellulose-bound arabinoxylan are branched, while 65% of the xylosyl residues in the arabinoxylan which does not bind are branched (Table II), and half of these xylosyl residues are doubly branched! Thus, the cellulose-bound arabinoxylan has regions of at least four contiguous unbranched 1,4-xylosyl residues and the arabinoxylan which does not bind to cellulose has regions of at least two contiguous branched xylosyl residues. These results are similar to those obtained by periodate oxidation studies of arabinoxylan from rye and wheat flour (4, 14) which demonstrated that arabinosyl residues are attached to isolated as well as to two and three contiguous xylosyl residues.

The results of the cellulose-binding experiments may also provide some insight into the interaction between arabinoxylan and cellulose within the aleurone wall. Since the arabinoxylan binds noncovalently to cellulose *in vitro*, the same binding probably occurs within the wall. Further, the binding of arabinoxylan to cellulose *in vivo* is probably "controlled" by the degree of arabinosyl branches because arabinose-rich arabinoxylan does not bind to cellulose *in vitro*.

It seems likely that the arabinoxylan binds not only to cellulose but also to itself for the aleurone cell wall contains only

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8% cellulose (Table I) but 85% arabinoxylan. This possibility is enhanced by the observation that plant arabinoxylans form aggregates in solution (6, 12). Dea *et al.* (12) have published evidence that, in solution, arabinoxylan is present as a mixture of random coils and aggregated linear chains. They (12) also present evidence that the aggregated linear chains are similar to those formed by xylans in the solid state, that is, linear twisted ribbons with a complete twist every three residues (17, 27, 30, 39). Dea *et al.* suggest, too, that, in solution, some of the arabinosyl side chains are incorporated into the aggregates by displacing water molecules which are present in pure xylan aggregates. Thus, arabinosyl side chains participate in the formation of the aggregates. This role of the arabinosyl side chains is in contrast to the usual role of polysaccharide side chains which is to disrupt aggregation by causing "termination of binding sites" (38).

A portion of the arabinoxylan in aleurone cell walls is probably present as straight linear chains which associate with each other much as described by Dea *et al.* (12). It is likely that some of the arabinosyl side chains are incorporated into the linear xylan aggregates. It is also possible that in regions containing a high frequency of arabinosyl side chains (especially regions with xylosyl residues which contain two separate arabinosyl side chains) the aggregation of arabinoxylan chains is disrupted. Hence, a single arabinoxylan molecule may contain regions which will bind to cellulose, regions which will bind to other arabinoxylan molecules, and random coil regions which will bind neither to cellulose nor other arabinoxylan molecules. These differently binding regions would lead to highly cross-linked walls, that is, walls which are very strong, but walls which do not lend themselves to elongation growth. Since neither aleurone cells nor endosperm cells undergo elongation growth, it is not surprising that they have very similar walls.

In conclusion, we believe that aleurone cell walls consist of a network of arabinoxylan chains and cellulose fibers. We think it is likely that strong noncovalent binding between the arabinoxylan chains, themselves, and between the arabinoxylan chains and the cellulose fibers are responsible for the strength of the walls. Evidence has been cited from the literature that arabinoxylan chains will form aggregates with each other. We have also shown, with *in vitro* cellulose-binding experiments, that the arabinoxylan chains will form aggregates with cellulose fibers and that this aggregation is controlled by the frequency of arabinosyl side chains.

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