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LIGNIN–PROTEIN COMPLEX IN CELL WALLS OF *PINUS ELLIOTTII*: AMINO ACID CONSTITUENTS*

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Abstract—Cell walls of *Pinus elliottii* callus contain *ca* 12% protein. Klason lignin prepared from the walls contained 9% protein and represented 4.5% of the wall. The lignin fraction was increased to 22% of the wall weight by reacting washed cell-wall tissue with coniferyl alcohol and H_2O_2, a reaction catalysed by peroxidase that remained bound to the wall. The augmented lignin preparation yielded 10% protein. The acid hydrolysate of whole wall tissue included five amino acids at a concentration higher than hydroxyproline. The hydrolysates of both natural and augmented lignin preparations yielded distributions of amino acids in which the concentration of hydroxyproline was higher than that of all other amino acids. The results suggest that polymerizing lignin links covalently with cell-wall glycoprotein, and that the bonds may be formed preferentially with hydroxyproline.

INTRODUCTION

According to many investigators [1] lignin is linked to cell-wall carbohydrates through covalent bonds. While direct evidence of these covalent bonds is not available, the difficulty of separating the two constituents is a strong indicator of their presence. Model systems, however, have yielded direct evidence of covalent bonds between phenolic structures and carbohydrate [2, 3].

Lignin preparations of non-woody plant tissues also contain significant amounts of protein. There is evidence that lignin developing in cell walls enters into covalent bonds with cell-wall protein, and that hydroxyproline may have an exceptional tendency to participate in this bonding [4, 5].

This report extends the previous arguments for lignin–protein structural bonds by comparing the amino acid distribution of proteins associated with lignin with that of the whole cell wall. In these experiments, both whole cell-wall tissue and Klason lignin prepared from the walls were hydrolysed and the distribution of amino acids in each was observed. In addition, lignin was augmented by incubation of the walls with coniferyl alcohol and H_2O_2. Klason lignin, prepared from treated wall tissue, was hydrolysed and its amino acids were likewise separated.

RESULTS

The yield of Klason lignin from whole wall tissue was 4.5% based upon the dry wt of the tissue (Table 1). When wall tissue was reacted with coniferyl alcohol at the rate of 0.25 mg/mg of tissue, and H_2O_2, the yield of lignin increased *ca* five-fold to 22.2%.

In the lignin-forming reaction, peroxidase (EC 1.11.1.7) remaining with the wall tissue after washing with detergent and salt catalyses the dehydrogenation of coniferyl alcohol, which polymerizes into lignin. The reaction is the same as that occurring in intact cells, although the degree of polymerization is probably less than that in living cells. Lignin formed in this manner is spectrophotometrically similar to lignin formed naturally [4].

During the formation of artificial lignin, bonds apparently are formed with protein of the wall. Augmentation of lignin with coniferyl alcohol increased the proportion of lignin in the wall from 4.5% to 22.2% (Table 1). However, the percentage of protein in the augmented lignin remained approximately the same as in natural lignin, 10.1% vs 9.3%, respectively. Since the percentage of protein did not decrease in the augmented lignin, new complexing between artificial lignin and cell-wall protein must have occurred.

The amino acid distribution in the hydrolysates of both augmented and natural lignins was quite different from that in the hydrolysate of washed cell-wall tissue (Table 2). Threonine, alanine, valine, leucine, and lysine were all present in the whole wall hydrolysate at higher concentrations than hydroxyproline. In the lignin hydrolysates, however, all other amino acids were at a lower concentration than hydroxyproline, in most cases substantially so. Hydroxyproline comprised 7.3% of the amino acids in the hydrolysate of wall tissue; in coniferyl alcohol-augmented lignin it was 16.5% (Table 1). Similar proportions were obtained in a preliminary experiment using a different method [6].

To determine whether or not the binding of coniferyl alcohol dehydrogenation products (or lignin) is simply the result of acid-catalysed co-condensation with the cell-wall protein during lignin isolation, a comparison was made between the amount of protein extracted with lignin

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Table 1. Lignin, protein and hydroxyproline content of cell-wall fractions of *Pinus elliottii*

Fraction	Lignin (% of wall)	Protein (% of wall)	Hydroxyproline (% protein)
Washed cell walls, whole tissue	4.5	13.0	7.3
Cell walls augmented with coniferyl alcohol	22.2	—	—
Lignin from untreated cell walls	—	9.3	12.2
Lignin from cell walls augmented with coniferyl alcohol	—	10.1	16.5

Callus cells were homogenized, washed with Triton X-100, NaCl at increasing concentrations and H_2O. Lignin was isolated by H_2SO_4 digestion of washed, untreated cell walls or cell walls in which lignin had been augmented by incubation with coniferyl alcohol and H_2O_2. (Peroxidase remains bound to the wall and catalyses lignin formation.) Dry cell-wall tissue was weighed; protein and hydroxyproline were determined after hydrolysis and separation of amino acids on ion-exchange resin columns.

when coniferyl alcohol was incubated with wall tissue before extraction and when dehydrogenation products were added to the wall tissue just prior to extraction. Wall protein was labelled by incubating callus tissue with [U-^{14}C]proline. In this experiment, as in an earlier one [6], essentially all the radioactivity in the resulting cell-wall protein was in proline or hydroxyproline residues. Relative amounts of protein complexed to lignin were determined by counting the lignin preparations.

Acid hydrolysis resulted in some binding through co-condensation when the coniferyl alcohol dehydrogenation products were added just before lignin isolation. The increase in counts was *ca* 20% more than the unreacted control. Formation of dehydrogenation products in the presence of wall tissue, however, gave an increase of 64% in counts over the control. This experiment suggests that binding of lignin to protein does occur during the polymerization of lignin units.

Table 2. Amino acid composition (residues/100 residues Hyp) of hydrolysates from washed callus cell walls and lignin of *Pinus elliottii*

Amino acid	Washed cell walls	Lignin from untreated cell walls	Lignin from cell walls augmented with coniferyl alcohol
Hyp	100	100	100
Asp	85	39	17
Thr	113	49	33
Ser	91	71	34
Glu	71	67	40
Pro	81	93	50
Gly	90	58	37
Ala	106	70	45
Val	150	55	77
Met	13	8	0
Ile	67	34	40
Leu	112	67	57
Tyr	14	11	2
Phe	45	39	1
Lys	129	57	39
His	38	13	15
Arg	53	0	18

Washed cell walls were hydrolysed directly with 6 M HCl. Lignin was prepared from untreated cell walls and from cell walls incubated with coniferyl alcohol and H_2O_2. Dried lignin was hydrolysed in 6 M HCl. Hydrolysates were separated into constituent amino acids by ion-exchange resin columns.

DISCUSSION

In spite of the common occurrence of protein in Klason lignin preparations [1], little attention has been given to the possibility that protein and lignin may exist as a covalently bonded, structural association in the middle lamella and primary wall, especially in cells that form lignified, secondary walls. The occurrence of protein in lignin preparations is generally attributed to co-condensation during extraction with strong mineral acids. On the other hand, considerable attention has been given to the lignin–carbohydrate bond.

It is generally agreed that if covalent bonds link lignin with carbohydrate, they are ether bonds through the 2-position of a guaiacyl or other phenylpropane moiety and a hydroxyl of a saccharide moiety [1]. In the formation of such a bond, coniferyl alcohol or some other phenylpropanoid is converted to a free radical by dehydrogenation in the presence of peroxidase and H_2O_2. The radical is stabilized by addition of carbohydrate. Recent work has shown that the preferred hydroxyl group of glucose for this bond is located on C-6 [3]. Stabilization of the radical may also take place by addition of another phenylpropanoid radical or water.

If the pine callus cell-wall protein has repeating hydroxyproline residues similar to that of other plant species, its molecular conformation may be the same as polyproline II [7] in the regions of repeating hydroxyproline. In polyproline II, the rigid, planar pyrrolidine ring holds C-4 out away from the peptide backbond [8]. Thus, if C-4 is hydroxylated to form 4-hydroxyproline, the hydroxyl may be in just as favorable a steric position to enter into an ether linkage with a quinone methide as a glycosidic link with carbohydrate [9].

I have already demonstrated that dehydrogenated coniferyl alcohol enters into strong bonds more easily with free hydroxyproline or polyhydroxyproline than with serine, proline, glycine, sucrose, polygalacturonic acid, polyserine, polyproline, or cellulose [5]. Whether or not the bonds are actually through hydroxyl groups has not been determined.

For the hydroxyls of hydroxyproline to enter into linkages with phenolics, they would have to be unsubstituted. Some tissues, however, have most hydroxyls blocked by glycosidic bonds to oligosaccharides. About 90% of the hydroxyproline hydroxyls were found to be involved in glycosidic linkages in *Acer*

pseudoplatanus suspension cultures [10] and in *Phaseolus coccineus* parenchyma [11]. In these tissues only a small proportion of hydroxyproline hydroxyls would be free to enter into ether bonds with phenolic compounds. Nonetheless, a small number of phenolic cross-links might be sufficient to bind the glycoprotein tightly to the rest of the wall matrix.

O'Neill and Selvendran [11] used acidified sodium chlorite, an efficient lignin solvent, to remove a soluble glycoprotein that was rich in hydroxyproline. They suggested that, since this fraction is generally resistant to removal by other reagents except degradative ones, it may be connected to the wall structure by phenolic cross-links. The results of the present study are in agreement with this suggestion.

Klason lignin is isolated by the hydrolysis of polysaccharides with H_2SO_4, first with cold 72% acid, followed by heating to 100° after dilution to 3%. The conditions are sufficient to hydrolyse essentially all polysaccharides. Proteins are only partially dissolved, leaving insoluble lignin and protein. Protein can be a serious contaminant in lignin preparations from tissues containing a large proportion of cell-wall proteins in relation to lignin.

As shown in Table 1, the protein component was 13% of the cell-wall tissue. The lignin preparation from untreated wall was 4.5% of the wall tissue and contained 9% protein. The amount of protein remaining bound to lignin was 9% × 4.5% = 0.4% of the wall tissue, or 3% of the original protein. Thus, 97% of the protein was solubilized during extraction. Of the amino acids in the protein remaining associated with lignin, the one in greatest concentration was hydroxyproline. When lignin was augmented with coniferyl alcohol, the proportion of protein to lignin remained about the same, but the total amount increased with the increase in lignin recovered. In this case, the proportion of hydroxyproline was even higher than in the lignin from untreated tissue.

It could be argued that part or all of the protein removed during the acid extraction was not cell-wall protein but cytoplasmic and membrane contaminants, and that the protein remaining with the lignin was the true wall protein as evidenced by its higher hydroxyproline content. When coniferyl alcohol was reacted with the washed cell-wall fragments, however, more lignin was formed, and more protein with a higher proportion of hydroxyproline was bound. If the lignin being polymerized from coniferyl alcohol had bound non-specifically with non-wall protein as well as with hydroxyproline-rich wall protein, one would expect the lignin-bound protein to resemble the amino acid distribution of the protein of the whole wall tissue. This was not the case.

It is clear that lignin has a greater affinity for, or forms stronger bonds with hydroxyproline or regions of protein containing hydroxyproline. This experiment does not prove that the bonds are formed preferentially with hydroxyproline residues. They could be formed through other residues that occur in sequences rich in hydroxyproline. Nevertheless, the high proportion of hydroxyproline in the lignin–protein complex, and other experiments cited above [4, 5], suggest a special affinity of lignin for hydroxyproline. If this is the case, the explanation may be the availability of the hydroxyproline hydroxyl group. It is interesting that the other amino acid residues with hydroxyls, namely threonine, serine, and tyrosine, exhibited no differences in distribution from the other amino acids having no OH groups.

EXPERIMENTAL

Callus. Cell-wall material was obtained from callus of *P. elliottii* 4 weeks from subculturing. Callus was grown on Murashige–Skoog medium [12], supplemented with 5 mg/l. 2,4-D and 0.5 mg/l. benzyladenine. The callus was derived from a single hypocotyl explant and had been subcultured at monthly intervals for *ca* 3 yr.

Preparation of cell-wall tissue. Calluses averaged *ca* 5 g and yielded *ca* 1% washed cell-wall tissue based upon fr. wt. About 85 g of callus tissue was collected and ground briefly in a blender with ice-cold K–Pi buffer, pH 7, 0.05 M. The slurry was filtered through two layers of Miracloth, then washed on the filter with cold H_2O. The solids were again ground for a few sec in the blender with ice-cold H_2O containing 0.1% Triton X-100. The mixture was then homogenized briefly in a Willems Polytron at the highest setting. The mixture was divided among 50-ml centrifuge tubes and washed twice with H_2O. Ppted wall tissue was combined and washed with stirring for 10 min with 0.1 M NaCl soln, then centrifuged. The NaCl washes were continued with 0.5, 1, and 2 M NaCl in the same way. After washing twice in H_2O, the tissue suspension was dialysed against H_2O overnight at 4°.

Reaction with coniferyl alcohol. After dialysis, the tissue was suspended in fresh H_2O, centrifuged, then resuspended. About 45% of the suspension was removed, centrifuged, then suspended in 100 ml of K-Pi buffer, pH 7, 0.05 M. Coniferyl alcohol (100 mg) was dissolved in 5 ml EtOH. This coniferyl alcohol soln (1 ml) and 30% H_2O_2 (0.1 ml) were added to the cell-wall suspension. The mixture was stirred at room temp. for 30 min and the same amounts of coniferyl alcohol and H_2O_2 were then added. Additions were repeated every 30 min until the coniferyl alcohol was consumed. 30 min after the final addition, the suspension was centrifuged, washed with H_2O_2, followed by EtOH and Me_2CO. The unreacted wall tissue was washed with the same solvents at this time. Both treated and untreated tissues were air-dried, then stored in a vacuum desiccator.

Preparation of lignin. Lignin was isolated from both treated and untreated tissue by a slight modification of the Klason method [1]. Enough 72% (w/w) H_2SO_4 was added to the dry tissue to make a thin slurry. After 2 hr at room temp., the acid was dil. to 3% with H_2O. The samples were autoclaved at 1 kg/cm^2 for 2 hr. Lignin was collected by centrifugation, washed on a filter with H_2O, then air-dried.

Amino acid analysis. Samples of whole wall tissue, lignin from coniferyl alcohol-treated tissue and lignin from untreated tissue were hydrolysed in a large excess of 6 M HCl at 105° for 18 hr. Acid was removed in a flash evaporator with repeated additions of H_2O. The condensed hydrolysates were evapd to dryness with a stream of N_2, then stored in a desiccator over solid NaOH. Amino acid analyses were made with the fraction collector method of ref. [13]. Resin was AG 50W-X8, minus 400 mesh (Bio-Rad).

Co-condensation experiment. Callus tissue weighing *ca* 10 g was incubated 20 hr in 5 ml K-Pi buffer, pH 7, 0.05 M, containing 0.1 g sucrose and 5 μCi [^{14}C]proline, sp. act. 285 mCi/mmol. Washed cell-wall material was prepared as above. Suspension of tissue (13 ml) in pH 7 buffer was apportioned among three treatments with two replicates each (2 ml/sample). To treatment 1 (control), nothing was added. In treatment 2 (incubation), 6 mg coniferyl alcohol and 50 μl 30% H_2O_2 were added to each replicate. In treatment 3, 6 mg coniferyl alcohol, 50 μl H_2O_2, and 0.1 mg horseradish peroxidase were mixed with 2 ml pH 7 buffer not

containing cell-wall tissue. The wall tissue for this treatment was set aside for a 1-hr period of incubation of the coniferyl alcohol mixtures. The coniferyl alcohol dehydrogenation product was added to the unreacted wall tissue with 3% H_2SO_4. Contents of all treatment flasks were washed into the centrifuge tubes with 3% H_2SO_4, the tubes centrifuged, the supernatants discarded and 1 ml conc. H_2SO_4 was then added to the pellets. Mixtures were hydrolysed at 20° for 2 hr, after which they were diluted to 3% H_2SO_4 and autoclaved at 1 kg/cm^2 for 2 hr. Lignin was collected on glass fiber filters, washed with cold H_2O and counted in a gas-flow detector. A sample of the labelled wall tissue was hydrolysed with 6 M HCl for 18 hr. Proline and hydroxyproline were separated by Si gel TLC in PrOH–28% NH_4OH (7:3).

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