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Biochemistry

Lignin-degrading enzyme from *Phanerochaete chrysosporium:* Purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase

(hemoprotein/stereospecificity/$^{18}O_2$ incorporation/white-rot fungi/wood decay)

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ABSTRACT An extracellular lignin-degrading enzyme from the basidiomycete *Phanerochaete chrysosporium* Burdsall was purified to homogeneity by ion-exchange chromatography. The 42,000-dalton ligninase contains one protoheme IX per molecule. It catalyzes, nonstereospecifically, several oxidations in the alkyl side chains of lignin-related compounds: C_α—C_β cleavage in lignin model compounds of the type aryl — C_αHOH — C_βHR — $C_\gamma H_2$OH (R = -aryl or ·*O*·aryl), oxidation of benzyl alcohols to aldehydes or ketones, intradiol cleavage in phenylglycol structures, and hydroxylation of benzylic methylene groups. It also catalyzes oxidative coupling of phenols, perhaps explaining the long-recognized association between phenol oxidation and linin degradation. All reactions require H_2O_2. The C_α— C_β cleavage and methylene hydroxylation reactions involve substrate oxygenation; the oxygen atom is from O_2 and not H_2O_2. Thus the enzyme is an oxygenase, unique in its requirement for H_2O_2.

We recently reported the discovery of a lignin-degrading enzyme from the basidiomycete *Phanerochaete chrysosporium* Burdsall (Aphylophorales, Corticiaceae) (1). This ligninase is extracellular and requires H_2O_2, for activity. This paper describes its purification and characterization.

The enzyme catalyzes C—C bond cleavage in the propyl side chains of two dimeric model compounds, as well as in spruce and birch lignins (1). This cleavage is prominent in the fungal degradation of lignin (2) and is the first reaction in the metabolism of dimeric models in cultures (3, 4). The studies here reveal that the enzyme is a heme-containing oxygenase, unique in that it requires H_2O_2. To study specific reactions we have used lignin substructure model compounds as substrates, rather than lignin. The two types of models chosen are of the ß-1 (1,2-diarylpropane-1,3-diol) and ß-*O*-4 (arylglycerol-ß-aryl ether) types. Together, the ß-1 and ß-*O*-4 linkages, represented by models I and II, respectively (Fig. 1), make up over 60% of the intermonomer linkages in lignins (5).

MATERIALS AND METHODS

Enzyme Production and Purification. *P. chrysosporium*, strain BKM-1767 (ATCC 24725), was maintained and spore inoculum was prepared and used as reported previously (6). The 10-ml cultures in 125-ml Erlenmeyer flasks were grown as described (7), with 10 mM 2,2-dimethylsuccinate, pH 4.5, as buffer. Enzyme activity appeared 3–4 days after culture initiation, was maximal in 6-day-old cultures, and was associated only with the extracellular culture fluid.

Cultures (130, 6-day) were combined and centrifuged (10,000 × *g*, 15 min, 4°C). To minimize proteolysis, *p*-methylsulfonyl fluoride (0.2 mM) (Sigma) was added lo the supernatant, which was concentrated (Amicon YM-10 filter; 10,000-dalton pore size) to 250 ml. This solution oxidized 0.16 μmol of 3,4-dimethoxybenzyl (veratryl) alcohol per min per ml (see assay procedure below). After overnight dialysis against 5 mM sodium tartrate buffer, pH 4.5, the sample was applied to a DEAE-Bio-Gel A column (1 × 16 cm) (Bio-Rad), previously equilibrated with the same buffer. The column was washed with 100 ml of buffer and a salt gradient was then applied (0–0.1M NaCl in 5 mM sodium tartrate, pH 4.5, total volume 400 ml). All steps in the purification were at 4°C. The enzyme solution (80 ml; activity: 0.24 μmol of veratryl alcohol oxidized per min per ml) was then dialyzed against distilled deionized H_2O and stored as a stable lyophilized powder at −20°C.

At pH 4.5, the enzyme did not consistently adhere to certain batches of DEAE-Bio-Gel A, a problem solved by increasing the pH of the buffer (5 mM $KHPO_4$, pH 7.0; 0–0.14 M NaCl).

Protein Determination. Protein content was routinely determined with Coomassie blue (8). The biuret method (9) was used in determining the extinction coefficient of the enzyme. Bovine serum albumin [$A^{1\%}_{280}$ = 6.6 (10)] was the standard in both procedures

Enzyme Assays. Enzyme activity was measured with two assays: quantitation of the [^{14}C]veratraldehyde produced on cleavage of model compound **I** (1), and quantitation, by UV spectroscopy, of veratraldehyde (ε_{310} = 9300 $M^{-1} \cdot cm^{-1}$) formed on oxidation of veratryl alcohol. The latter assay was also used to monitor oxidation of model **II.** In the assays, enzyme (1–5μg of protein per ml) was incubated with 0.54 mM H_2O_2. 0.1% Tween 80, and 0.4 mM veratryl alcohol or 1.15 mM model **II** in 0.1 M sodium tartrate, pH 3.0 at 37°C. Addition of H_2O_2 started the reaction.

Metal Analysis of the Enzyme. Transition metals (Cu, Zn, Mn, Fe, Mo, Co) were determined by atomic absorption spectroscopy. Prior dialysis against 10 mM sodium tartrate, pH 4.5, containing 0.1 mM 8-hydroxyquinoline-5-sulfonic acid (Sigma), for 20 hr at 4°C, eliminated extraneous metals. Dialysis had no effect on the activity.

Electrophoresis and Isoelectric Focusing. Purity of the enzyme was assessed by isoelectric focusing (11) and NaDodSO$_4$/polyacrylamide gel electrophoresis (12) (LKB, Uppsala, Sweden). The isoelectric focusing gel contained 5% acrylamide and 5% ampholytes (pH 2.5–4.2); the NaDodSO$_4$ gel contained 10% acrylamide. Protein bands were stained with Coomassie blue (13). M_r markers (Sigma) were lysozyme, ß-lactoglobulin, trypsinogen, pepsin, egg albumin, and serum albumin.

Pyridine Hemochromogen. The heme was quantitated by the absorption of the pyridine hemochromogen complex [ε_{557} = 32,500 $M^{-1} \cdot cm^{-1}$ (14)], after extraction of the heme (15) from the enzyme. The heme was dissolved in 3 M pyri-

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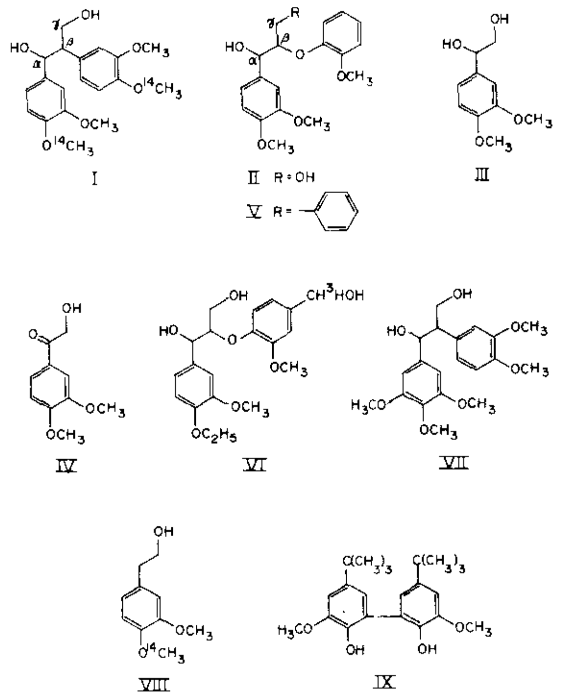

FIG. 1. Molecular formulae.

dine and 0.5 M NaOH. The spectrum of the dithionite-reduced complex was obtained immediately.

Synthesis of Model Compounds. 1,2-Bis-(3-methoxy-4-[^{14}C]methoxyphenyl)propane-1,3-diol **(I)** (Fig. 1), and unlabeled **I,** were prepared by methylating the corresponding phenolic compound (16) with $^{14}CH_3I$ (ICN), or with CH_3I, in *N,N*-dimethylformamide with excess K_2CO_3 at room temperature. Specific activity = 1.0 mCi/mmol (1 Ci = 37 GBq).

1-(3,4-Dimethoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol **(II)** was prepared by $NaBH_4$ reduction (95% ethanol, room temperature) of 3,4-dimethoxy-α-(2-methoxyphenoxy)-ß-hydroxypropiophenone (17).

1-(3′,4′-Dimethoxyphenyl)ethane-1,2-diol **(III)** was prepared by $NaBH_4$ reduction of compound **IV** in 95% ethanol.

α-Hydroxy-3′,4′-dimethoxyacetophenone **(IV)** was prepared by methods used for an analogous compound (7).

1-(3,4-Dimethoxyphenyl)-2-(2-methoxyphenoxy)-3-phenyl-propane-1-ol **(V)** was synthesized from 3′,4′-dimethoxy-α-(2-methoxyphenoxy)acetophenone (17) in two steps; (*i*) α-bromotoluene/NaH/*N,N*-dimethylformamide at room temperature and (*ii*) $NaBH_4$/95% ethanol at room temperature, ^{1}H NMR confirmed the structure: (C^2HCl_3) δ (ppm): 1.6–1.8 (1H, broad singlet, –OH), 2.63 (1H, two doublets, γ-CH_A, J = 14.44, 3.01 Hz), 3.03 (1H, two doublets. γ-CH_B, J = 14.29, 9.56 Hz), 3.88, 3.89, 3.90 (12H, three singlets, –OCH_3 × 3), 4.33 (1H, two triplets, ß-CH, J = 3.19, 3.22, 9.58 Hz), 4.85 (lH, d, α-CH, J = 2.93 Hz), 6.56 (1H, m, aromatic), 6.75–7.11 (7H, m, aromatic), 7.16–7.29 (5H, m, aromatic).

1-(3,4-Dimethoxyphenyl)-2-(2-methoxy-4-[^{3}H]hydroxymethylphenoxy)propane-1,3-diol **(VI)** was prepared by methylation of 3-methoxy-4-hydroxy-α(2-methoxy-4-formylphenoxyl-ß-hydroxypropiophenone (18) with CH_3I/K_2CO_3/*N,N*-dimethylformamide at room temperature, followed by reduction with NaB^3H_4 (Amersham) in 95% ethanol at room temperature. Specific activity = 45 mCi/mmol.

1-(3,4,5-Trimethoxyphenyl)-2-(3′,4′-dimethoxyphenyl) propane-1,3-dioI **(VII)** was prepared by methylation of the corresponding 4,4′-dihydroxy compound (16) with CH_3I/K_2CO_3 at room temperature.

2-(3-Methoxy-4-[^{14}C]methoxyphenyl)ethanol **(VIII)** was prepared by $^{14}CH_3I$ methylation of homovanillyl alcohol (Aldrich) as above.

The 6,6′-dehydrodimer **(IX)** of 4-*tert*-butylguaiacol was prepared by the horseradish peroxidase (Sigma)-catalyzed dimerization of 4-*tert*-butylguaiacol in the presence of 0.1 mM H_2O_2 in 50 mM phosphate buffer at pH 7.1 (room temperature). The structure was confirmed by ^{1}H NMR spectrometry and mass spectrometry. ^{1}H NMR (C2HCl$_3$) δ (ppm): 1.34 (18H, s, –CH, × 6), 3.94 (6H, s, –OCH_3 × 2), 6.94 (4H, s, aromatic). Mass spectrum: m/z (relative intensity): 358 (M+, 100), 343 (66), 287 (58), 164 (14).

The radiochemical purities of the labeled compounds were established by TLC (19).

Enzymatic Oxidation of Model Compounds. Model compounds ($\approx$ 50 μg/ml) were incubated in a total volume of 1 ml with the enzyme (5 μg/ml), 0.1% Tween-80, and 0.15 mM H_2O_2 in 0.1 M sodium tartrate, pH 3.0, at 37°C under air. Reactions were terminated by extraction with chloroform/acetone (1:1, vol/vol) (3, 7) 5–10 min after H_2O_2 addition.

Products from labeled compounds **I** and **VI,** after isolation by TLC. were identified by coelution of the ^{14}C-labeled products with added standards on TLC plates (19). Nonlabeled products from **II, III,** and **VII** were identified by gas chromatographic/mass spectrometric comparison with authentic samples, as trimethylsilyl derivatives (7) for hydroxyl-containing products.

Incubations under $^{18}O_2$ were in Warburg flasks with $H_2{}^{16}O_2$ initially in the side arm. After purging with dinitrogen, the headspace was filled with 97% $^{18}O_2$ (KOR, Cambridge, MA). and the reaction was started by H_2O_2 addition. Reaction mixtures were as above except that they contained 50 μg of enzyme and were terminated at 2 min. Extraction and work-up of products was done within 5 min to minimize exchange of ^{18}O with the oxygen of water in some products. Products were analyzed immediately, after trimethylsilylation (7), by gas chromatography/mass spectrometry.

Instrumentation. The following instruments were used: Packard (Downers Grove, IL) 3330 scintillation spectrometer; Perkin–Elmer (Norwalk, CT) 5000 atomic absorption spectrometer; Bruker (Billerica, MA) 250 MHz NMR spectrometer: Cary (Varian) 210 UV/visible spectrophotometer; and Finnigan MAT (San Jose, CA) 4510 gas chromatograph/mass spectrometer. Gas chromatography was with a 60-m, 0.25-μm film thickness DB-5 (nonpolar silicone polymer) fused silica capillary column (J & W Scientific, Rancho Cordova, CA), operated al various temperatures. Electron impact mass spectra were obtained at 70 eV.

RESULTS

Purification and Characterization

Purification. The elution profile of the ligninolytic enzyme from the DEAE-Bio-Gel A column is shown in Fig. 2. The major protein hand was coeluted with H_2O_2-requiring oxidative activity against veratryl alcohol (Fig. 2) and H_2O_2-requiring cleavage activity against models **I** and **II.** These activities were also associated with a minor protein peak that did not adhere to the column. Since this protein may be an isoenzyme or a proteolytic fragment, we focused our attention on the major protein. Isoelectric focusing (Fig. 2, *Inset* A) and $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 2, *Inset* B) confirmed its purity and revealed the isoelectric point of 3.5 and M_r of 42,000.

Purification (2-fold) resulted in 48% recovery of the activity. Up to 75% enzyme recovery was achieved in some preparations. Maximal activity is 8.4 μmol of veratraldehyde per min per mg of protein, based on veratryl alcohol oxidation, and 11.4 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, based on cleavage of compound I.

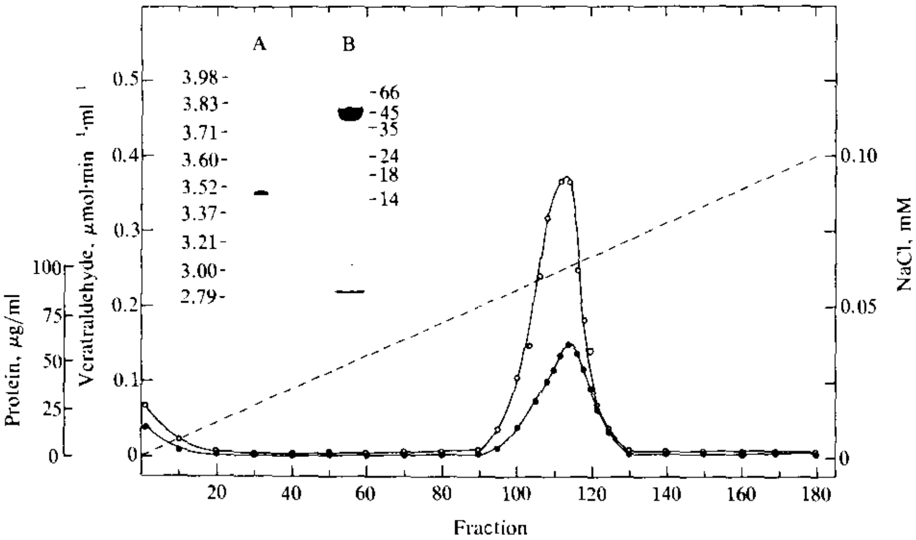

FIG. 2. Elution profile of the ligninase enzyme from a column of DEAE-Bio-Gel A. Fractions (2.2 ml) were assayed for H_2O_2-requiring activity for the oxidation of veratryl alcohol (() and for protein content (!). Purity is assessed by isoelectric focusing (*Inset A*) and NaDodSO$_4$/polyacrylamide gel electrophoresis (Inset B). Numbers adjaacent to get A are pH values and numbers adjacent to *B* are $M_r \times 10^{-3}$.

Metal Analysis. Atomic absorption spectroscopy indicated that the enzyme contains Fe (1.02 ± 0.02 atom per enzyme molecule). The enzyme dies not contain Cu, Zn, Mn, Mo, or Co.

Spectral Properties. The absorption spectrum of the enzyme revealed maxima at 409 and 502 nm (Fig. 3). The enzyme does not give a distinct peak at 280 nm. The millimolar extinction coefficients are 102 at 409 nm and 5 at 502 nm.

Addition of dithionite (anaerobic) or aromatic substrates (aerobic or anaerobic) to the enzume has no effect on its UV/visible spectrum. In contrast, addition of 21 μM H_2O_2 (3 × stoichiometric, aerobic) results in a red shift and an absorbance decrease. Thus the 409- and 502-nm bands are shifted to 420 and 544 nm, and their millimolar extinction coefficients are decreased to 55 and 3.4. Addition of dithionite to this latter solution shhifts the relatively stable spectrum back to the original. Substrates (veratryl alcohol, **I, II;** 50 μM) also cause this reversion.

The spectrum of the enzyme suggested that it is a hemoprotein; this was verified by formation of a diagnostic pyridine hemochromogen complex (14). Or the bases of the absorbance at 557 nm a heme content of 0.80 molecule per enzyme molecule was calculated.

pH Optima. Activities for veratryl alcohol oxidation and for cleavage of models I and II are maximal near pH 3.0.

H_2O_2 Optimum. For both veratryl alcohol and model **II,** maximal activity is at 0.15 mM H_2O_2 or above (Fig. 4). Although H_2O_2 is essential for activity, high concentrations (>5 mM) are inhibitory. The K_m for H_2O_2 is approximately 30 μM.

Reactions Catalyzed

Cleavage of ß-1 Models. The enzyme catalyzes C_α—C_β cleavage in model **I** and in several ß-1 compounds related to

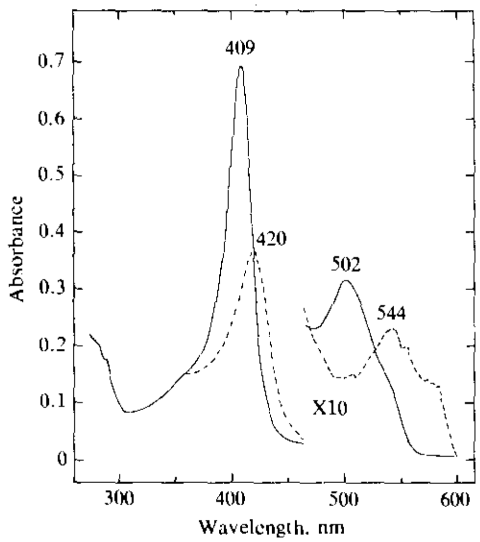

FIG. 3. Absorption spectrum of ligninolytic enzyume. The spectrum of the purified enzyme (9.3 mg/ml in 5 mM sodium tartrate, pH 4.5) was recorded in the presence (---) and absence (—) of 21 μM H_2O_2. Numbers above peaks denote wavelength maxima.

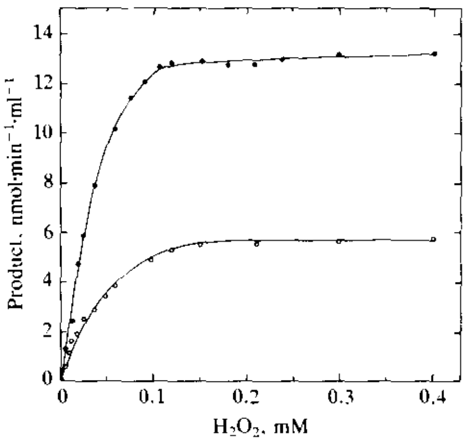

FIG. 4. Effect of H_2O_2 on enzyme activity. H_2O_2 was added to reaction mixtures containing enzyme at 5 μg/ml, 0.1% Tween 80. 0.1 M sodium tartrate at pH 3.0, and 0.4 mM veratryl alcohol (!) or 1.15 mM model **II** ((). The formation of veratraldehyde from veratryl alcohol and the formation of aryl-conjugated carbonyl from model II was monitored by the associated increase in absorbance at 310 nm.

it, including anisyl models (7). Cleavage of **I** and **VII** yields veratraldehyde or 3,4,5-trimethoxybenzaldehyde from the C_α moiety and phenylglycol product **III** from the C_β moiety as the initial products. Product **III** is further oxidized by the enzyme to yield ketol **IV** and in part it is cleaved (predominant reaction) to yield veratraldehyde and a C_1 fragment. Under substrate-limiting concentrations, cleavage of model **I** proceeds to completion. Oxidation of model **VII** is shown in Fig. *5A*

The cleavage of β-1 model **VII** under $^{18}O_2$ and with $H_2^{16}O_2$ resulted in 91% incorporation of ^{18}O into the benzyl alcohol group of C_β-derived product **III** (Fig. 6*A*) and 0% ^{18}O into the C_α-derived product, 3,4,5-trimethoxybenzaldehyde (Fig. 6*B*). Further oxidation of product **III** under $^{18}O_2$ produced ketol **IV** and veratraldehyde, which retained 91% and 51% ^{18}O, respectively (Fig. 6 *C* and *D*).

Cleavage of β-*O*-4 Models. The β-*O*-4 model **III** is cleaved between C_α and C_β with formation of veratraldehyde from the C_α moiety. We could not, however, identify any fragments from the C_β moiety by using model **II.** To facilitate such identification, model **V,** which contains a γ-phenyl group in place of a γ-hydroxyl group. was studied. Model **V,** like model **II,** is readily cleaved by the enzyme, with formation of veratraldehyde from the C_α moiety. From the C_β moiety we identified phenylacetaldehyde [*m/z* (relative intensity): 120 (M^+, 22), 92 (24), 91 (100), 65 (19)].

Another product, benzaldehyde [*m/z* (relative intensity): 106 (M^+, 100), 105 (94), 77 (96)], is also formed, and it is in fact the dominant product from the C_β-derived portion of model **V.** Subsequent study showed that benzaldehyde is not derived from phenylacetaldehyde (which is not a substrate). This indicates that the enzyme also catalyzes hydroxylation of C_γ in model **V** and that subsequent C_γ—C_β cleavage (a reaction now analogous to C_α—C_β cleavage) results in formation of benzaldehyde.

Cleavage of β-*O*-4 model **V** under $^{18}O_2$ resulted in no incorporation of ^{18}O into the C_α product, veratraldehyde. The C_β product phenylacetaldehyde also contained no ^{18}O. Experiments with $H_2^{18}O$ (20% enriched, KOR) and phenylacetaldehyde demonstrated, however, that exchange of oxygen between the aldehyde and H_2O is too fast to permit trapping of ^{18}O. The C_γ-derived product benzaldehyde contained 27% ^{18}O.

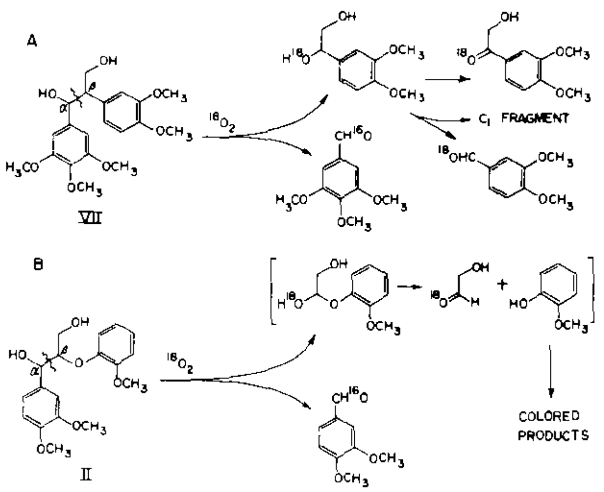

FIG. 5. Scheme showing ligninase action on β-1 (*A*) and β-*O*-4 models (*B*). β-1 and β-*O*-4 models are represented by **VII** and **II,** respectively. Compounds in brackets (*B*) have been deduced from studies with three β-*O*-4 models (see text). Incorporation of ^{18}O from $^{18}O_2$ into the C_β-derived product **(III)** from model **VII** is established. Similar incorporation of ^{18}O is presumed in the case of model **II,** as shown.

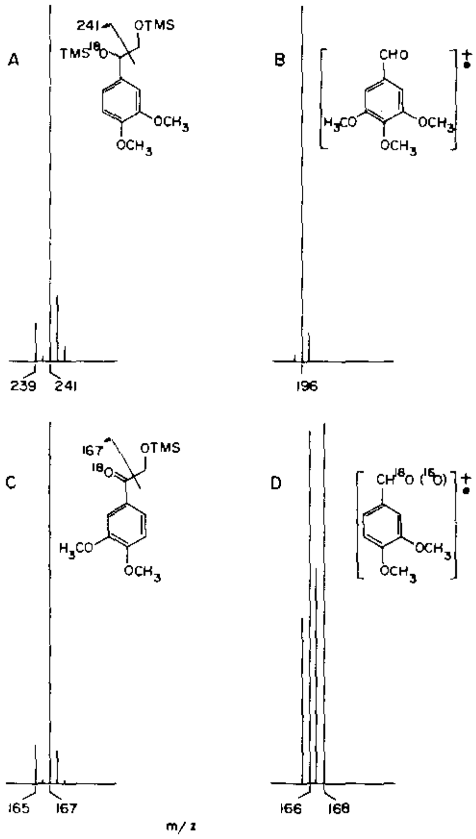

FIG. 6. Diagnostic portions of mass spectra of products formed on enzymatic oxidation of model **VII.** The reaction was under $^{18}O_2$ and with $H_2{}^{16}O_2$. The major gragments from the trimethylsilyl (TMS) derivatives are shown for products **III** (*A*) and **IV** (*C*). The molecular ion regions are shown for 3,4,5-trimethoxybenzaldehyde (*B*) and veratraldehyde (*D*). Regions shown contain the base peaks (100%, >3600 ion counts). Molecular ions were present for the trimethylsilyl derivatives of compounds **III** and **IV** at *m/z* = 344 (1.3%) and 270 (10.6%), respectively.

As observed with β-1 model **I,** cleavage of β-*O*-4 model **II** under substrate-limiting concentrations proceeds to completion.

Hydroxylation of Benzylic Methylene Groups. The finding that benzaldehyde is produced from model **V** indicated that The enzyme is capable of hydroxylating certain aromatic methylene groups. This reaction was confirmed by using coumpound **VIII,** which is hydroxylated to phenylglucol product **III; III** in turn is further degraded as discussed above, to ketol **IV** and veratraldehyde.

Oxidation of Phenols. Another product, guaiacol, was expected to be formed indirectly (see *Discussion*) from the β-ether-linked aromatic moiety of both models **II** and **V,** but it was not detected. Further study revealed that guaiacol is rapidily oxidized to inidentified colored products, suggesting that oxidative coupling and polymerization occur. That such reactions do result from the enzyme action was shown with 4-*tert*-butylguaiacol, which is divereized (via oxidative radical coupling), forming predominantyl the 6,6′-dehydrodimer **(IX).**

To facilitate detection of the suspected phenolic product from β-*O*-4 models, we prepared model **VI** labeled with tritium in the ß-ether-linked vanilly alcohol moiety. Reaction with the enzyme resulted in formation of [^{3}H] vanillin. No vanillyl alcohol was detected; evendently it was oxidized to vanillin before or after C_α—C_β cleavage in a reaction analogous to the oxidation of the benzyl alcohols.

Enzymatic cleavage of model **II** is shown in Fig. 5.

Oxidation of Benzyl Alcohols. The *ß-O*-4 model **II** undergoes another reaction in addition to C_α—C_β cleavage: it is oxidized at C_α to form the corresponding ketone. This reaction is analogous to the oxidation of veratryl alcohol; it accounts for 15% of the model **II** oxidized. In addition to veratryl alcohol, the 4-methoxy- and 3,4,5-trimethoxy analogues are also oxidized to aldehydes.

DISCUSSION

The ligninase has spectral properties similar to those of the hemoproteins catalase, horseradish peroxidase, and *c*-type cytochromes (20). The spectrum of the pyridine hemochromogen complex, in conjunction with the metal analysis of the enzyme, indicates one protoheme **IX** per enzyme molecule. The spectrum of the H_2O_2-treated enzyme is similar to that of the two-electron-oxidized compound **I** intermediate of catalase and peroxidase in exhibiting a similar decrease in absorbance. The enzyme shows no catalase activity; however, like peroxidase, it oxidizes phenols in the presence of H_2O_2. Like catalase (21), the enzyme does not undergo any spectral changes upon the anaerobic addition of dithionite. However, the fact that dithionite as well as organic substrates shift the absorption spectrum of the H_2O_2-treated enzyme back to that of the resting enzyme suggests that the reaction sequence involves oxidation of the enzyme by H_2O_2 followed by oxidation/oxygenation of substrates.

The enzyme catalyzes a variety of oxidations: (i) C_α—C_β cleavage of *ß*-1 and *ß-O*-4 models; (*ii*) oxidation of benzyl alcohols: (*iii*) oxidation of phenols leading to radical coupling; (*iv*) hydroxylation of certain benzylic methylene groups; and (*v*) intradiol cleavage of phenylglycol structures. All of these reactions were discovered in studies with *ß*-1 and *ß-O*-4 models. The predominant reaction in oxidation of these models is cleavage of the C_α—C_β bond. Hydroxylation at C_β probably occurs simultaneously with, or after cleavage, as it does in cultures (7).

The complete cleavage of models **I** and **II,** under substrate-limiting conditions, demonstrates an absence of stereospecificity in the catalysis. These synthetic models each contain two chiral carbons (C_α and C_β); thus both models consist of a mixture of four stereoisomers. Their complete cleavage is significant because lignin is not a stereoregular polymer (22).

Our most recent results (not shown) have revealed that the enzyme also catalyzes the hydroxylation of C_α and C_β of the C_α=C_β olefinic bond in styryl structures, including 3,4-dimethoxycinnamyl alcohol, to produce the corresponding phenylglycol products [1-(3,4-dimethoxyphenyl)glycerol from the cinnamyl alcohol]. This latter reaction, and the intradiol cleavage reaction (as in **III**), were reported by Glenn *et al.* (23) for the crude culture filtrate of ligninolytic cultures.

Results here indicate that *ß-O*-4 models are cleaved by the same mechanism as the *ß*-1 models. We propose that model **II,** like the *ß*-1 models, is hydroxylated at C_β during cleavage io yield a hemiacetal intermediate, which is analogous to product **III** from the *ß*-1 models. This intermediate spontaneously decomposes to glycolaldehyde and guaiacol (Fig, 5*B*). This interpretation is based on identifying phenylacetaldehyde from the cleavage of model V and identifying tritiated vanillin from cleavage of model VI. The rapid exchange between the carbonyl oxygen in phenylacetaldehyde and the oxygen of water precluded our establishing C_β-hydroxylation from $^{18}O_2$ by using model V. The experiment with model V under $^{18}O_2$ did reveal aromatic methylene hydroxylation with O_2-derived oxygen; thus [^{18}O]benzaldehyde was identified as a product.

An unexplained association of phenol oxidation with lignin degradation has long been recognized (24). H_2O_2-dependent phenol-oxidizing activity has been reported in several lignin-degrading fungi (25). Results here help resolve this puzzling association by demonstrating that phenol oxidation is catalyzed by peroxidase activity intrinsic to the ligninase.

In this report we have described the purification, characterization, and catalytic properties of a ligninase. This oxygenase is unique in requiring H_2O_2 for activity. The enzyme has characteristics required for lignin degradation: it is extracellular, allowing it to contact the lignin polymer; it has low substrate specificity, in keeping with the heterogeneity and nonstereoregularity of its substrate, lignin; and it is oxidative, allowing it to degrade a poorly hydrolyzable substrate.

Note Added In proof. We have now shown that the ligninase is a glycoprotein (approximately 13% by weight carbohydrate). The M_r of 42,000 as determined by gel electrophoresis may be high.

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