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12. Serum tryptophan concentrations in control sheep ranged from 22 to 32 μ*M*. After an injection of L-tryptophan a large and sustained increase in serum tryptophan was observed, with concentrations reaching 290 to 370 μ*M*; pineal tryptophan levels increased about fivefold (to 50 to 60 ng/mg, wet weight) over 5 hours.
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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 nonenzyme-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[U^{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 **3**, 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

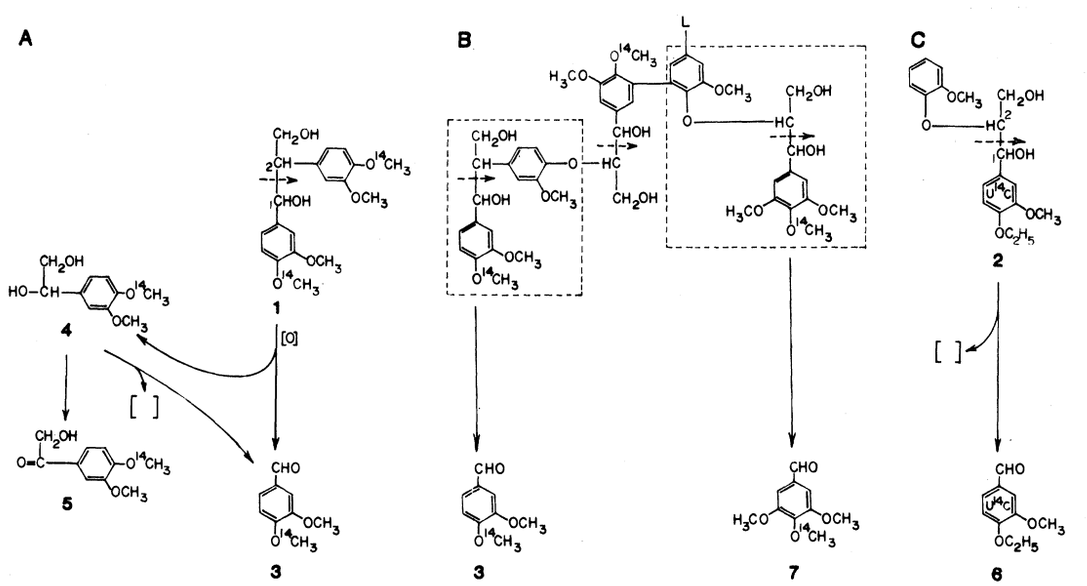

Fig. 1. A portion of a lignin macromolecule and substructure model compounds **1** and **2**. The fungal degradation products are shown. (A) Model compound **1**. (B) Lignin with phenolic hydroxyl groups ^{14}C-methylated. Dashed boxes enclose the 1,2-diarylpropane (left) and arylglycerol-β-aryl ether (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.

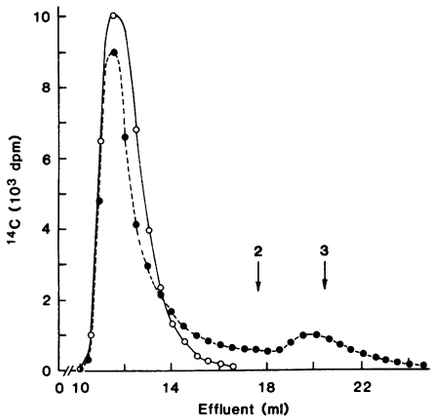

Fig. 2. Gel filtration of ^{14}C-methylated spruce lignin on a Sephadex LH-20 column (1 by 22 cm) in *N*,*N*-dimethylformamide (DMF) (*20*). The calibrants were **2** (molecular weight, 348) and **3** (molecular weight, 166). Reaction mixtures (1 ml) contained lignin (1×10^5 dpm), boiled (–○–) or active (--●--) enzyme (concentrated culture fluid, 50 μg of protein per milliliter), in the presence of 0.2 m*M* H_2O_2 and 0.1 percent Tween 80 in 100 m*M* sodium tartrate, *p*H 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 engelmanii* Parry (*17*), and milled wood lignin (*18*) of birch (*Betula verrucosa* L.). Free phenolic hydroxyl groups were methylated with $^{14}CH_3I$ (*19*) to facilitate detection and product identification. The largest molecules ($\gtrsim$ 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 μ*M* (*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 unimolecularity and confirmed the molecular size of 42,000 daltons (Fig. 4B).

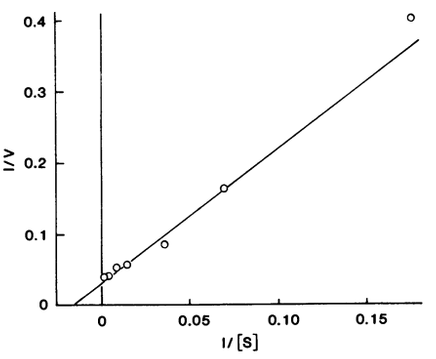

Fig. 3. Lineweaver-Burk analysis showing an apparent K_M of 55 μ*M* for **1** by formation of aldehyde **3** (*22*). Reaction mixtures contained 5 μg of protein per milliliter of concentrated culture fluid, 0.2 m*M* H_2O_2 (saturating), 0.1 percent Tween 80, and the indicated concentrations of **1** (diluted with unlabeled **1** to about 5×10^4 dpm per reaction) in 100 m*M* sodium tartrate, *p*H 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 = nmole $\cdot$ $min^{-1} \cdot ml^{-1}$; substrate concentration = μ*M*).

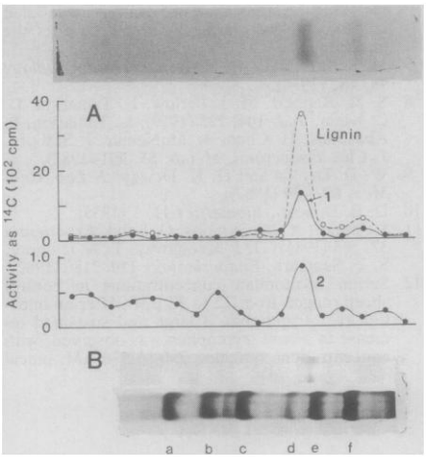

Fig. 4. Analysis of extracellular proteins by gel electrophoresis. (A) Proteins (20 μg per well) were subjected to electrophoresis in a 10 percent polyacrylamide slab gel with the use of a nondissociating continuous buffer system (100 m*M* sodium phosphate, *p*H 7.2) at 6°C (origin, cathode, right). 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 μg/ml), **2** (380 μg/ml), and spruce lignin (50 μg/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).

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.

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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/mmole.
14. Cultures were grown as described (*9*), except that they were buffered at *p*H 4.3 with 10 m*M*, 2,2-dimethylsuccinate. [Ligninolytic activity appears as a part of idiophasic metabolism, triggered by nitrogen starvation (*3*).] Replicate cultures were pooled and centrifuged (10,000*g*; 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-μm filter (T = 4°C throughout). This preparation contained 80 to 100 μg 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-hydroxypropiophenone, labeled uniformly with ^{14}C in the propiophenone 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/mmole.
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19. About 2 mg of each lignin was methylated with $^{14}CH_3I$ 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 m*M* sodium acetate, *p*H 4.5 and calibrated with glucose oxidase, serum albumin, ovalbumin, cytochrome c and vitamin B_{12}.
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Transcriptional Enhancer Elements in the Mouse Immunoglobulin Heavy Chain Locus

Abstract. *Two regions in the immunoglobulin heavy chain locus were tested for their ability to enhance transcription of the SV40 early promoter. A portion of the intervening sequence between the heavy chain joining region (J_h) and the constant region of the μ chain (Cμ) can enhance transcription when it is cloned either 5′ or 3′ to the SV40 early promoter. The region between Cα and the alpha switch site, which occurs 5′ to the translocated c-myc oncogene in many murine plasmacytomas, does not show transcriptional enhancer activity in this assay.*

Efficient transcription from certain eukaryotic viral promoters requires the presence of positive regulatory sequences that have been called enhancer sequences (*1*). Enhancer sequences have been found in several papovaviruses and retroviruses (*2*). Although there is no striking sequence similarity among enhancers from various viruses, they generally have several properties in common: (i) they act on promoters only in *cis*, (ii) they can act from a location either 3′ or 5′ to the promoter, (iii) they usually work in either orientation with respect to the promoter, and (iv) they can act on heterologous promoters. The activity of certain mammalian promoters, such as the rabbit β-globin promoter, is significantly increased in the presence of a viral enhancer sequence (*3*). It is not yet known whether DNA sequences similar in function to the viral enhancers also occur in mammalian DNA and whether they might play a role in gene regulation. Several lines of evidence discussed below have led us to ascertain whether enhancer-like elements are located at two particular sites in the immunoglobulin heavy chain gene region.

During ontogeny of antibody-producing B lymphocytes, functional heavy chain genes are formed as a result of a DNA rearrangement called VDJ joining (*4*). Unrearranged variable (V) gene segments contain the necessary 5′ sequences for transcription but are not actively transcribed even in fully differentiated B cells (*5*). Regulatory regions 5′ to the V gene cap (initiation) site remain unaltered during B-cell development (*6*). Thus, enhancer elements located between J_h (heavy chain joining region) and Cμ (constant μ chain region), brought into functional proximity with V_h promoters after VDJ joining, could activate V_h transcription. Two observations support this suggestion: (i) Cμ gene segments are transcribed in lymphoid cells in the absence of VDJ joining (*7*, *8*) and (ii) a pre-B-cell lymphoma line which suffered a deletion in the J_h-Cμ intron shows a decrease in Cμ transcripts (*8*).

Most murine plasmacytomas and human Burkitt's lymphomas have characteristic chromosomal alterations which involve translocation of c-myc (the cellular homolog of the avian myelocytomatosis virus transforming gene) to the immunoglobulin heavy chain locus (*9*). In murine plasmacytomas, the c-myc gene is often translocated a few kilobases 5′ to the nonexpressed Cα gene segment (*10*). Translocation leads to an increase in c-myc transcripts, many of which are initiated at aberrant sites (*11*). Since enhancer sequences associated with the Cα gene segment could be responsible for this altered c-myc transcription, we set out to determine if such elements were located 5′ of Cα.

We used a convenient vector system (*12*) to test for enhancer activity in the immunoglobulin heavy chain locus. The pA10CAT-2 vector contains the SV40 early promoter directing transcription of the bacterial chloramphenicol acetyltransferase (CAT) gene but does not contain a functional SV40 72-bp enhancer sequence. Since CAT activity is normally absent from mammalian cells, the enhancer activity of fragments cloned into the vector may be quantified by measuring CAT activity in transfected cells. A positive control, pSV2-CAT, contains the SV40 enhancer sequence.

Portions of the J_h-Cμ intron and of the 5′ flanking region of the Cα gene, which were cloned into pA10CAT-2, are illustrated in Fig. 1b. The clones were transfected into COS cells (SV40 transformed monkey kidney line constituitively expressing T antigen), and the CAT activity was determined. The J_h-Cμ intervening sequence contained on a 7.6-kb Bam HI fragment is capable of enhancing transcription from the SV40 early promoter when it is cloned 3′ to the CAT gene in the "antisense" orientation (Fig. 2a). The immunoglobulin sequence had 60 percent activity of the SV40 enhancer element, which represents a 20-fold increase over the enhancer-deleted negative control.

Since there is a low amount of CAT gene transcription from the pA10CAT-2 vector lacking an enhancer, it was possible that the high levels of CAT activity observed with immunoglobulin sequence cloned into pA10CAT-2 were the result of preferential replication of these vectors to levels 20-fold higher than pA10CAT-2 itself. Therefore, we determined the approximate copy number of the various vectors in the extrachromosomal DNA fraction (Hirt supernatant) (*13*) of transfected cells by probing quantitative dot blots (*14*) with a pA10CAT-2 probe. Although this experiment does not distinguish between replicated and nonreplicated molecules, nor can we be sure that all of the copies detected are