Original

Oxidation of Methoxylated Benzyl Alcohols by Laccase of *Coriolus versicolor* in the Presence of Syringaldehyde*1

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Abstract——Nonphenolic lignin model monomers, 3,4,5-trimethoxybenzyl alcohol **I** and veratryl alcohol **II** were oxidized by laccase of *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.

Key words: Biodegradation, *Coriolus versicolor*, laccase, veratryl alcohol, syringaldehyde

1. Introduction

Laccase is commonly distributed in white-rot fungi and is known to cause Bavendamm's reaction. *Coriolus versicolor* (Fr.) Quél. is a powerful lignin degrading fungus and excretes both laccase[1] and lignin peroxidase[2].

Laccase mediates one-electron oxidation of phenolic substrates to form many degradation products via various pathways[3~9], but the enzyme can not oxidize nonphenolic substrate. However, an earlier papar reported that veratrylglycerol-β-guaiacyl ether was converted to its α-carbonyl derivative by laccase in the presence of spruce MWL[3]. They concluded that free radicals participated in the oxidation of veratrylglycerol-β-guaiacyl ether. Recently, the formation of guaiacol from the mixture of guaiacoxyethanol and syringaldehyde by laccase was reported[6].

In the present paper, we report the oxidation of nonphenolic monomers, 3,4,5-trimethoxybenzyl alcohol **I** and veratryl alcohol **II**, by laccase of *C. versicolor* in the presence of syringaldehyde and discuss the mechanisms of the oxidation. We further examined the oxidation of nonphenolic β-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[7,10].

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 Na_2SO_4 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. Deutrated 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 Na_2SO_4, and evaporated under reduced pressure. The extract was acetylated and analyzed by GC-MS.

Table 1. Reaction conditions of the degradation of nonphenolic β-1 substructure compounds **V** and **VI** by laccase in the presence of syringaldehyde or bamboo MWL

Nonphenolic compound	Phenolic compound	Medium		Time
V	syringaldehyde	acetate buffer	pH 4.0	0.5 h
VI	syringaldehyde	acetate buffer	pH 5.3	0.5 h
VI	syringaldehyde	acetate buffer	pH 4.0	0.5 h
VI	syringaldehyde	tartrate buffer	pH 3.0	0.5 h
V	MWL	acetate buffer	pH 4.0	2.0 h
V	MWL	dioxane/water	9/1	2.0 h
V	MWL	dioxane/acetate buffer, pH 5.3	3/1	2.0 h

2.4 Syntheses of Substrates and Authentic and Deutrated Compounds

3,4,5-Trimethoxybenzaldehyde **III** was prepared from syringaldehyde by methylation with CH_3I and K_2CO_3 in DMF at room temperature. C^2H_3I (^2H: 99.5%, Merck) was used for the preparation of [4-OC^2H_3]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 $NaBH_4$ in methanol at 0°C. MS *m/z* (%) 241 (15), 240 (M^+, 100), 198 (43), 197 (12), 193 (17), 181 (67), 169 (19).

[4-OC^2H_3]veratraldehyde **IV-D** was prepared from vanillin by methylation with C^2H_3I 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).

Veratryl alcohol **II**, veratraldehyde **IV** and syringaldehyde were commercailly available (Tokyo Chemical Inductry 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[5] by methylation with diazomethane. 1,2-Bis(4-ethoxy-3,5-dimethoxyphenyl)-1,3-propanediol **VI**[5], 2,6-dimethoxy-*p*-hydroquinone **VII**[7] and 2,6-dimethoxy-*p*-benzoquinone (**VIII**[5] 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 *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 7.4×10^{-3} μmol (1.5 μg, average of experiments 1 and 2). While the amount of **III** formed in the absence of syringaldehyde was 9.7×10^{-4} μmol (0.2 μg).

As degradation products of the mixture of veratryl alcohol **II** and syringalde-

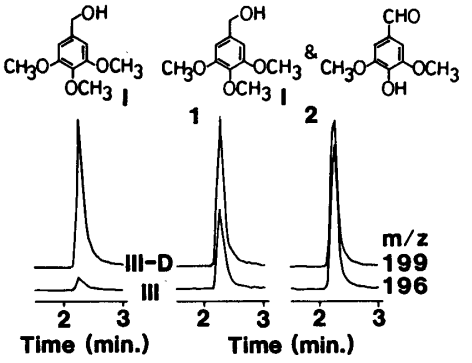

Fig. 1. Mass chromatograms of the M^+ regions of the degradation product **III** (*m/z* 196) and internal standard **III-D** (*m/z* 199). Internal standard (2 μg) was added to the culture before extraction.

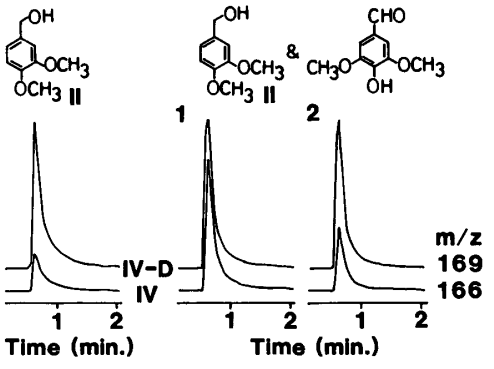

Fig. 2. Mass chromatograms of the M^+ regions of the degradation product **IV** (*m/z* 166) and internal standard **IV-D** (*m/z* 169). Internal standard (2 μg) was added to the culture before extraction.

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 7.6×10^{-3} μmol (1.3 μg, average of experiments 1 and 2). While the amount of **IV** formed in the absence of syringaldehyde was 3.1×10^{-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-Trimethoxybenzaldehyde **III** was little formed as a Cα-Cβ 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 laccase[1] and lignin peroxidase[2]. The lignin peroxidase catalyzes both nonphenolic and phenolic lignin substructure models[2,11] similar to those of *Phanerochaete chrysosporium*[12]. On the other hand, it has been recognized that laccase is unable to oxidize nonphenolic lignin dilignols[3,5]. Lignin macromolecules are composed of phenolic (10~20%) and nonphenolic (80~90%) moieties[13,14]. The content of phenolic hydroxyl groups could be increased during side chain cleavage by lignin peroxidase[12] and laccase[4,9]. Hence, if laccase or radical intermediates formed by laccase could catalyze the oxidation of nonphenolic moieties of lignin, the degradation rate of lignen 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-*p*-hydroquinone **VII** and 2,6-dimethoxy-*p*-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.

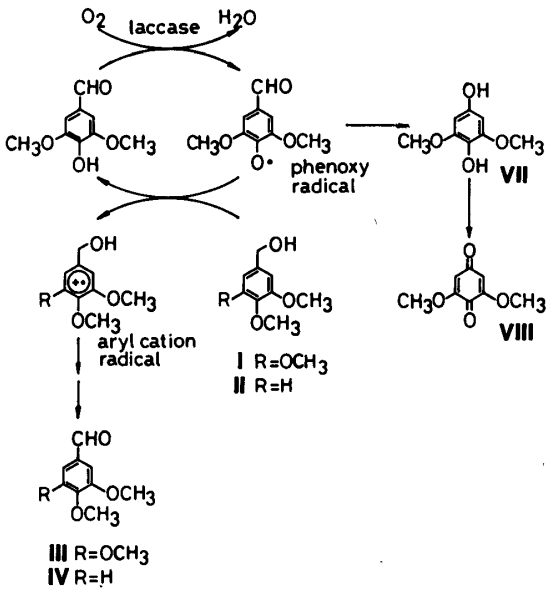

Fig. 3. The oxidation mechanisms of nonphenolic monomers **I** and **II** by laccase of *Coriolus versicolor* in the presence of syringaldehyde.

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 Cα-Cβ cleavage products, the formation mechanism of which is very similar to that of Cα 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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