Lignin Formation in Wheat Coleoptile Cell Walls

A POSSIBLE LIMITATION OF CELL GROWTH

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ABSTRACT

Four growth-influencing compounds—hydroxyproline, 2,2'-dipyridyl, 2-chloroethylphosphonic acid, and indoleacetic acid—were used to examine the relationship between lignin formation and growth of wheat coleoptile sections. Hydroxyproline and 2-chloroethylphosphonic acid, at low concentrations, inhibited growth and increased lignin content. Dipyridyl, which promoted coleoptile elongation, decreased lignin content. Indoleacetic acid caused a 300% increase in growth at 0.1 mM but resulted in lignin content no different from controls with no auxin. Chemical and anatomical evidence is given which indicates that lignin is present in the epidermal cell walls of the wheat coleoptile. It is thus possible that bonding between lignin and hemicellulose may have some influence on coleoptile growth.

WARDROP (20) has hypothesized that lignification may limit enlargement of plant cells by immobilizing the hemicellulose matrix, thereby preventing surface growth. His suggestion pertained to woody cells which undergo secondary wall thickening, and in which lignin forms a major component of the cell wall. Coleoptile cells, however, also have a secondary type of wall layer which is formed at the end of the extension phase (1). Experiments described in this report show that lignin may be synthesized in the epidermal cells of the wheat coleoptile. As a result of bonding between lignin and hemicellulose (5, 7), factors which control lignification may thereby influence the termination of elongation of coleoptile cells. The relationship between lignin biosynthesis and coleoptile elongation was investigated.

MATERIALS AND METHODS

Incubation and Nitrobenzene Oxidation. Wheat seeds (Triticum vulgare cultivar Knox or Redcoat) were grown for 72 hr in the dark in vermiculite. Coleoptile sections 9 mm long were cut 3 mm from the tips, and the leaves were removed. The sections were floated in water for 1 to 2 hr before the treatment incubation. Twenty-five to 50 sections were incubated in 50-ml Erlenmeyer flasks in 2- to 4-ml solutions. The basic medium for incubation was 50 mM sucrose; 2.5 mM potassium maleate buffer, pH 4.8; the lignin precursor U-14C-L-phenylalanine, 15 mc/mmmole, 1 to 5 mc per flask; and hydroxy-

proline, 2,2'-dipyridyl, 2-chloroethylphosphonic acid (Achmea Ethrel 68–240), and IAA as specified in the “Results” section. Incubation temperature was 25 C. Flasks were gently shaken for 20 hr in a darkroom.

At the end of the incubation period, length of the sections was measured. Sections were ground in a mortar, washed twice with 80% (v/v) ethanol, centrifuged, then washed once with ethanol-ether (1:1, v/v). To isolate vanillin, a characteristic oxidation product of lignin, the dried tissue was placed in a glass or Teflon centrifuge tube with 0.3 ml of nitrobenzene and 2 ml of 2 N NaOH and heated in an iron bomb for 2 hr at 160 C. The cooled solution was filtered and extracted 3 times with ether. The aqueous solution was acidified to pH 3 with HCl and extracted 3 times with ether. The ether extract was evaporated to dryness and dissolved in ethanol. Portions of the ethanol solution were spotted on 500 µl silica gel thin layer plates and developed in benzene-glacial acetic acid, 9:1, v/v. The plates were sprayed with 0.2% 2,4-dinitrophenylhydrazine to locate marker and extracted vanillin. Vanillin spots were scraped off and eluted with ethanol, portions of the eluates were dried on planchets, and radioactivity was measured. Vanillin from the tissue was identified by cochromatography of authentic samples in two other solvent systems: ethyl acetate-2-propanol-water, 65:24:11, v/v; and 1-butanol-acetic acid-water, 63:10:27, v/v. The benzene-acetic acid system was used exclusively in most experiments.

Acid Hydrolysis of Whole Sections. Coleoptile sections incubated in 14C-phenylalanine were placed in a sealed glass tube containing 6 N HCl and heated to 110 C for 18 hr. A few pieces of the tissue residue were washed, applied to microscope slides with gelatin, then coated with autoradiographic stripping film for exposure. The remainder of the residue was counted after drying on a planchet. The residue was removed from the planchet and hydrolyzed in 72% H2SO4, by weight, for 1 hr, at 23 C, to check for any cell wall carbohydrates which might have incorporated 14C from phenylalanine.

The acid was diluted to 3% and the sample was autoclaved at 15 psi for 2 hr. The hydrolysate was neutralized with Ba(OH)2, then separated by paper chromatography with ethyl acetate-pyridine-water, 8:2:1, v/v, and a running time of 24 hr. Spots were detected with 3% o-aminodiphenyl hydrochloride in 95% ethanol (18). Authentic glucose, galactose, mannose, arabinose, and xylose were included on the chromatograms for identification of the labeled cell wall sugars.

Differentiation Extraction of Vascular and Epidermal Tissue. Fifty coleoptile sections were incubated with 20 µc of 14C-phenylalanine. With the aid of a dissecting microscope, the two vascular bundles were removed from each section and kept separate from the remaining tissue. The two fractions, designated as vascular and epidermal, were ground in a mortar with 80% ethanol. The tissues were washed in a 0.5% solution

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of Tween 20, water, 80% and 100% ethanol, with centrifugation between washings. Finally suspended in 3 ml of ethanol, each tissue fraction was divided into 3 approximately equal parts by pipetting. The portions were dried on planchets and their radioactivity was counted. One portion of each fraction was then hydrolyzed in 6 N HCl for 5 hr at 15 psi in an autoclave, and one portion was hydrolyzed in 72% H2SO4 for 1 hr at room temperature, then diluted to 3% acid and autoclaved at 15 psi for 2 hr. The third portion of each fraction was extracted with 16 ml of water, 0.15 g of sodium chloride, and 1 drop of glacial acetic acid at 100 C for 4 hr, with fresh reagents added every hour (11). Extracts from the epidermal fraction by all 3 hydrolyses were tested for the presence of radioactive phenylalanine and tyrosine by separation on silica gel thin layer chromatographic plates, using 1-propanol-ammonia, 70:30, v/v, as the solvent. The dried plates were placed in contact with x-ray film for exposure. Radioactive spots were compared with known phenylalanine and tyrosine spots run concurrently and detected with ninhydrin spray.

Isolation of Epidermal Cell Constituents. One hundred coleoptile sections were incubated 48 hr in 20 μCi of 2C-phenylalanine, after which the vascular bundles were excised and discarded. The remaining epidermal and other tissues were ground in a mortar with water, centrifuged, and washed 4 times with water. The water extract and washings were combined and heated to boiling to precipitate protein. After the protein was washed with hot water, it was suspended in 10 ml of water with 0.05 ml of Tween 20, from which one portion of each fraction was filtered, and a portion was refluxed with water for 18 hr to extract epidermal and other tissues. The remaining epidermal and other tissues were extracted with 80% ethanol followed by ethanol. The ethanol extract was removed for counting. One portion of each tissue remaining after the initial KOH extraction, the tissue was subjected to separation of water-insoluble cellulose, as described in previous work. The remainder was subjected to separation of radioactive phenylalanine and tyrosine by separation on silica gel thin layer chromatographic plates, using 1-propanol-ammonia, 70:30, v/v, as the solvent. The dried plates were placed in contact with x-ray film for exposure. Radioactive spots were compared with known phenylalanine and tyrosine spots run concurrently and detected with ninhydrin spray.

Microautoradiography. Coleoptiles which had been incubated in the basic medium, including 1 μCi of 2C-phenylalanine per ml, were dehydrated, embedded in paraffin, and sectioned transversely at 5 μ thickness. Microautoradiographs were prepared with Kodak AR-10 stripping film (10). Exposure was 1 to 6 weeks.

Determination of Radioactivity. Cell wall residues remaining after hydrolyses were filtered onto cellulose acetate filters and repeatedly washed with water followed by ethanol. The filters were cemented to planchets and counted in a gas flow detector with an efficiency of about 25%. Liquid extracts were dried on planchets and counted in the same way.

RESULTS

Lignin Formation in Epidermal Cells. Microautoradiographs made from transverse sections of coleoptiles incubated in labeled phenylalanine showed that radioactive compounds, insoluble in water and organic solvents used in preparation of the sections, were most concentrated in the vascular bundles and the inner and outer epidermal cell walls (Fig. 1). The radioactive compounds resulting from phenylalanine presumably were both protein and lignin. In order to remove protein, several whole sections were hydrolyzed in 6 N HCl. Hydrolysis destroyed the structure of the coleoptile cylinder, leaving particles which appeared upon microscopic examination to be epidermal wall fragments. The fragments stained very lightly in safranin. Microautoradiographs showed uniformly distributed radioactivity (Fig. 2).

To check the possibility that the residual radioactivity in the cell wall particles might have been contained in cellulose or other HCl-resistant carbohydrate, a sample was hydrolyzed in H2SO4, resulting in further destruction of the fragments. Glucose was the only sugar detected in the hydrolysate after chromatographic separation, and it contained less than 0.5% of the radioactivity originally in the HCl-hydrolyzed fragments. The lack of radioactivity in carbohydrate was not surprising since the coleoptiles were furnished with adequate non-labeled sucrose during incubation. The radioactivity remaining in the fragments after protein hydrolysis was therefore presumed to be lignin. This conclusion was supported by isolation of radioactive vanillin from epidermal peels.

The stability of radioactive compounds in epidermal and vascular tissue formed from labeled phenylalanine was investigated further. Vascular bundles were excised from the coleoptile sections and the two resulting tissue fractions, vascular and epidermal, were subjected to three treatments which differ in their attack on protein, carbohydrate, and lignin. The vascular fraction contained some epidermal tissue, but the epidermal fraction was entirely free of vascular tissue. Autoclaving the tissue in 6 N HCl, which hydrolyzes protein as well as noncellulosic carbohydrate, removed about 40% of the radioactivity from the epidermal fraction and about 25% from the vascular fraction (Table I). Primary hydrolysis with 72% H2SO4, followed by autoclaving in 3% acid, removes nearly all carbohydrate and some cell wall protein. This treatment decreased the radioactivity of the epidermal tissue by 65%, and the vascular tissue by about 50%. The delignifying agent sodium chloride reduced the radioactivity of both fractions to less than 1%. Most of the radioactivity removed by HCl and H2SO4 was in protein, as evidenced by identification of nearly all the radioactivity in the hydrolysates as phenylalanine and tyrosine. Protein also was hydrolyzed by sodium chloride, but the large reduction in residual radioactivity by this treatment is presumed to result from the removal of lignin. The mineral acids are poor lignin solvents.

Further evidence for the presence of lignin in epidermal cell walls was obtained by the isolation of lignin degradation products in several fractions of the tissue after extraction by the procedures of Lamport (13). Table II summarizes the radioactivity found in eight major fractions of tissue obtained after sections had been incubated in 2C-phenylalanine. Before extraction, all the vascular bundles were removed and discarded, so that no xylem elements were included.

After the initial grinding of the tissue in water to extract cytoplasmic contents, samples of the residue were counted. The total water-insoluble radioactivity was 1,004,000 cpm. A portion of the tissue remaining after hot water extraction was
FIG. 1. Microautoradiograph of part of a transverse section of wheat coleoptile. The section was cut from a 72-hr-old coleoptile and incubated with $^{14}$C-phenylalanine for 20 hr. The most intense radioactivity was located in the xylem elements of the vascular bundle (v), and in the inner and outer epidermal-cells (e).
Fig. 2. Microautoradiograph of a cell wall fragment isolated after an 18 hr of hydrolysis in 6 N HCl at 110 C from a coleoptile section which had been incubated previously for 20 hr in 14C-phenylalanine. Radioactivity is distributed fairly uniformly; the darker streaks are folds.
subjected to nitrobenzene oxidation. Vanillin isolated from this degradation contained the equivalent of 80,730 cpmp for the entire sample; hence about 8% of the radioactive carbon in the cell walls was found in vanillin.

The largest amount of radioactivity, more than half of the total contained in the tissue, was removed by 6% KOH. This fraction contained hemicellulose and some protein. More than half the radioactivity in the 6% KOH fraction was soluble in ether when neutralized and therefore was not protein. Ferulic acid, commonly released by lignin in alkali extraction (5), was the major radioactive product in the ether fraction.

The second hemicellulose fraction, extracted with 24% KOH and 4% H₂BO₃, yielded less than 1% of the ether-soluble radioactivity obtained by extraction with 6% KOH. Most of the coleoptile lignin thus is soluble in 6% KOH.

The cellulose fraction, extracted with 72% H₂SO₄, contained very little radioactivity. The residue from this extraction contained both lignin and protein. In order to remove protein, the residue was hydrolyzed in 6 N HCl, which removed 2,600 cpmp, leaving a solid residue which contained 12,250 cpmp, presumed to be lignin. The residue was subjected to nitrobenzene oxidation, yielding only 60 cpmp in vanillin. In studies on wood lignin, it has been found that yields of vanillin from sulfuric acid lignins are very low, apparently because of changes in the phenylpropane structures during acid extraction (5).

The results given in Table II strongly indicate the presence of lignin in epidermal cells, especially because of the relatively high yield of radioactive ferulic acid in the 6% KOH extract. This ferulic acid could not have existed in the tissue as a free compound, because it would have been removed earlier by 80% ethanol, benzene-ethanol, or hot water.

### Table II. Radioactivity in Fractions of Coleoptile Tissue after Incubation in ¹⁴C-Phenylalanine

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Main Components</th>
<th>Radioactivity cpmp</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Water-soluble, heat-precipitated</td>
<td>Cytoplasmic protein</td>
<td>202,500</td>
</tr>
<tr>
<td>II. Benzeno-ethanol</td>
<td>Fats, waxes</td>
<td>4,340</td>
</tr>
<tr>
<td>III. Hot water</td>
<td>Pectic material, protein</td>
<td>75,600</td>
</tr>
<tr>
<td>IV. 6% KOH</td>
<td>A. Ether-soluble</td>
<td>Phenolic aldehydes, acids</td>
</tr>
<tr>
<td></td>
<td>Ferulic acid</td>
<td>65,600</td>
</tr>
<tr>
<td></td>
<td>B. Water-soluble</td>
<td>Carbohydrate, protein</td>
</tr>
<tr>
<td>V. 24% H₂BO₃</td>
<td>A. Ether-soluble</td>
<td>Phenolic aldehydes, acids</td>
</tr>
<tr>
<td></td>
<td>B. Water-soluble</td>
<td>Carbohydrates, protein</td>
</tr>
<tr>
<td>VI. 72% H₂SO₄</td>
<td>Cellulose, protein</td>
<td>1,100</td>
</tr>
<tr>
<td>VII. 6% HCl</td>
<td>Protein</td>
<td>2,600</td>
</tr>
<tr>
<td>VIII. Residue</td>
<td>Lignin</td>
<td>12,250</td>
</tr>
<tr>
<td>Total, major fractions</td>
<td></td>
<td>740,030</td>
</tr>
</tbody>
</table>
LIGNIN FORMATION IN COLEOPTILE CELL WALLS

Fig. 3. Relationships between lignin content and growth of coleoptile sections after incubation in four growth-influencing compounds. Included in all media were 2 μC of 14C-phenylalanine, 50 mM sucrose, and 2.5 mM potassium maleate buffer, pH 4.8. Sample units contained 25 coleoptile sections each and were incubated at 25°C for 20 hr. Growth was expressed as the difference between the length of the sections after 20 hr and the initial length of 9 mm. Lignin was estimated by determining the cpm in vanillin isolated after nitrobenzene oxidation of the sample of 25 sections. The points located off the curves and connected by vertical lines represent individual samples of 25 sections; thus, each point for growth is the mean of 25 sections, and each point for lignin is the total cpm in vanillin for 25 sections. Controls are the zero levels of each compound, and their values are set at 100%. The actual mean values for controls in all the experiments were 8.4 mm per section for growth and 1240 cpm in vanillin per section.

Table III. Uptake of Phenylalanine

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Soluble in 80% Ethanol</th>
<th>Insoluble in 80% Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4,590</td>
<td>11,325</td>
</tr>
<tr>
<td>IAA, 0.1 mM</td>
<td>7,340</td>
<td>13,515</td>
</tr>
<tr>
<td>Dipyridyl, 0.1 mM</td>
<td>3,470</td>
<td>11,685</td>
</tr>
<tr>
<td>2-Chloroethylphosphonic acid, 0.1 μl/liter</td>
<td>4,520</td>
<td>11,960</td>
</tr>
<tr>
<td>Hydroxyproline, 1 mM</td>
<td>4,310</td>
<td>10,825</td>
</tr>
</tbody>
</table>

Samples of 20 coleoptile sections were incubated in 2.5 mM potassium maleate buffer, pH 4.8, 50 mM sucrose, 0.33 μC 14C-phenylalanine, and the treatment compound, for 4 hr at 20°C. After washing and flushing out the leaf cavities with water, the sections were ground in 80% ethanol. Counts were made on the 80% ethanol extracts and the insoluble residue which included cell walls. Each value is the mean of 2 samples of 10 sections.

content was reduced to less than half that of the controls. The curves of Figure 3D are similar to those of Figure 3B (dipyridyl) in that lignin content relative to the control is always lower than relative growth. IAA, like dipyridyl, decreases the activity of peroxidase (22), but certainly not by a general decrease in respiration.

The significance of the results in Figure 3 is that the growth inhibitors hydroxyproline and 2-chloroethylphosphonic acid result in lignin contents, expressed as a percentage of controls, which are higher than growth values relative to controls, at all treatment levels. Conversely, the growth promoters dipyridyl and IAA give relative lignin contents which are consistently lower than relative growth values.

The effect of the growth-influencing substances on uptake of phenylalanine are shown in Table III. Except for IAA, none of the compounds exerted much effect on total uptake in the 4-hr period. The increase in uptake of phenylalanine caused by IAA is similar to that reported for glucose in auxin-induced growth (3). No parallel increase in lignin content resulted (Fig. 3D). The soluble fraction of the dipyridyl treatment was lower in radioactivity than the control; however, the insoluble fraction was about the same as the control. With the possible exception of dipyridyl, then, it seems unlikely that the synthesis of lignin could be controlled by uptake of the precursor from the medium.

In none of the experiments of Figure 3 were the vascular bundles removed. It is extremely difficult to excise vascular bundles in such a way as to leave uniform amounts of epidermal tissue in each section. Lignin estimates based on vanillin therefore include xylem elements. Because of this, the possibility cannot be eliminated that some of the influence of the treatments in terms of lignin formation was exerted on the xylem elements.

DISCUSSION

Several points must be raised concerning the relationship of the experiments to the hypothesis that lignification limits cell expansion. First, there is the question of whether the isolation of vanillin arising from labeled phenylalanine is a good indication of lignin formation. The widely used lignin estimation based on weighing Klasson lignin after acid hydrolysis (5) is not suitable for living cells which have a fairly high protein content, because the sulfuric acid digestion does not remove all the cell wall protein (13). Lignin cannot be isolated in a pure form with present methods (14). The isolation of
vanillin after nitrobenzene oxidation is generally accepted as a reliable indicator of the presence of lignin (18).

There is no doubt that L-phenylalanine is a good precursor for lignin (14); however, its introduction into tissue may increase the pool of the free amino acid to the extent that more lignin is formed than would be under normal physiological levels of the precursor. The facts that lignin is formed from the precursor and the amount varies with treatment indicate that a biosynthetic pathway exists in epidermal cells and that it is sensitive to control, whether or not the normal condition is one of limited phenylalanine.

Finally, there is the question of whether the lignified part of the epidermal wall is in a structural position to limit extension. The outer epidermal wall in wheat and oat coleoptiles is very thick and is covered with a thin cuticle (16). Wall fragments isolated after HCl hydrolysis (Fig. 2) may be cuticle, for their areas were much too great to be walls of individual epidermal cells. The fact that the fragments contain glucan and lignin, however, suggest that they might be pieces of the thick outer wall noted by O'Brien (15). If this is the case, the outer wall fragments would have to be common to several epidermal cells, because of their large areas. An electron microscopic study of developing coleoptiles, perhaps with autoradiography of lignin deposition, may help clarify this point.

A central question is whether lignification begins before the cell has reached its final size. If it does not, then lignin formation cannot limit the size of the cell, but only make the cell wall more rigid. If lignin formation does limit the size of the cell, it could do so by controlling the rate of wall expansion over the entire extension phase or by causing the termination of cell growth without influencing the earlier rate of expansion. Studies by Adamson et al. (1) suggest that the latter situation may be the more likely, since the appearance of secondary wall layers coincides closely with the end of cell growth. A process controlling only the termination of cell growth such as lignification might, however, influence the rate of elongation of the entire coleoptile. As Avery and Burkholder (2) have shown, regions of the coleoptile elongate at different rates. This could be caused by the termination of extension of increasing numbers of cells in the coleoptile regions having decreasing rates of elongation.

If lignin indeed is formed in epidermal cells of monocot coleoptiles, it should be considered a possible factor in the system controlling elongation of this organ. Hemicelluloses seem to be the carbohydrate wall constituents most directly involved in controlling cell expansion (3). It is with hemicellulose that lignin is believed to form covalent bonds, probably ether linkages, at least in woody cells (7). Lignin may thus form stabilizing cross-linkages, similar to those proposed for extension (13), within the hemicellulose matrix of coleoptile epidermal cells.

In an analysis of differential growth in coleoptiles, Thimann and Schneider (19) found that the epidermal cells were under tension, while the inner cells were under compression during elongation. This means that a growth-limiting phenomenon was more advanced in the epidermal cells than in the inner cells, and it is the outer cells which apparently contain lignin. Of course, the xylem elements also contain lignin, but their secondary walls are in the form of spiral thickenings allowing for extension.

The results of these experiments do not confirm sufficiently the hypothesis of limitation of coleoptile growth by lignin formation. No causal relationship between lignification and growth limitation was established. The results, however, are consistent with those predicted by the hypothesis. Lignin formation is increased by treatments which increase peroxidase activity and which reduce elongation of coleoptiles, but both could be the result of some other process limiting cell growth, such as the metabolism of hydroxyproline-rich protein (13).

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LITERATURE CITED


