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SCIENCE

**Lignin-Degrading Enzyme from the Hymenomycete
Phanerochaete chrysosporium Burds**

Ming Tien and T. Kent Kirk

Lignin-Degrading Enzyme from the Hymenomycete *Phanerochaete chrysosporium* Burds.

Abstract. The extracellular fluid of ligninolytic cultures of the wood-decomposing basidiomycete *Phanerochaete chrysosporium* Burds. contains an enzyme that degrades lignin substructure model compounds as well as spruce and birch lignins. It has a molecular size of 42,000 daltons and requires hydrogen peroxide for activity.

Lignin biodegradation plays a key role in the earth's carbon cycle. Not only is lignin the most abundant renewable organic material next to cellulose, but it also encrusts and, until degraded, prevents access of degradative enzymes to the cellulose and hemicelluloses in woody plant tissues (1). Lignin is decomposed preeminently by higher basidiomycetous fungi that cause the white-rot type of wood decay (2).

Past research has shown that oxidizing agents with low specificity are involved in the biodegradation (3), but has not revealed the nature of these agents. Recent indirect evidence indicates that non-enzyme-bound activated oxygen species derived from H_2O_2 , rather than enzymes, are the actual degradative agents

(4, 5), and that H_2O_2 has a role in lignin degradation (4-7).

Essential to defining the biochemical mechanism is the identification of individual reactions of the lignin degradation process. Because of the complexity of the lignin polymer (Fig. 1), lignin substructure model compounds such as 1,2-bis-(3-methoxy-4-[^{14}C methoxyphenyl]propane-1,3-diol (1) and 1-(4-ethoxy-3-methoxy[^{14}C]phenyl)-2-(*o*-methoxyphenoxy) propane-1,3-diol (2) have been used to define specific reactions. Compound 1 represents the 1,2-diarylpropane substructure (Fig. 1A), which accounts for ~7 percent of the linkages in lignins, and 2 represents the arylglycerol- β -aryl ether type of substructure (Fig. 1C), which is the dominant one in lignins,

accounting for 50 to 60 percent of the interunit linkages (8). The degradative pathways of these and related model compounds in cultures of the white-rot fungus *Phanerochaete chrysosporium* Burds. have been partially elucidated (9-12). An oxidative C-C bond cleavage, which initiates the degradation of 1 (Fig. 1A) and related structures in cultures (9-11), has been described. We report the discovery of an extracellular enzyme from *Phanerochaete chrysosporium* which, in the presence of added H_2O_2 , catalyzes that cleavage, not only in 1, but also in compound 2 and in spruce and birch lignins.

The enzyme activity was detected by incubating 1 (13), in the presence of added H_2O_2 , with the concentrated extracellular fluid from 6-day-old ligninolytic cultures (14); this compound (1) was cleaved between C-1 and C-2, with formation of vanillin methyl ether (3) from the C-1 moiety, and 1-(3',4'-dimethoxyphenyl)ethane-1,2-diol (4) from the C-2 portion (Fig. 1A). The ^{14}C -labeled products were extracted and identified by coelution, after isolation by thin-layer chromatography (TLC), with unlabeled standards on TLC plates (15). They are the same products formed initially in intact cultures (9). Both intact cultures (9-11) and the reconstituted system (concentrated culture fluid + H_2O_2) further cleave the diol product (4) to form the aldehyde 5, and both also oxidize diol 4 to ketol 5 as a minor reaction (Fig. 1A). Thus 3 is produced from both aromatic moieties.

The reconstituted system was active also against model compound 2 (16), which differs from 1 in having an aryl ether rather than an aryl substituent at C-2 (Fig. 1C). Like 1, compound 2 is

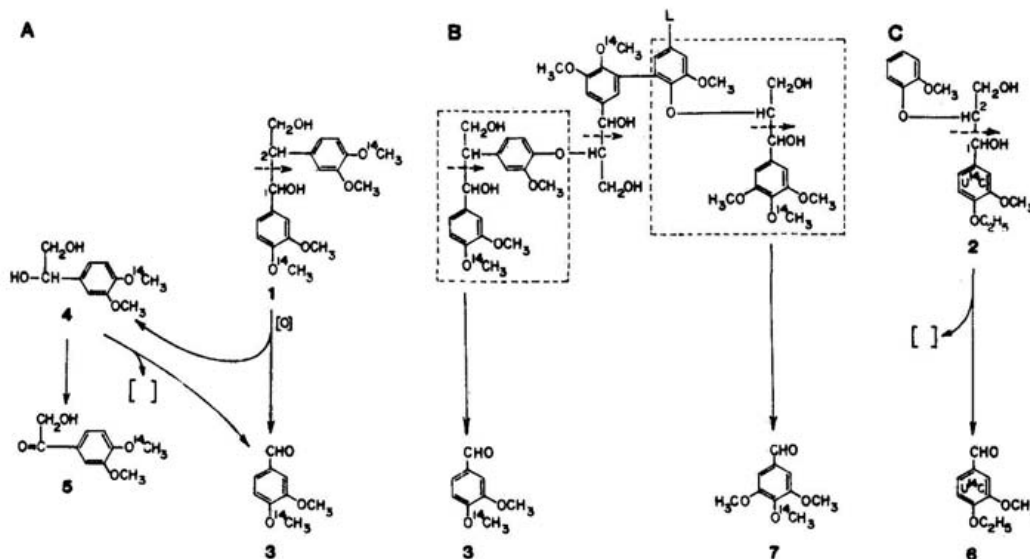


Fig. 1. A portion of a lignin macromolecule and substructure model compounds 1 and 2. The fungal degradation products are shown. (A) Model compound 1. (B) Lignin with phenolic hydroxyl groups ^{14}C -methylated. Dashed boxes enclose the 1,2-diarylpropane (left) and arylglycerol- β -aryl ether (right) substructures in the lignin corresponding to model compounds 1 and 2. L, continuation of lignin polymer. (C) Model compound 2. Dashed arrows indicate sites of C-C bond cleavages. Empty brackets in (A) and (C) indicate unidentified degradation products.

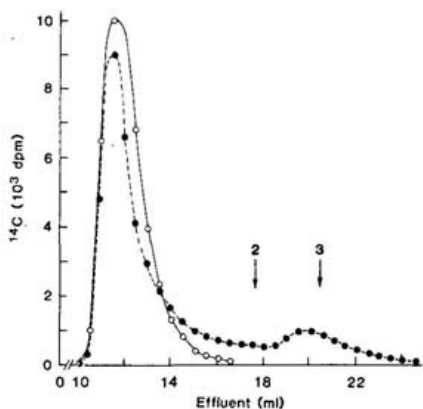


Fig. 2. Gel filtration of ^{14}C -methylated spruce lignin on a Sephadex LH-20 column (1 by 22 cm) in *N,N*-dimethylformamide (DMF) (20). The calibrants were 2 (molecular weight, 348) and 3 (molecular weight, 166). Reaction mixtures (1 ml) contained lignin (1×10^5 dpm), boiled (—○—) or active (—●—) enzyme (concentrated culture fluid, 50 μg of protein per milliliter), in the presence of 0.2 mM H_2O_2 and 0.1 percent Tween 80 in 100 mM sodium tartrate, pH 3.0, 37°C. The reaction was terminated at 1 hour by addition of 2 ml of DMF and immediate evaporation to reduce the volume to about a half milliliter; this removed most of the H_2O . Samples were then filtered through glass wool and applied to the column. Reaction with active enzyme but without H_2O_2 gave the same results as boiled enzyme.

cleaved between C-1 and C-2 with formation of an aromatic aldehyde product, in this case, vanillin ethyl ether (6), from the C-1 portion (Fig. 1C). [The alcohol formed on reduction of 6 is the dominant product detected in intact cultures (12).]

Because 1 and 2 represent more than 50 percent of the substructures in lignin (8), we considered it likely that cleavage might be demonstrable with lignin itself as substrate. To assess this possibility, we used spruce lignin, purified from an aqueous acetone extract of the wood of *Picea engelmannii* Parry (17), and milled wood lignin (18) of birch (*Betula verrucosa* L.). Free phenolic hydroxyl groups were methylated with $^{14}\text{CH}_3\text{I}$ (19) to facilitate detection and product identification. The largest molecules (≥ 1500 daltons) (20) were used. Incubation with the reconstituted system yielded vanillin methyl ether (3) from both the spruce and birch lignins, and birch yielded syringaldehyde methyl ether (7) as well. Our results with the model compounds indicate that 3 and 4 were formed by cleavages between C-1 and C-2 in end groups (Fig. 1B). Aldehyde 3, isolated by TLC, accounted for 4.5 percent of the original ^{14}C in the spruce lignin, and aldehydes 3 and 7, also isolated by TLC, contained 0.6 and 0.4 percent of the ^{14}C from the birch lignin, a result in accord with the known chemistry of spruce and birch

lignins. The birch lignin is a copolymer of guaiacyl (monomethoxyphenyl) and syringyl (dimethoxyphenyl) units, which gave rise to products 3 and 7, whereas spruce lignin is comprised only of guaiacyl units (8).

The reconstituted system also partially depolymerized the lignins. As determined by LH-20 column chromatography, depolymerization products accounted for approximately 22 and 6 percent of the original ^{14}C in the spruce and birch lignins, respectively (Fig. 2). Cleavage of internal bonds between C-1 and C-2 (Fig. 1B) probably contributed to the partial depolymerization.

That the cleavage reactions were enzyme-catalyzed became apparent with further study. Activity against the models and lignins was destroyed by heating the concentrated culture fluid at 100°C for 10 minutes. Activity against all substrates eluted from a Bio-Gel P-100 column as a single, Coomassie blue-staining (protein) peak, corresponding to a molecular size of 42,000 daltons (21). More important, activity against 1 exhibited saturation kinetics, with an apparent K_M of 55 μM (22) (Fig. 3).

Results with the Bio-Gel P-100 column suggested that the activity against all substrates resides in a single enzyme. The fact that activity against 1 and 2, and against lignin resides in a single enzyme was further supported by polyacrylamide gel electrophoresis. A single band contained all three activities (Fig. 4A). Sodium dodecyl sulfate gel electrophoresis of the protein recovered from the active band also indicated unimolecular-

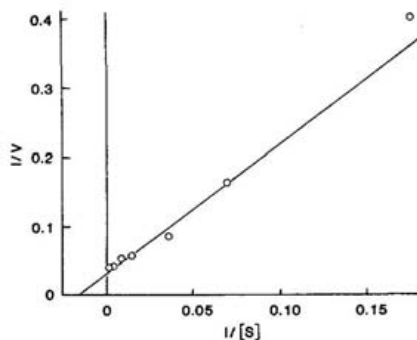


Fig. 3. Lineweaver-Burk analysis showing an apparent K_M of 55 μM for 1 by formation of aldehyde 3 (22). Reaction mixtures contained 5 μg of protein per milliliter of concentrated culture fluid, 0.2 mM H_2O_2 (saturating), 0.1 percent Tween 80, and the indicated concentrations of 1 (diluted with unlabeled 1 to about 5×10^4 dpm per reaction) in 100 mM sodium tartrate, pH 3.0, in a total volume of 1 ml at 37°C. Addition of H_2O_2 started the reaction. Aldehyde 3 was isolated (15) and quantified by scintillation spectrometry ($V = \text{nmole} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$; substrate concentration = μM).

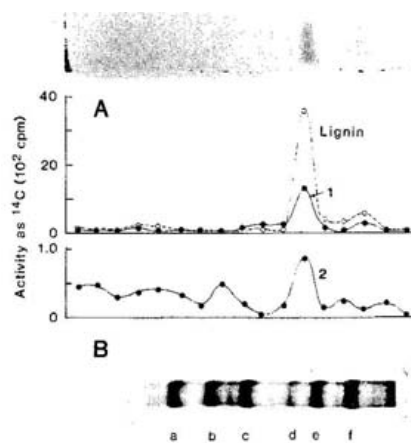


Fig. 4. Analysis of extracellular proteins by gel electrophoresis. (A) Proteins (20 μg per well) were subjected to electrophoresis in a 10 percent polyacrylamide slab gel with the use of a nondissociating continuous buffer system (100 mM sodium phosphate, pH 7.2) at 6°C (origin, cathode, right). In one of the lanes the proteins were stained with Coomassie blue (top). The other lanes were cut into 17 slices, each 0.5 cm wide, and assayed for cleavage activity against 1 (18 $\mu\text{g}/\text{ml}$), 2 (380 $\mu\text{g}/\text{ml}$), and spruce lignin (50 $\mu\text{g}/\text{ml}$). The incubation time was 30 minutes; aldehyde formation was assayed as described (legend to Fig. 3). (B) The fluid of the crushed gel fractions containing the cleavage activity was filtered, concentrated, and analyzed by sodium dodecyl sulfate gel electrophoresis (23). The adjacent lane contained markers for molecular size (a, lysozyme, 14.3K; b, β -lactoglobulin, 18.4K; c, trypsinogen, 24K; d, pepsin, 34.7K; e, egg albumin, 45K; f, albumin, 66K).

ity and confirmed the molecular size of 42,000 daltons (Fig. 4B).

Our results demonstrate that one of the key (3) reactions of lignin biodegradation is catalyzed by an oxidative, H_2O_2 -requiring enzyme, rather than being due to a nonenzyme-bound activated oxygen species derived from H_2O_2 .

MING TIEN*
T. KENT KIRK

Forest Products Laboratory,
U.S. Department of Agriculture, Forest
Service, Madison, Wisconsin 53705

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 13. Compound **1** was prepared earlier [F. Nakatsubo, I. D. Reid, T. K. Kirk, *Biochem. Biophys. Res. Commun.* **102**, 484 (1981)]. Specific activity = 2.4×10^9 dpm/mole.
 14. Cultures were grown as described (9), except that they were buffered at pH 4.3 with 10 mM, 2,2-dimethylsuccinate. [Ligninolytic activity appears as a part of idiophasic metabolism, triggered by nitrogen starvation (3).] Replicate cultures were pooled and centrifuged (10,000g; 15 minutes) and the mycelial pellet was discarded. The supernatant was concentrated about tenfold by means of a 10,000-dalton filter, and then freed of any residual cells or spores by passage through a 0.45- μ m filter ($T = 4^\circ\text{C}$ throughout). This preparation contained 80 to 100 μ g of protein per milliliter.
 15. Products were extracted with a mixture of chloroform and acetate (1:1). Radioactive products were isolated by TLC and quantified by scintillation spectrometry (9). TLC procedures: C.-L. Chen, H.-m. Chang, T. K. Kirk, *Holzforschung* **36**, 3 (1982). Unlabeled standards for compounds **4** and **5** were prepared as described for related compounds (9).
 16. This compound was prepared from 3-methoxy-4-hydroxy- α -(2-methoxyphenoxy)- β -hydroxy-propiofenone, labeled uniformly with ^{14}C in the propiofenone ring [L. L. Landucci, S. A. Geddes, T. K. Kirk, *Holzforschung* **35**, 67 (1981)], by ethylation with ethyl iodide and K_2CO_3 in *N,N*-dimethylformamide, and subsequent reduction with NaBH_4 . Specific activity was 7.2×10^8 dpm/mole.
 17. T. K. Kirk and H.-m. Chang [*Holzforschung* **28**, 217 (1974)] isolated and characterized this sample. It was chosen here for its high phenolic hydroxyl content [0.44 hydroxyls per monomer (C_6) unit], indicative of a large number of end groups.
 18. A. Björkman, *Sven. Papperstidn.* **59**, 477 (1956). This lignin was provided by Dr. K. Lundquist, Chalmers University, Gothenburg, Sweden.
 19. About 2 mg of each lignin was methylated with $^{14}\text{CH}_3\text{I}$ and K_2CO_3 in *N,N*-dimethylformamide. Specific activities: spruce, 1.9×10^8 dpm/mg; birch, 1.1×10^8 dpm/mg.
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 21. A column (2.5 by 43 cm) of Bio-Gel P-100 was eluted with 100 mM sodium acetate, pH 4.5 and calibrated with glucose oxidase, serum albumin, ovalbumin, cytochrome c and vitamin B_{12} .
 22. The K_M probably reflects formation of **3** via two competing reactions; therefore, the value of 55 μM is not a true K_M . Nevertheless, the results demonstrate saturation kinetics.
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 24. We thank M. D. Mozuch for technical assistance.
- * On assignment from Department of Wood and Paper Science, North Carolina State University, Raleigh 27650.

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