Characterization of an Extracellular Lignin Peroxidase of the Lignocellulolytic Actinomycete Streptomyces viridosporus†

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Previously we reported production of an extracellular lignin-inducible peroxidase by Streptomyces viridosporus (M. Ramachandra, D. L. Crawford, and A. L. Pometto III, Appl. Environ. Microbiol. 53:2754–2760, 1987). This peroxidase was shown to oxidize 3,4-dihydroxyphenylalanine, 2,4-dichlorophenol, homoprotocatechuic acid, caffeic acid, and N,N,N',N'-tetramethylphenylenediamine and was found in higher than normal levels in strains enhanced for lignocellulose degradation. In the present study, we used a pure extracellular enzyme preparation with high peroxidase isoform F3 activity to oxidize lignin substructure model compounds of both the 1,2-diaryl propane and arylglycerol-β-aryl ether types and containing C6-carbonyl and C6′-hydroxy groups. The reactions were monitored by gas chromatography-mass spectrometry and high-pressure liquid chromatography techniques. In the presence, but not the absence, of hydrogen peroxide, the enzyme preparation catalyzed C6-C6′ bond cleavage in the side chains of the diaryl ethers 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol (I) and 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propan-1-one (II) and the diaryl ethane 1-(4-methoxyphenyl)2-(phenyl)ethan-1-one (III). Rapid hydrogen peroxide consumption was observed when the enzyme preparation was added to either milled corn lignin or lignocellulose. Additional characterizations showed that this enzyme is a heme protein (Soret band, 408 nm) and a major component of the ligninolytic system of S. viridosporus T7A. This is the first report of a lignin peroxidase in a bacterium. We have designated this new lignin peroxidase as ALIP-P3.

Lignin is a complex polymer consisting of phenylpropane units interconnected by a variety of carbon-carbon bonds and ether linkages (1). In nature, lignin physically encrusts cellulose and is resistant to degradation by most microorganisms (21). Streptomyces viridosporus T7A (ATCC 39115) depolymerizes lignin while degrading cellulose (6) and produces a modified water-soluble, acid-precipitable polymeric lignin (APPL) as a major lignin degradation product (7). A similar degradative mechanism has been reported in another actinomycete, Thermomonospora mesophila (26). Along with APPL, the release of several single-ring aromatic phenols occurs during lignocellulose degradation by Streptomyces species (7).

In the white rot fungus Phanerochaete chrysosporium, the enzymology of lignin degradation is partially understood (21, 27, 36); extracellular lignin peroxidases are thought to catalyze the initial catabolism of lignin (21, 22, 27, 28, 36–38). In a previous publication (32), we proposed the involvement of an extracellular, lignin-inducible peroxidase in lignocellulose degradation by Streptomyces spp. The peroxidase catalyzes hydrogen peroxide-dependent oxidation of various phenolic compounds and is found in higher than normal levels in strains enhanced for lignin degradation. The finding that APPL-overproducing strains also overproduce peroxidase (32) is indirect evidence implying that peroxidase plays a role in lignin biodegradation.

Various lignin substructure model compounds have been used successfully to elucidate fungal lignin peroxidase reaction mechanisms (4, 21). Lignin substructures of the 1,2-diaryl propane (β-1) and arylglycerol-β-aryl ether (β-O-4) types together represent nearly 60% of intermonomeric linkages in a typical softwood lignin (10). Cleavage of C6-C6′ side chain bonds in lignin may be the most important reaction involved in its initial degradation (4). For catalyzing such a reaction, lignin peroxidase of P. chrysosporium requires the presence of a C6-hydroxyl group (4, 14, 22, 34, 38). Chemical characterizations of lignin-derived aromatic degradation products of Streptomyces spp. have suggested that catabolism of lignin by the actinomycete involves substantial initial cleavages of the lignin C6-C6′ and β-O-4 ether linkages, concomitant with other lignin oxidation reactions (8). This results in an increase in the C6-carbonyl content in partially degraded lignins (25). Reduction of the C6-carbonyls of model dimers by S. viridosporus has also been reported (35). In a mixed ligninolytic bacterial population, C6 oxidation before C6-C6′ cleavage was reported (15). The ability of bacteria to degrade lignin model compounds, natural and synthetic lignins, and chemically polymerized lignins has been established (2, 11, 15, 18, 19, 27, 35). Recently, in S. cyanus an extracellular protein fraction responsible for the solubilization of lignocellulose has been reported (24). However, no specific enzyme(s) involved in lignin biodegradation by any bacterium has been described.

In this study, we determined how selected β-O-4 and β-1 type lignin substructure model compounds containing C6-hydroxy or C6′-carbonyl groups were degraded by a purified, extracellular peroxidase preparation from S. viridosporus T7A. The results showed oxidative cleavage of C6-C6′ bonds of both types of model compounds in the presence of hydrogen peroxide. Further characterization indicated that this heme enzyme is a major component of the ligninolytic system of S. viridosporus.

MATERIALS AND METHODS

Culture conditions. S. viridosporus T7A (ATCC 39115) stock cultures were maintained at 4°C on yeast extract-malt extract-glucose agar (32). Spores from stock slants were

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inoculated into 2-liter flasks containing 1 liter of 0.6% (wt/vol) yeast extract (Difco Laboratories, Detroit, Mich.) in mineral salt solution (31) plus 0.5% larchwood xylan (Sigma Chemical Co., St. Louis, Mo.) (YMX) and grown for 3 days at 37°C with shaking at 125 rpm.

**Enzyme purification.** After 3 days of growth, culture supernatant solutions were harvested by filtration through glass wool. Proteins in the filtrate were concentrated 10-fold by ultrafiltration and then precipitated with ammonium sulfate (70% saturation). The precipitate was then suspended in 30 ml of 20 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 5.5). This preparation was loaded onto a Sepharose CL-6B-200 (Pharmacia Inc., Piscataway, N.J.) gel filtration column (bed volume, 150 cm³) and chromatographed at a flow rate of 0.25 ml/min with MES buffer as the eluent. After chromatography, the fractions exhibiting peroxidase activity were pooled and concentrated again by ultrafiltration.

**Enzyme assay.** Peroxidase activity was routinely assayed with 2,4-dichlorophenol (2,4-DCP) (Sigma) as the substrate (17). A final volume of 1.0 ml of the reaction mixture contained 100 mM sodium succinate buffer (pH 5.5), 82 mM 4-aminoantipyrine (Sigma), 1.0 mM 2,4-DCP, 4.0 mM hydrogen peroxide, and 100 µl of the enzyme preparation. The reaction was initiated by addition of hydrogen peroxide, and the increase in A₅₁₀ was monitored for 1 min at 37°C. One unit of enzyme activity was expressed as the amount of enzyme required for an increase in absorbance of 1.0 U/min. Peroxidase activity was also assayed with 1-3,4-dihydroxyphenylalanine (l-DOPA; Sigma) (32) and veratryl alcohol (20) as substrates.

Catalase activity was assayed (9) by measuring oxygen production at 37°C with an oxygen electrode upon addition of 10 µl of 10 mM hydrogen peroxide to 1.0 ml of a reaction mixture containing 100 µl of the enzyme and 100 mM sodium succinate buffer (pH 5.5).

**PAGE and peroxidase staining on gels.** Proteins in the enzyme preparation were analyzed by nondenaturing, discontinuous polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)-PAGE on vertical slab gels (7.5% polyacrylamide) (16, 32). After electrophoresis, protein bands were visualized by silver staining (16). Peroxidase bands on nondenaturing PAGE gels were developed by activity staining with l-DOPA, 2,4-DCP, caffeic acid, homoprotocatechuic acid, and N,N,N',N'-tetramethylphenylenediamine (all from Sigma) as substrates (32).

**HPLC of proteins.** Proteins in the purified and crude enzyme preparations were analyzed on a Hewlett-Packard 1090A high-pressure liquid chromatograph (HPLC) equipped with an HP-1040A diode array detector using a pharmacia fast-protein liquid chromatography Mono Q anion-exchange column (20). The mobile phase consisted of a gradient of 10 mM to 1 M sodium acetate, pH 6.0, over a period of 40 min at a flow rate of 1 ml/min. The peaks were monitored at 280 and 409 nm, and the absorbance spectrum (250 to 600 nm) of each peak was recorded.

**Lignin substructure model compounds.** 1-[(3,4-Dimethoxyphenyl)-2-(2-methoxyphenoxo)propane-1,3-diol (I) was prepared by sodium borohydride reduction of 1-[(3,4-dimethoxyphenyl)-3-hydroxy-2-[(2-methoxyphenoxo)propan-1-one, which was synthesized as described by Landucci et al. (23). The model compounds 1-(4-methoxyphenyl)-2-(phenylethyl)an-1-one (III) and 1-(4-hydroxy-3-methoxyphenyl)-2(2-methoxyphenoxo)propan-1-one (II) were kindly provided by R. L. Crawford (Department of Bacteriology and Biochemistry, University of Idaho, Moscow).

**Oxidations of lignin model compounds.** The model compounds were added to reaction mixtures at a final concentration of 0.02% (wt/vol). Reactions were performed in a total volume of 3.0 ml containing 0.1 mM hydrogen peroxide or a peroxide-generating system consisting of 0.02 U of glucose oxidase (Sigma) per ml-3 mM glucose (14), 0.1 M sodium tartrate buffer (pH 5.5), and 300 µl of the enzyme preparation. Control reactions were performed in a boiled enzyme preparation. Some reaction mixtures were flushed with oxygen, and all were incubated at 37°C for 18 h on a shaker in slanting tubes. The reaction mixtures were then acidified to pH 2 to 3 with 12 M HCl and extracted once with ether and once with ethyl acetate (29). Specific products were identified by gas chromatography (GC)-mass spectrometry (MS) by comparing retention times with those of authentic standards, by examination of their MS fragmentation patterns alone when authentic standards were unavailable, or by HPLC.

**Chromatographic analysis.** For GC, trimethylsilyl derivatives of extracted samples were prepared with 100 µl of p-dioxane-10 µl of pyridine-50 µl of N,O-bis(trimethylsilyl) acetamide. Each sample was kept at 35°C for 2 h before injection. GC was performed on a Hewlett-Packard 5890 GC with a flame ionization detector and an HP Ultra 2 capillary column (30 m by 0.2 mm) (Hewlett Packard Co., Santa Clara, Calif.). Column conditions were as follows. The oven temperature was 120°C for 2 min, followed by a 15°C/min gradient to 280°C, which was maintained for 15 min. The injector temperature was 240°C, and the detector temperature was 280°C. Mass spectral analysis was performed on a VG-7070 HG mass spectrometer (VG International) at 70 eV, which was coupled to the GC.

HPLC was performed on a Hewlett-Packard 1090A HPLC with an HP-1040A diode array detector (29). During each run, chromatograms for 258, 280, and 310 nm were recorded and the UV absorbance spectrum (250 to 350 nm) of each peak was recorded at its front side, apex, and trailing side. A 100-mm Hewlett-Packard microbore reverse-phase column of Hypersil ODS with a 5-µm particle diameter was used with a 40°C column temperature, a 5-µl sampling loop, and a 0.4-ml/min flow rate. The gradient used for solvent delivery was a mobile phase consisting of water adjusted to pH 3.2 with H₂SO₄ and acetoni trile. The percentage of acetonitrile was 10% for 2 min, which was increased to 50% over the next 10 min and 100% over the following 5 min. The mobile phase was at 100% acetonitrile for the final 15 min; the total run time was 30 min. Products were identified by their specific retention times and comparison of UV spectra with those of available standard compounds.

**Peroxidase reaction with MCL and lignocellulose.** The enzyme preparation (300 µl) was added to a 3.0-ml reaction mixture containing either 10 mg of (i) milled corn lignin (MCL) (7, 10), extracted corn stover lignocellulose (7), and cellulose (Whatman cellulose powder; W and R Balston Ltd.), or (ii) no substrate, 0.1 M sodium succinate buffer (pH 5.5), and 1 mM hydrogen peroxide. The reaction mixtures were next incubated at 37°C, while hydrogen peroxide consumption was determined at regular intervals as described by Frew et al. (13). The above-described reaction mixtures, without addition of the enzyme, served as controls. At different time intervals, a known volume (100 µl) of the reaction mixture was added to 4-aminoantipyrine-phenol reagent (13) and the A₅₉₅ of the resulting solution was measured. Hydrogen peroxide content was calculated from a standard curve.
FIG. 1. PAGE analysis of peroxidase isoforms in concentrated culture filtrates. Activity staining was performed with L-DOPA as the substrate. Only isoform P3 reacted with 2,4-DCP.

RESULTS

After growth of S. viridosporus for 3 days in YMX medium, extracellular peroxidase-specific activity of about 0.30 U/mg of protein was typically observed in unconcentrated culture filtrates. This was much higher than maximum peroxidase activities in the same medium with larchwood xylan (0.15 U/mg of protein after 4 days) in medium supplemented with 0.5% corn stover lignocellulose (0.24 U/mg of protein after 3 days) or in medium with oat spelt xylan (0.14 U/mg of protein after 3 days). PAGE, followed by activity staining for peroxidase, revealed a higher level of peroxidase isoform P3 (Fig. 1) in media with xylan than in media with lignocellulose. Hence, YMX was used as the medium of choice for large-scale production of peroxidase.

Figure 1 shows the presence of four peroxidase isoforms from crude, concentrated supernatant fluids when PAGE gels were stained for peroxidase activity with L-DOPA. None of the bands were seen when staining was performed with reaction mixtures containing no hydrogen peroxide. Among the four isoforms, only isoform P3 was also active against 2,4-DCP, homoprotocatechuc acid, caffeic acid, and N,N,N',N'-tetramethylphenylenediamine. PAGE gel activity staining with L-DOPA as the substrate also showed only P3 in the purified enzyme preparation, as opposed to all four in the original culture fluid.

Purification of peroxidase. A 1,000-ml volume of a 3-day YMX-grown culture supernatant fluid was concentrated 10-fold by ultrafiltration and then subjected to ammonium sulfate precipitation (70% saturation). The precipitate was redissolved in 30 ml of 20 mM MES buffer (pH 6.0). This concentrated preparation was applied to a Sepharose CL-6B-200 gel filtration column (bed volume, 150 cm³). Proteins were eluted from the column with 20 mM MES buffer (pH 6.0). The elution profile of the peroxidase from the gel filtration column is shown in Fig. 2. Only the major protein peak exhibited peroxidase activity. Fractions containing the major peak were pooled and concentrated again by ultrafiltration.

By following this purification procedure, an enzyme preparation with a 36-fold increase in specific activity was obtained. The specific activities in the enzyme preparation, as determined by spectrophotometric assays with 2,4-DCP and L-DOPA as substrates, are presented in Table 1. Without hydrogen peroxide in the reaction mixtures, the specific peroxidase activities in the enzyme preparations were negligible (0.005 and 0.000 U/mg of protein with 2,4-DCP and L-DOPA, respectively). Both 2,4-DCP and L-DOPA oxidizing activities were inhibited with known peroxidase inhibitors, such as potassium cyanide and sodium azide (Table 1). Similar inhibition of activity was noticed during activity staining of PAGE gels. The purified enzyme preparation exhibited a positive but relatively low specific activity of 0.03 U (micromoles of veratraldehyde formed per minute)/mg of protein with veratryl alcohol. The enzyme preparation did not catalyze production of oxygen from hydrogen peroxide in catalase assays. In contrast, the crude concentrated culture supernatants exhibited low levels of catalase activity.

Silver staining of SDS-PAGE gels revealed that the purified peroxidase preparation contained one protein band with one very low-molecular-weight polypeptide impurity (Fig. 3). The polypeptide traveled with the dye front. A molecular weight of 17,800 was estimated for the enzyme by SDS-PAGE.

HPLC analysis of proteins. The absorption spectrum of the single, pure peroxidase eluted from the column was recorded with the diode array detector. The absorption spectrum of this active enzyme exhibited two distinct peaks at 280 and 408 nm (Fig. 4).

Model compound oxidation. Figure 5 shows the lignin substructure model compounds which were oxidized by peroxidase and the products that were formed. Only vera-

![Graph showing elution profile of proteins from a Sepharose CL-6B gel filtration column.](image)

**FIG. 2. Elution profile of the proteins from a Sepharose CL-6B gel filtration column. A₂₈₀ values (A) indicate protein contents in the fractions (2.5 ml each). Peroxidase activity (○), as indicated by A₅₁₀ values, was assayed in each fraction with 2,4-DCP as the substrate. Samples (100 μl each) from each fraction were added to 900 μl of the assay mixture (see Materials and Methods) and incubated for 10 min at 37°C, and the A₅₁₀ was recorded.**

<table>
<thead>
<tr>
<th>TABLE 1. Inhibition of peroxidase P3 activities by cyanide and azide</th>
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<td><strong>Treatment</strong></td>
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<td>----------------</td>
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<tr>
<td></td>
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<tr>
<td>No inhibitor (control)</td>
</tr>
<tr>
<td>Potassium cyanide</td>
</tr>
<tr>
<td>Sodium azide</td>
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* Inhibitors were added at a final concentration of 1 mM.
* Protein content was determined by the Lowry method (28). Values in parentheses indicate percent inhibition.
tramaldehyde, anisaldehyde, and vanillin were detected by HPLC, GC, and GC-MS. Guaiacol, formed in trace amounts, was detected only by GC and GC-MS. Small amounts of products IV and V were detected only by MS fragmentation. Minor peaks observed with oxidations of all three model compounds were suspected to be products derived from the ring proximal to C₆. None of the above-described products were detected in the original substrate. The β-aryl ether dimer I was cleaved at the C₆-C₈ bond to yield veratraldehyde (VI) and guaiacol (VIII). Vanillin (X) and guaiacol were formed from the diaryl ether (II), indicating similar C₆-C₈ cleavages. The diaryl ethane (III) was cleaved between the C₆ and C₈ carbons to yield p-anisaldehyde (XIII) as a major product. Mass spectra of model compound oxidation products are presented in Table 2.

Oxidations of the above-described lignin substructure model compounds occurred with or without flushing of

![Image](http://aem.asm.org/)

**FIG. 3.** Protein bands on an SDS-PAGE gel. Protein bands were developed by silver staining. The bands in the right two lanes indicate proteins present in the purified enzyme preparation, and the bands in the leftmost lane are molecular weight standards.

**TABLE 2.** Mass spectra of major products obtained from oxidation of lignin model compounds by peroxidase

<table>
<thead>
<tr>
<th>Product</th>
<th>m/z (intensity in %)&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Anisaldehyde (XIII)</td>
<td>136 (M⁺, 10.0), 135 (100), 107 (10.3), 92 (26.9), 77 (34.2)</td>
</tr>
<tr>
<td>Benzyl alcohol (XIV)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>180 (M⁺, 17.3), 165 (35.4), 164 (100), 149 (16.6), 136 (35.3), 135 (15.2), 107 (45.2), 91 (67.3), 82 (16.9), 77 (17.2), 73 (TMS⁺, 69.1)</td>
</tr>
<tr>
<td>Guaiacol (VIII)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>196 (M⁺, 0.6), 180 (0.3), 165 (3.0), 149 (1.5), 135 (42.8), 91 (6.2), 77 (20.2), 73 (TMS⁺, 100)</td>
</tr>
<tr>
<td>Vanillin (X)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>224 (M⁺, 10.1), 223 (100), 209 (4.4), 179 (4.0), 165 (8.4), 77 (11.6), 73 (TMS⁺, 12.6)</td>
</tr>
<tr>
<td>Veratraldehyde (VI)</td>
<td>166 (M⁺, 100), 165 (40.4), 151 (17.2), 135 (22.1), 121 (2.5), 95 (52.4), 77 (29.9)</td>
</tr>
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</table>

<sup>a</sup> M⁺, Molecular ion; TMS⁺, trimethylsilyl ion.

<sup>b</sup> As a trimethylsilyl derivative.

**FIG. 4.** Absorption spectrum of the purified peroxidase preparation. mA, Milliabsorbance units.

**FIG. 5.** Proposed pathways for the degradation of model compounds by peroxidase ALiP-P3. The compounds designated in parentheses were not identified.
TABLE 3. Rate of hydrogen peroxide consumption during peroxidase P3 reaction with different lignocellulosic substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hydrogen peroxide consumption (μmol/mg of enzyme per min)*</th>
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<tbody>
<tr>
<td></td>
<td>With enzyme</td>
</tr>
<tr>
<td>Lignocellulose</td>
<td>1.51</td>
</tr>
<tr>
<td>MCL</td>
<td>0.75</td>
</tr>
<tr>
<td>Cellulose</td>
<td>0.76</td>
</tr>
<tr>
<td>None</td>
<td>0.40</td>
</tr>
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</table>

* The rate of hydrogen peroxide consumption was measured by a spectrophotometric assay as described in Materials and Methods.

oxgen through the reaction mixture, which was incubated on a shaker. Hydrogen peroxide was necessary for the reaction. Addition of hydrogen peroxide twice (once at time zero and once after 9 h) during the incubation period or generation of hydrogen peroxide by means of the glucose-glucose oxidase system yielded similar results. Enzyme activity was tested with acetate, tartrate, and succinate buffers at pH 3.5 to 6.5. Among these buffers, sodium tartrate buffer (pH 5.5) was superior for model compound oxidations, which did not occur above pH 5.5.

**Oxidation of lignin.** The enzyme preparation was incubated with extractive-free corn stover lignocellulose, MCL, or pure cellulose under the same conditions used for enzyme assays. The rate of hydrogen peroxide consumption during reactions was monitored as an indirect measurement of the oxidation of lignin structures (Table 3). Hydrogen peroxide was rapidly utilized when lignocellulose was present in the reaction mixture. Hydrogen peroxide consumption was also higher in the presence of MCL and cellulose than in the absence of a substrate and was at very low levels in control mixtures containing substrates but lacking an enzyme.

**DISCUSSION**

The culture conditions used in this study are different from those we used in our previous reports (29, 32). Addition of larchwood xylan to yeast extract-mineral salts medium enhanced extracellular 2,4-DCP peroxidase activity. Use of such a non-lignin inducer enabled us to obtain a culture supernatant that did not contain APPL and other water-soluble, partially degraded lignin polymers. These polymers interfere with enzyme assays and complicate purification. Interestingly, when xylan from oat spelt (Sigma) was used, there was no increase in specific activity. This indicates that the actual lignin peroxidase inducer is an impurity (aromatic?) present in larchwood xylan. In another ligninolytic actinomycete, *S. cyaneus*, such an induction of an extracellular protein(s) responsible for the degradation of lignocellulose was noticed in the presence of ball-milled straw (24).

In our previous report (32), 1-DOPA was used to measure peroxidase activity. 1-DOPA was readily oxidized by all four peroxidase isoforms. In the present study 2,4-DCP was used in developing a modified peroxidase assay which was highly sensitive (Table 1) and specific for peroxidase isoform P3. The assay with 2,4-DCP can be used to detect activity in unconcentrated culture supernatant solutions and fractions from chromatography columns (Fig. 2). Therefore, it is preferred over the veratryl alcohol oxidation assay because of its sensitivity. The reagents for this assay also can be stored for a longer period of time than 1-DOPA solutions.

Among four peroxidase isoforms produced by *S. viridosporus* (Fig. 1), only isoform P3 was found at elevated levels when cultures were grown in media supplemented with either lignocellulose or larchwood xylan and in culture supernatant of the APPL-overproducing protoplast fusion recombinant SR-10 (32). Also, because of its inducibility and its wider substrate specificity, the involvement of this isoform in oxidizing lignin and lignin substructure model compounds was considered likely. Hence, an enzyme preparation active only in isoform P3 was used in assays to examine the oxidation of lignin substructure model compounds and lignin preparations. These experiments confirm that the peroxidase is a lignin peroxidase.

By denaturing SDS-PAGE, a molecular weight of 17,800 was estimated for the peroxidase. Interestingly, another laboratory has recently reported that a low-molecular-weight protein of *S. cyaneus* (apparent molecular weight, 20,000) catalyzes the solubilization of [*4C]*lignin in [*14C]*lignin-labeled lignocelluloses (24). The enzymatic properties of that protein have not been reported in detail. Such relatively low molecular weights were estimated by SDS-PAGE for other peroxidases (3).

Peroxidase P3 was inhibited by known heme protein inhibitors (Table 1). The absorption spectrum of the enzyme showed distinct peaks at 280 and 408 nm (Fig. 4), which are also characteristic of heme proteins (33, 36). The absorption spectrum is remarkably similar to that of a peroxidase purified from *Escherichia coli* (5). These observations strongly suggest that this lignin peroxidase is a heme protein.

The proposed pathways for degradation of model compounds used in this study are shown in Fig. 5. The β-O-4 model compound (I) was cleaved at a Cα-Cβ bond in a manner similar to that seen with the lignin peroxidase of *P. chrysosporium* (22, 33, 34, 38). Compound I was probably hydroxylated at Cα during cleavage to give the hemiacetal intermediate (compound VII), which spontaneously decomposed to glycolaldehyde (compound IX) and guaiacol (compound VIII) (22). Both the diaryl ether and diaryl ethane derivatives (compounds II and III) containing Cα-carboxyls were either reduced at the Cα position first to obtain compounds IV and V, respectively, or directly cleaved at the Cα-Cβ bonds. Reduction of the carbonyl groups of 1,2-diarylethane model compounds by *S. viridosporus* T7A has been shown previously (35). Jokela et al. (18) have also reported such Cα oxidation before Cα-Cβ cleavage by a mixed bacterial population. This is in contrast to the action of lignin peroxidase of *P. chrysosporium*, which readily cleaves similar compounds only if they contain a hydroxyl group at the Cα position (4, 14, 22). The Cα-carboxyl groups render aromatic nuclei resistant to oxidation by fungal lignin peroxidase (21). Kirk et al. (22) proposed the involvement of other enzymes in the degradation of such carbonyl-bearing structures by *P. chrysosporium*. Degradation of such Cα-carboxyl-containing compounds during actinomycete-mediated lignin degradation is significant because the Cα-carboxyl content of the lignin increases during degradation by both *Streptomyces* spp. and *Thermomonospora* spp. (11, 25, 26). Enhanced carbonyl and free phenolic hydroxyl groups on APPL and the residual decayed lignin (7) also support this cleavage pattern.

Model compound oxidation were performed in a buffer of pH 5.5 at 37°C. These conditions were ideal for both 2,4-DCP and model compound oxidations. These results agree with our previous findings that relatively acidic pH conditions were optimal for lignin mineralization by *S. viridosporus* (30). Hydrogen peroxide was absolutely necessary for the reactions. A mixture of glucose oxidase and glucose could be used to provide a continuous low concen-
tration of hydrogen peroxide. However, addition of hydrogen peroxide, once to initiate the reaction and once more halfway through the reaction, resulted in similar activity.

Rapid consumption of hydrogen peroxide in the presence of the enzyme and lignocellulose indicates that the peroxidase oxidizes lignocellulose to a greater extent than MCL and cellulose. The relatively low amount of hydrogen peroxide consumption without a substrate could be attributed to hydrogen peroxide-dependent conversion of the enzyme to a higher oxidation state (36). Since the pure enzyme showed no catalase activity in a specific catalase assay, hydrogen peroxide consumption by catalase rather than peroxidase was ruled out. In addition, an active enzyme plus a lignin substrate led to significant hydrogen peroxide uptake, while none of the substrates consumed comparable levels of hydrogen peroxide in the absence of an enzyme.

In *Streptomyces* and other genera of actinomycetes, lignin degradation occurs during primary growth and is presumed to be the result of primary metabolic activity (25). High concentrations of both organic and inorganic nitrogen do not inhibit lignin degradation (25). Peroxidase production by *S. viridosporus* is also growth associated, is not inhibited by high nitrogen levels, and is subject to glucose catabolite repression (unpublished data). This is in contrast to the ligninolytic system in *P. chrysosporium*, in which both lignin degradation and lignin peroxidase production are secondary metabolic events which follow nitrogen or other nutrient depletion (12).

Ruttimann et al. (35) grew our strain of *S. viridosporus* with dimeric lignin model compounds and found no cleavage of 1,2-diaryl propane or arylglycerol-β-aryl ether structures. Although a hydrogen peroxide-generating aromatic aldehyde oxidase activity has been reported in *S. viridosporus* (9), the results of Ruttimann et al. (35) may be attributable to the absence of an efficient hydrogen peroxide-generating system or suboptimal conditions for peroxidase production.

We propose that this new lignin-oxidizing enzyme of *S. viridosporus* T7A be designated as actinomycete lignin peroxidase P3 (ALiP-P3).

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LITERATURE CITED


