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BINDING OF FERULIC ACID TO CELL WALLS BY PEROXIDASES OF *PINUS ELLIOTTII*

FRANK W. WHITMORE

Department of Forestry, Ohio Agricultural Research and Development Center, Wooster, OH 44691, U.S.A.

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Key Word Index—*Pinus elliottii*; Pinaceae; slash pine; peroxidase; binding; ferulic acid.

Abstract—Lignin is formed abundantly in the maturing walls of slash pine cambial cells, but very little in slash pine callus cell walls. Peroxidases removed from the cytoplasm of callus or cambial cells with phosphate buffer (soluble peroxidase), from the walls with NaCl (ionically bound peroxidase), and from the walls with cellulase (covalently bound peroxidase) differed in their capacity to catalyze bond formation between carbohydrate and ferulic acid or its condensation products. Bond formation per unit of enzyme was highest in the peroxidases of cambium, especially in those attached ionically or covalently to the cell walls. The wall-bound peroxidases also catalyzed the strongest linkages between lignin monomers and carbohydrates as estimated by their resistance to hydrolysis by NaOH.

INTRODUCTION

Peroxidase (E.C. 1.11.1.7) is commonly associated with plant cell walls by ionic and covalent bonds [1]. The function of this enzyme in cell wall physiology is unclear, although it is presumed to catalyze the dehydrogenation of coniferyl alcohol and related phenylpropane compounds in the process of lignin formation [2]. Peroxidase does occur, however, in cell walls which do not form lignin [3].

Results presented here show that peroxidase bound to cell walls catalyzes the formation of strong linkages between lignin monomers and carbohydrate. Such binding may be a normal function of peroxidase in the cell walls in addition to the dehydrogenation of phenol compounds.

RESULTS AND DISCUSSION

A comparison was made between soluble, ionically, and covalently bound peroxidases of both slash pine callus and the cambial tissue of slash pine seedlings in terms of their ability to catalyze binding of ferulic acid to carbohydrate of the walls. Cell walls of slash pine callus do not give a phloroglucinol HCl staining reaction for lignin. The callus does, however, have a weak capacity for transforming ferulic acid to coniferyl alcohol, as evidenced by experiments with a crude enzyme preparation used in the system of Mansell *et al.* [4]. In addition, tissue incubated with cinnamic acid-[^{14}C] produces vanillin-[^{14}C] upon nitrobenzene oxidation. Yields of thioglycolic acid lignin from callus tissue were about 1% of the dry wt of the cell walls. Cambial scrapings from 2-yr-old slash pine seedlings give a strong phloroglucinol–HCl reaction, indicating abundant lignification of the developing tracheids. These two tissues of the same species were chosen for the investigation because of their differing capacities for lignin formation.

Ferulic acid, rather than coniferyl alcohol, was used for the binding experiments. Ferulic acid is relatively stable, except for conversion from *trans* to *cis* form by UV radiation [5], whereas coniferyl alcohol is easily oxidized by air and light. Ferulic acid-[^{14}C] can be prepared quite easily from cinnamic acid-[^{14}C] by the use of wheat coleoptiles. Ferulic acid is considered a true monolignol and may be a principal phenolic precursor for some grass lignins [6].

In the initial investigation of binding of ferulic acid to carbohydrate by peroxidase, carboxymethylcellulose (CMC) and horseradish peroxidase (HRP) were used. After incubation of ferulic acid-[^{14}C] with CMC, HRP, and H_2O_2, a large proportion of the radioactivity was associated with carbohydrate, as shown in the elution pattern from a Bio-Gel column (Fig. 1). After treatment of the incubation mixture with cellulase, both the phenol component and the carbohydrate were shifted to a

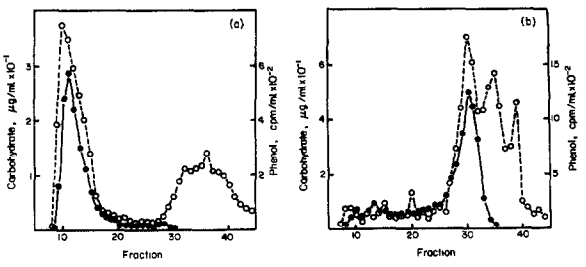

Fig. 1. Association of carbohydrate and phenol before and after digestion with cellulase. CMC, onto which had been dried HRP, was incubated with ferulic acid-[^{14}C] and H_2O_2 and separated on a column of Bio-Gel P-100 before (A) and after (B) incubation with cellulase. ●—carbohydrate; ○—phenol.

smaller range of MW, but the largest peak of radioactivity occurred in the same elution range as the peak of carbohydrate. This indicates that the fast-running radioactive phenolic compounds appeared as large molecules (about 100000 daltons) because of association with carbohydrate, not because of polymerization. If the elution pattern in Fig. 1A had occurred because of polymerization of ferulic acid and not because of association with carbohydrate, then the cellulase treatment would not be expected to alter the pattern of radioactivity unless the cellulase preparation contained enzymes which could depolymerize the ferulic acid complex. That this was not the case was shown by incubating ferulic acid-[^{14}C] with peroxidase and H_2O_2 but without CMC and separating the mixture on the same Bio-Gel column. The elution pattern showed some polymerization of ferulic acid, as expected, but not into the high MW complex obtained with CMC. A sample of the mixture was digested with cellulase and separated on the column. No change in the elution pattern was obtained, showing that the cellulase did not contain enzymes capable of hydrolyzing a ferulic acid polymer.

The bonds established between ferulic acid and CMC in the experiment may be similar to those established between sucrose and coniferyl alcohol by the action of laccase in an experiment by Freudenberg and Harkin [7].

The CMC experiment described above and several earlier experiments on model systems of lignification with peroxidase and filter paper or other solid substrates used HRP, a soluble peroxidase [6,8]. A major interest in the present work was to determine the relative effectiveness of soluble and wall-bound peroxidases in lignin formation. To this end, the efficiency of soluble peroxidase was compared with peroxidases bound ionically and covalently to cell walls in binding or polymerizing ferulic acid on a solid carbohydrate support. The three classes of peroxidase were extracted from slash pine callus and slash pine seedling cambium, then dried in mixture with microcrystalline cellulose. After incubation with ferulic acid-[^{14}C] and H_2O_2, the mixtures were washed with water and ethanol, then compared in terms of radioactivity bound to the cellulose. The cellulose samples were later extracted successively with 5% $NaHCO_3$ and 2% NaOH to evaluate the solubility of the phenolic component or the stability of the phenolic–carbohydrate linkages. Estimates of activity with guaiacol as substrate were made for all enzyme preparations before they were applied to cellulose.

Table 1. Peroxidase activity and efficiency of binding of ferulic acid to microcrystalline cellulose in enzyme fractions of callus and cambial tissue

Tissue	Soluble peroxidase	Ionically bound peroxidase	Covalently bound peroxidase
(Peroxidase units/sample*)			
Callus	207	21	62
Cambial	7	1	0·5
(Cpm bound to cellulose/unit of peroxidase)			
Callus			
Total	183	722	17
After $NaHCO_3$ extraction	59	550	6
After NaOH extraction	5	248	2
Cambial			
Total	2239	3467	1075
After $NaHCO_3$ extraction	1278	2752	475
After NaOH extraction	312	1320	160

* Tissue samples had fr. wts of 0·350 g. One unit of peroxidase was an increase of 1·0 A/min, with guaiacol substrate.

Table 1 shows that most of the total peroxidase occurs in the soluble fraction of both callus and seedling cambium. Callus tissue yielded more activity than seedling cambium in all three forms of peroxidase. All three callus peroxidases also catalyzed the binding of more ferulic acid to cellulose than their counterpart fractions from cambium; however, when expressed as amounts of radioactivity bound per unit of enzyme activity, callus peroxidases showed lower efficiencies than cambium peroxidases. Furthermore, the stability of the complexes to basic extraction was greater in the cambial fractions. The greatest efficiency of total binding was found in the ionically bound peroxidase of seedling cambium. In terms of NaOH-resistant complexing, the same fraction was more effective.

Experiments with slash pine peroxidase and microcrystalline cellulose yielded information on efficiencies of complexing ferulic acid with a non-specific carbohydrate but not on complexing of ferulic acid with native cell wall carbohydrates by these peroxidases. A further experiment was carried out in which portions of finely ground callus and cambial tissue were washed to remove soluble peroxidase, leaving both wall-bound forms, and further treated with NaCl, leaving only the covalently bound peroxidase.

Very similar results were obtained to those from the artificial substrate (Table 2). Cambial peroxidase was far more effective than callus peroxidase in causing the binding of ferulic acid to cell walls. However, in this exper-

Table 2. Activity of native wall bound peroxidase of callus and cambial tissue and efficiency of binding of ferulic acid to cell walls by the attached enzymes

Tissue	Non-NaCl extracted cell walls	NaCl extracted cell walls
(Peroxidase units/sample)		
Callus	3·25	0·78
Cambial	0·34	0·23
(Cpm bound to cell walls/unit of peroxidase)		
Callus		
Total	4085	2813
After $NaHCO_3$ extraction	1130	1323
After NaOH extraction	495	710
Cambium		
Total	18742	21369
After $NaHCO_3$ extraction	12939	16174
After NaOH extraction	8085	9783

Non-NaCl extracted cell walls contained ionically and covalently bound peroxidase; NaCl extracted cell walls contained only covalently bound oxidase. One unit of peroxidase was an increase of 1·0 A/min, with guaiacol substrate. Tissue samples had fr. wts of 0·215 g.

iment, the efficiency of binding was highest in the covalently bound fraction of cambial peroxidase rather than the ionically bound fraction. In callus tissue, the higher efficiency of total ferulic acid binding was obtained by the ionically-bound peroxidase, similarly to the results of the microcrystalline cellulose experiment, but the resistance to basic reagents of binding was greater in the NaCl washed tissue (covalently bound peroxidase). Since the activity of ionically bound peroxidase could not be observed alone, it was assumed that the effects of both forms were additive, and the activity of the ionically bound fraction could be obtained by subtracting the activity of the NaCl washed tissue from that of the non-washed tissue.

A clear separation between peroxidase-mediated bonding between phenol–carbohydrate and phenol–phenol was not obtained. Probably both types of linkages were formed. The CMC experiment indicates that phenol–carbohydrate bonding does occur. Some binding of ferulic acid to protein of the wall may also occur, although the amount may be small. In the experiment shown in Table 2, a sample of non-NaCl-washed cambial tissue was further extracted for 6 hr with pronase after the NaOH extraction. About 13% of the phenolic radioactivity was removed by this treatment.

Clearly shown are differing capacities for the complexing of carbohydrate and ferulic acid by differing fractions of peroxidases. Not only do they differ by cell types in the same species, but by their occurrence within the cell, with the wall-bound fractions exhibiting the greater efficiencies.

Electrophoretic studies of peroxidase in this laboratory and by others show differences in isozymes between cellular fractions [1]. More work needs to be done to see if specific isozymes are responsible for binding or polymerizing ferulic acid or other monolignols [9].

EXPERIMENTAL

Preparation of labeled ferulic acid. Coleoptiles were collected from wheat plants (*Triticum aestivum* cv. Redcoat) grown in vermiculite for 3 days at 27°, in darkness. One section 9 mm long was cut from each coleoptile at 3 mm from the tip. The sections were incubated for 20 hr in a mixture containing 1% sucrose, 0·05 M K-maleate buffer, pH 4·8, and 2·5 μCi cinnamic acid-[2-^{14}C] (ICN) of sp. act. 4 μCi/μmol in a total volume of 6 ml. Leaves were removed from the segments, of which 200 were used for each preparation. Segments were rinsed with H_2O, ground in a mortar, washed 3× with H_2O and 3× with EtOH by suspension and centrifugation. The tissue was extracted 18 hr with 4 ml 2% NaOH at 25°, centrifuged with the supernatant saved, then washed with 4 ml H_2O which was added to the original supernatant. The soln was acidified with HCl to pH 1·0 and extracted with Et_2O. The Et_2O was removed by evaporation and the residue dissolved in EtOH for spotting on TLC plates of Si gel with fluorescent indicator. After separation in C_6H_6–HOAc (9:1), the ferulic acid was located with UV by a reference spot, eluted with EtOH, and used in binding experiments. The yield of radioactive ferulic acid was about 20% of the cinnamic acid starting material with a specific activity of about 2 μCi/μmol [10].

CMC experiment. Sodium carboxymethylcellulose (Nutritional Biochemicals), 10 mg, was mixed with 0·2 mg horseradish peroxidase (Nutritional Biochemicals, 400 units/mg) in 1 ml H_2O and dried with a stream of N_2. The mixture was incubated at 25° for 18 hr with about 10 μg ferulic acid-[^{14}C], 0·01 ml 30% H_2O_2 in 4 ml 0·05 M phosphate buffer, pH 5·4. A 2 ml portion of the mixture was separated into 4 ml fractions on a column of Bio-Gel P-100 with 0·02 M ammonium formate buffer, pH 7·0, as solvent. A 1 ml portion was incubated at 25° for 16 hr in 2 ml 0·05 M Pi buffer, pH 5·4, containing 10 mg cellulase (Cellulysin, Calbiochem, 10 units/mg). This mixture was separated on a Bio-Gel column as described above. Carbohydrate was determined by a phenol–sulfuric acid method [11].

Preparation of enzymes. The bark of 2-yr-old slash pine (*Pinus elliottii*) was removed and cambial tissue scraped off. For each experiment, 150–350 mg of cambial tissue and an equal amount of slash pine callus was used. The callus had been subcultured at monthly intervals for about 5 yr on Brown and Lawrence's medium [12]. Tissue was ground in a mortar with sand in 0·067 M Pi buffer, pH 7·0, centrifuged at 2000 × g for 10 min and the supernatant was used as the soluble enzyme. Tissue was then washed with 1% Tween 80 and centrifuged at 800 × g for 1 min, followed by washes with H_2O until no peroxidase activity could be detected in the washings. Bound peroxidase was released with 3 successive washes with 1 ml 1M NaCl. The NaCl washings were combined and used as the ionically bound fraction. Covalently bound peroxidase was released by incubating the NaCl-washed tissue for 18 hr at 25° with 10 mg cellulase in 2 ml 0·05 M Pi buffer, pH 5·4. After centrifugation, the supernatant was used directly. The soluble and ionically bound peroxidases were dialyzed against H_2O overnight at 4°. All the enzyme solns were mixed with 50 mg microcrystalline cellulose and freeze dried.

Preparation of washed cell walls. Cambial and callus tissues were collected, ground, and washed in exactly the same way as in the preparation of peroxidase fractions given above except that the enzymes removed by the initial buffer extraction and subsequent NaCl extraction were discarded and the tissues, containing bound peroxidase, were directly incubated with ferulic acid-[^{14}C].

Measurement of peroxidase activity. Activity of enzymes in soln was determined with guaiacol as substrate [3]. Peroxidase bound to cell walls was determined by the method of Ridge and Osborne [1].

Incubation with ferulic acid-[^{14}C]. All cell wall and microcrystalline cellulose preparations were incubated at 25° for 18 hr in a mixture of various amounts of ferulic acid-[^{14}C] and 0·01 ml 30% H_2O_2 in 2–4 ml 0·05 M phosphate buffer, pH 5·4.

Preparation for counting. Microcrystalline cellulose and cell wall samples after incubation with labeled ferulic acid were washed 3× with H_2O and 3× with EtOH. Samples were removed for counting. This was followed by extraction for 1 hr with 5% $NaHCO_3$ at 25°, 3 washes with H_2O and removal of a sample for counting. The remainder of the material was extracted at 25° for 1 hr with 2% NaOH, washed 3× with H_2O, and counted. Cambial cell wall sample not initially extracted with NaCl, and after successive extractions with $NaHCO_3$ and NaOH, was extracted with 5 mg Pronase (Calbiochem, 45 units/mg) for 6 hr at 25°. After washing with H_2O and centrifugation, a portion of the tissue was removed for counting. Radioactivity was counted with a gas-flow planchet system or a liquid scintillation spectrometer.

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