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Oxidation of Methoxylated Benzyl Alcohols by Laccase of \textit{Coriolus versicolor} in the Presence of Syringaldehyde*1

Shingo Kawai*2,3, Toshiaki Umezawa*2 and Takayoshi Higuchi*2

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Abstract—Nonphenolic lignin model monomers, 3,4,5-trimethoxybenzyl alcohol I and veratryl alcohol II were oxidized by laccase of \textit{Coriolus versicolor} in the presence of syringaldehyde. However, when syringaldehyde was not added to the reaction mixture, these substrates I and II were scarcely oxidized. These results indicate that the phenoxy radical of syringaldehyde formed by laccase mediates the oxidation of these nonphenolic substrates.

\textit{Key words}: Biodegradation, \textit{Coriolus versicolor}, laccase, veratryl alcohol, syringaldehyde

1. Introduction

Laccase is commonly distributed in white-rot fungi and is known to cause Bavendamm's reaction. \textit{Coriolus versicolor} (Fr.) Quel. is a powerful lignin degrading fungus and excretes both laccase1) and lignin peroxidase2).

Laccase mediates one-electron oxidation of phenolic substrates to form many degradation products via various pathways3-9), but the enzyme can not oxidize nonphenolic substrate. However, an earlier paper reported that veratrylglycerol-\(\beta\)-guaiacyl ether was converted to its \(\alpha\)-carbonyl derivative by laccase in the presence of spruce MWL3). They concluded that free radicals participated in the oxidation of veratrylglycerol-\(\beta\)-guaiacyl ether. Recently, the formation of guaiacol from the mixture of guaiacoxethanol and syringaldehyde by laccase was reported6).

In the present paper, we report the oxidation of nonphenolic monomers, 3,4,5-trimethoxybenzyl alcohol I and veratryl alcohol II, by laccase of \textit{C. versicolor} in the presence of syringaldehyde and discuss the mechanisms of the oxidation. We further examined the oxidation of nonphenolic \(\beta\)-1 lignin model compounds

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by laccase in the presence of syringaldehyde or bamboo MWL. However, β-1 substrates could not be oxidized under the present experimental conditions.

2. Materials and Methods

2.1 Laccase Preparation

A purified homogeneous laccase preparation from C. versicolor IFO-30340 was kindly provided from Oji Paper Co., Ltd., Japan. It was stored at −20°C as a 50% glycerol solution. The enzyme activity was determined spectrophotometrically by measuring the absorption at 525 nm using syringaldazine (Aldrich Chemical Co., Inc.) as substrate at 30°C.

2.2 Enzyme Reactions of Methoxylated Benzyl Alcohol in the Presence of Syringaldehyde

Substrate, 3,4,5-trimethoxybenzyl alcohol I or veratryl alcohol II (0.5 μmol in 5 μl N,N-dimethylformamide (DMF) solution), and syringaldehyde (2 μmol in 5 μl DMF solution) were incubated in a total volume of 1 ml with the enzyme (15–25 nkat) in 0.2 M acetate buffer (pH 4.0) at 30°C for 30 min under air. In a control experiment, no syringaldehyde was added to the flask. The reaction mixture was extracted with 10 ml ethyl acetate. The organic layer was washed with saturated NaCl solution, dried over anhydrous Na2SO4, and evaporated under reduced pressure. The extract was acetylated with acetic anhydride and pyridine (1/1, v/v) in ethyl acetate for 10 h, and analyzed by gas chromatograph-mass spectroscopy (GC-MS, instrument: Shimadzu GCMS-QP 1000 gas chromatograph-mass spectrometer (EI, 70 eV), column: Shimadzu capillary column Hicap CBP1-W12-100 (methyl silicone), 12 m × 0.53 mm (i.d.), temperature program: Initial temp. at 130°C was held for 2 min, then elevated to 150°C at 5 °C/min.). Degradation products were identified by comparison of the mass spectra (MS) and retention times with those of the authentic compounds.

The amounts of benzaldehydes III and IV formed were calculated quantitatively by stable isotope dilution method. Deuterated internal standard, III-D or IV-D, (2 μg) was added to the flask before extraction, and analyzed by GC-MS.

2.3 Enzyme Reactions of Nonphenolic β-1 Lignin Substructure Model Compounds in the Presence of Syringaldehyde or Bamboo MWL

Reaction conditions of β-1 lignin model compounds V and VI by laccase are listed in Table 1. Reaction mixture was extracted with ethyl acetate (10 ml, twice), and the organic layer was washed with saturated NaCl solution, dried over anhydrous Na2SO4, and evaporated under reduced pressure. The extract was acetylated and analyzed by GC-MS.
Table 1. Reaction conditions of the degradation of nonpheno1ic β-1 substructure compounds V and VI by laccase in the presence of syringaldehyde or bamboo MWL.

<table>
<thead>
<tr>
<th>Nonphenolic compound</th>
<th>Phenolic compound</th>
<th>Medium</th>
<th>Time</th>
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</thead>
<tbody>
<tr>
<td>V</td>
<td>syringaldehyde</td>
<td>acetate buffer pH 4.0</td>
<td>0.5 h</td>
</tr>
<tr>
<td>VI</td>
<td>syringaldehyde</td>
<td>acetate buffer pH 5.3</td>
<td>0.5 h</td>
</tr>
<tr>
<td>VI</td>
<td>syringaldehyde</td>
<td>acetate buffer pH 4.0</td>
<td>0.5 h</td>
</tr>
<tr>
<td>VI</td>
<td>syringaldehyde</td>
<td>tartrate buffer pH 3.0</td>
<td>0.5 h</td>
</tr>
<tr>
<td>V</td>
<td>MWL</td>
<td>dioxane/water pH 4.0</td>
<td>2.0 h</td>
</tr>
<tr>
<td>V</td>
<td>MWL</td>
<td>dioxane/acetate buffer, pH 5.3</td>
<td>3/1</td>
</tr>
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</table>

2.4 Syntheses of Substrates and Authentic and Deutrated Compounds

3,4,5-Trimethoxybenzaldehyde III was prepared from syringaldehyde by methylation with CH₃I and K₂CO₃ in DMF at room temperature. C₂H₃I (99.5%, Merck) was used for the preparation of [4-OC₃H₃]3,4,5-trimethoxybenzaldehyde III-D. III: MS m/z (%) 199 (0), 198 (2.3), 197 (12), 196 (M⁺, 100), 195 (5.5), 181 (41), 125 (19), 110 (14). III-D: MS m/z (%) 200 (13), 199 (M⁺, 100), 198 (8.7), 196 (3.0), 181 (34), 125 (13).

3,4,5-Trimethoxybenzyl alcohol I was obtained by reduction of III with NaBH₄ in methanol at 0°C. MS m/z (%) 241 (15), 240 (M⁺, 100), 198 (43), 197 (12), 193 (17), 181 (67), 169 (19).

[4-OC₃H₃]veratraldehyde IV-D was prepared from vanillin by methylation with C₂H₃I in DMF at room temperature. MS m/z (%) 170 (19), 169 (M⁺, 100), 168 (52), 167 (1.7), 166 (0.4), 98 (15), 95 (12), 79 (11).

Veratral alcohol II, veratraldehyde IV and syringaldehyde were commercially available (Tokyo Chemical Industry Co., Ltd.). II: MS (acetate) m/z (%) 211 (10), 210 (M⁺, 81), 168 (36), 153 (12), 152 (10), 151 (100), 150 (10), 137 (16), 135 (12), 107 (16). IV: MS m/z (%) 169 (0.6), 167 (15), 166 (M⁺, 100), 165 (52), 151 (13), 95 (28), 77 (18).

1,2-Bis(3,4,5-trimethoxyphenyl)-1,3-propanediol V was prepared from 1,2-bis(4-hydroxy-3,5-dimethoxyphenyl)-1,3-propanediol by methylation with diazomethane. 1,2-Bis(4-ethoxy-3,5-dimethoxyphenyl)-1,3-propanediol VI and 2,6-dimethoxy-p-hydroquinone VII and 2,6-dimethoxy-p-benzoquinone VIII were prepared previously. Bamboo MWL was kindly provided by Dr. M. Tanahashi, Wood Research Institute, Kyoto University.
3. Results

3.1 Degradation of 3,4,5-Trimethoxybenzyl Alcohol I and Veratryl Alcohol II

As degradation products of the mixture of 3,4,5-trimethoxybenzyl alcohol I and syringaldehyde by laccase of \textit{Coriolus versicolor}, 3,4,5-trimethoxybenzaldehyde III, hydroquinone VII and benzoquinone VIII were identified by GC-MS analysis. When no syringaldehyde was added to the flask, very little 3,4,5-trimethoxybenzaldehyde III was detected. Then, the quantification of degradation product III was conducted. Figure 1 shows the mass chromatograms of M+ regions of degradation product III (m/z 196) and internal standard III-D (m/z 199). The amount of product III formed in the presence of syringaldehyde was $7.4 \times 10^{-3}$ pmol (1.5 pg, average of experiments 1 and 2). While the amount of III formed in the absence of syringaldehyde was $9.7 \times 10^{-4}$ pmol (0.2 pg).

As degradation products of the mixture of veratryl alcohol II and syringalde-
hyde by laccase, veratraldehyde IV, hydroquinone VII, and benzoquinone VIII were identified. In the control experiment, however, veratraldehyde IV was formed little. Figure 2 shows the mass chromatograms of the M+ regions of degradation product IV (m/z 166) and internal standard IV-D (m/z 169). The amount of product IV formed in the presence of syringaldehyde was $7.6 \times 10^{-3}$ μmol (1.3 μg, average of experiments 1 and 2). While the amount of IV formed in the absence of syringaldehyde was $3.1 \times 10^{-3}$ μmol (0.5 μg).

3.2 Degradation of Nonphenolic β-1 Lignin Substructure Model Compounds

The acetylated degradation products of the mixture of nonphenolic β-1 model compound V and syringaldehyde by laccase were submitted to GC-MS. 3,4,5-Tri-methoxybenzaldehyde III was little formed as a Ca-Cα cleavage product of V, but almost equal amount of III was found in the degradation products of V by laccase in the absence of syringaldehyde. The results obtained on the other experimental conditions in Table 1 were the same.

4. Discussion

Coriolus versicolor excretes laccase1 and lignin peroxidase2. The lignin peroxidase catalyzes both nonphenolic and phenolic lignin substructure models2,3,11 similar to those of Phanerochaete chrysosporium12. On the other hand, it has been recognized that laccase is unable to oxidize nonphenolic lignin dilignols3,5. Lignin macromolecules are composed of phenolic (10~20%) and nonphenolic (80~90%) moieties13,14. The content of phenolic hydroxyl groups could be increased during side chain cleavage by lignin peroxidase12 and laccase4,9. Hence, if laccase or radical intermediates formed by laccase could catalyze the oxidation of nonphenolic moieties of lignin, the degradation rate of lignin macromolecules could be increased.

The present investigation showed that nonphenolic trimethoxybenzyl alcohol I or veratryl alcohol II was oxidized to the corresponding benzaldehyde III or IV by laccase of C. versicolor in the presence of syringaldehyde. These results indicate that the addition of syringaldehyde induces the oxidation of the nonphenolic monomers I and II. Hence, we proposed the oxidation mechanisms of nonphenolic methoxylated benzyl alcohol I and II by laccase in the presence of syringaldehyde as shown in Fig. 3. Laccase mediates one-electron oxidation of syringaldehyde to give the phenoxy radical. Considerable amounts of the phenoxy radical are converted to 2,6-dimethoxy-β-hydroquinone VII and 2,6-dimethoxy-β-benzoquinone VIII. However, it seems that some of the radical mediates one-electron oxidation of nonphenolic monomer I or II to give the corresponding aryl cation radical. The aryl cation radical is converted to benzaldehyde III or IV via several steps.
We further attempted the oxidation of nonphenolic β-1 lignin substructure model compounds V and VI by laccase in the presence of the phenolic substances, syringaldehyde or bamboo MWL. Kirk et al.\(^3\) reported that incubation of veratrylglycerol-β-guaiacyl ether (25 mg), spruce MWL (50 mg), and laccase in acetate buffer (pH 5.0) for 46 h resulted in the formation of a small amount (<1 mg) of its α-carbonyl derivative. However, in the present experiment Ca-Cβ cleavage products, the formation mechanism of which is very similar to that of Ca oxidation products, of β-1 model compounds could not be detected.

Kirk et al.\(^3\) also reported the conversion of veratrylglycerol-β-guaiacyl ether to its α-carbonyl derivative by 2,4,6-triphenylphenoxyl dimer, which dissociated to phenoxy radical monomers, in benzene. These results suggest that the possibility of the oxidation of nonphenolic dilignols by phenoxy radicals formed by laccase is still remained.

**Acknowledgments**

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