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Oxidation of syringic acid by extracellular peroxidase of white-rot fungus, *Pleurotus ostreatus*

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The oxidative degradation of syringic acid by the extracellular peroxidase of *Pleurotus ostreatus* was studied. Three products formed in the oxidation of syringic acid by the peroxidase in the presence of O_2 and H_2O_2 were identified as 2,6-dimethoxyphenol, 2,6-dimethoxy-1,4-dihydroxybenzene, and 2,6-dimethoxy-1,4-benzoquinone. A free radical was detected as the reaction intermediate of the extracellular peroxidase-catalyzed oxidation of acetosyringone. These results can be explained by mechanisms involving the production of a phenoxy radical and subsequent decarboxylation. This is the first time that 2,6-dimethoxyphenol has been identified in extracellular peroxidase-catalyzed reactions.

Key Words——extracellular peroxidase; *Pleurotus ostreatus*; syringic acid.

Introduction

Lignin is a complex biopolymer consisting of phenylpropane units interconnected by a variety of carbon-carbon bonds and ether linkages (Freudenberg, 1968). The complex structure of lignin prevents the economical use of plant polysaccharides or the potential use of lignin derivatives as chemical feedstocks. The most effective lignin degraders are the white-rot fungi belonging to the Basidiomycotina. *Phanerochaete chrysosporium* Burds., the most potent lignin degrader, secretes two types of extracellular peroxidase which are thought to constitute the major components of its lignin degradative system. Lignin peroxidase (LiP) has been shown to catalyze a wide variety of reactions important for the degradation of lignin, such as C_α-C_β cleavage, cleavage of β-O-4 ether bonds, oxidation of C_α-carbinol to C_α-oxo compounds, hydroxylation of benzylic methylene groups and styrene olefinic bonds, decarboxylation of phenylacetic acid, and aromatic ring opening (Kirk et al., 1986; Kirk and Farrell, 1987). Manganese peroxidase (MnP) oxidized Mn (II) to Mn (III), and the Mn (III) in turn oxidized monomeric phenols, phenolic lignin dimers, and synthetic lignin via the formation of a phenoxy radical (Glenn and Gold, 1985; Wariishi et al., 1989; Wariishi et al., 1991; Tuor et al., 1992).

In a previous publication, we reported the purification and characterization of an extracellular peroxidase from another white-rot fungus, *Pleurotus ostreatus* (Jacq.: Fr.) Kummer. The enzyme consisted of two identical subunits with a molecular mass of 72 kDa and had affinity towards various phenolic comounds containing methoxyl and *p*-hydroxyl groups, directly attached to the benzene ring. However, the enzyme did not react with veratryl alcohol and showed no affinity for nonphenolic compounds. The catalytic mechanism of the enzyme reaction was of the Ping-Pong type. The enzyme contained 1 mole of iron protoporphyrin IX heme per mole of one subunit of the enzyme (Kang et al., 1993). Although there have been many studies on the oxidation of substructural lignin model compounds, this study was concerned with the mode of action of *P. ostreatus* peroxidase. Syringic acid is representative of phenolic carboxylic acid occurring in the soil, and it is one of the chemicals which contribute to the formation of humic substances (Flaig et al., 1975). It appears as an intermediate in the degradation of lignin by white-rot fungi and serves as a monomeric model for lignin degradation studies (Haider and Trojanowski, 1975; Eriksson et al., 1984). It acted as a good substrate for *P. ostreatus* peroxidase but did not react with horseradish peroxidase (Shin, 1990). So, I determined how syringic acid is degraded by this enzyme.

Materials and Methods

Enzyme preparations Extracellualr peroxidase was extracted and purified from *P. ostreatus* as described previously (Kang et al., 1993). The electrophoretically homogenous enzyme was used.

Chemicals Syringic acid (I), 2,6-dimethoxyphenol (II) and acetosyringone were purchased from Aldrich Chemical Co. 2,6-Dimethoxy-1,4-dihydroxybenzene (III) and 2,6-dimethoxy-1,4-benzoquinone (VI) were prepared as previously reported (Wariishi et al., 1989).

Oxidation of syringic acid Oxidation was carried out at 30°C for 30 min in the air. The reaction mixture contained 5.0 μmol of syringic acid, 10 μg of peroxidase in 5 ml of 20 mM sodium acetate buffer (pH 4.0). Reaction

was run with five sequential additions (every 5 min) of H_2O_2 (0.5 mmol/addition). The reaction mixture was then acidified with 6 N HCl and extracted three times with an equal volume of ethyl acetate. The combined organic phase was dried over anhydrous sodium sulfate, the solvent was evaporated under N_2, and the products were analyzed.

Analytical methods Oxidation products were identified by GC/MS or ^{1}H-NMR spectrometry by comparison of the spectra with those of authentic compounds. GC/MS was performed at 70 eV on a VG-Quattro mass spectrometer combined with HP 5890 Series II gas chromatography (Hewlett Packard) and SPB-1 capillary column (Supelco; 0.32 × 30 m). The temperature was programmed at 120°C for 2 min, followed by a 10°C/min gradient to 280°C, which was maintained for 20 min. ^{1}H-NMR spectra were obtained on a 200.1-MHz nuclear magnetic resonance spectrometer (Varian VXR-200S). UV spectra were taken in 20 mM sodium acetate buffer (pH 4.0) on a Shimadzu UV-265 spectrophtometer. EPR spectroscopic measurement was performed at 255 k in quartz cells with a Bruker ESP 300S EPR system operating at X-band (9.5 GHz) and 100 kHz field modulation. Reaction mixture contained 0.8 μmol of substrate, 0.04 nmol of enzyme, and 0.08 μmol of H_2O_2 in 0.2 ml of 20 mM sodium acetate buffer (pH 4.0). Reaction was started by addition of H_2O_2 and after 1 min the reaction mixture was frozen with liquid nitrogen.

Results and Discussion

UV-visible spectral studies The spectral changes of the reaction mixture of peroxidase/H_2O_2 and syringic acid are shown in Fig. 1. The oxidation resulted in biphasic spectral changes, with the formation of an intermediate having a well defined peak at about 280 nm. Isobestic points at 232, 247 and 276 nm indicate that this intermediate is converted to final products without detectable accumulation of any other additional intermediates. Changes in the visible region of 360-500 nm probably reflect formation of other oxidized products, as *p*-benzo-

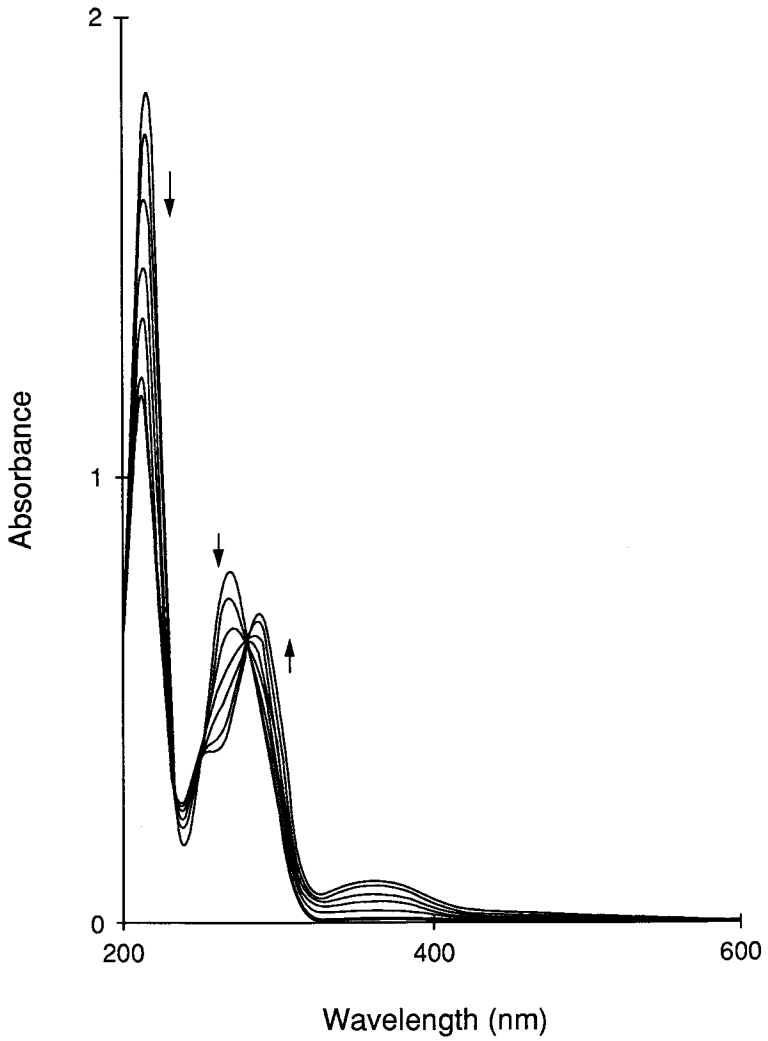

Fig. 1. Spectral changes in syringic acid upon incubation with *P. ostreatus* extracellular peroxidase and H_2O_2. The reaction was initiated by the addition of H_2O_2 and followed in repetitive scans between 600 and 200 nm, with a cycle time of 1 min. Other experimental conditions were described in the text.

Table 1. GC retention times and mass spectra of substrate and reaction products[a].

Compound	GC retention time (min)	Mass spectrum (%)
I	12.0	198 (M^+, 100), 183 (27.3), 127 (44.6), 109 (22.3), 79 (21.3), 67 (30.9), 66 (23.3), 53 (36.0), 45 (41.7), 39 (43.9), 31 (11.5)
II	6.2	154 (M^+, 98.5), 139 (47.5), 111 (48.7), 96 (64.0), 93 (36.2), 79 (25.2), 68 (52.2), 65 (38.8), 55 (33.3), 53 (41.3), 51 (47.1), 50 (31.9), 39 (100), 38 (24.5), 32 (85.8)
III	9.8	170 (M^+, 44.9), 155 (30.1), 127 (39.4), 69 (34.9), 55 (22.7), 53 (23.2), 41 (21.7), 32 (100)
IV	8.5	168 (M^+, 74.3), 138 (25.7), 125 (16.2), 97 (14.3), 80 (46.2), 76 (20.9), 69 (100), 59 (21.4), 53 (26.2), 45 (13.3), 41 (14.7)

[a] Products were analyzed as described in the text. Retention times and mass spectra of reaction products were identical to those of the authentic samples.

quinone shows weak absorbance at about 432 nm. The UV-visible maxima of purified compounds II, III, and IV were 228 and 268, 200 and 278, and 280 and 400 nm, respectively. In the case of control sample without syringic acid, no spectral changes occurred.

Products analyses The reaction products were subjected directly to GC/MS. When syringic acid (I) was oxidized by peroxidase in the presence of oxygen, three reaction products were obtained. Using their mass and ^{1}H-NMR spectral data compared with those of authentic compounds, I assigned compounds II, III and IV the structures 2,6-dimethoxyphenol, 2,6-dimethoxy-1,4-dihydroxybenzene and 2,6-dimethoxy-1,4-benzoquinone, respectively. The mass spectral data and GC retention times are listed in Table 1. The structures of compounds II and IV were confirmed by ^{1}H-NMR spectrometry. ^{1}H-NMR (compound II, $CDCl_3$) δ (ppm): 3.90 (6H, s, $-OCH_3 \times 2$), 5.52 (1H, s, -OH), 6.58 (2H, d, Ar-2H), 6.77 (1H, t, Ar-H). ^{1}H-NMR (compound IV, $CDCl_3$) δ (ppm): 3.82 (6H, s, $-OCH_3 \times 2$), 5.88 (2H, s, proton on quinoid nucleus).

Syringic acid was readily oxidized by peroxidase to produce a variety products via decaboxylation. Harvey et al. (1985) reported that the lignin degradation reactions were brought about by an initial single-electron transfer process. To determine whether a free radical derived from lignin substructures is formed during the oxidation process, I conducted EPR experiments with syringic acid and acetosyringone as substrates. I could not detect a free radical from the syringic acid oxidation process because of its low stability and small amount. The free radical detected in acetosyringone is shown in Fig. 2. The magnetic parameters for the acetosyringone radical are as follows: $a^H = 1.45$ G (6H), $a^H = 1.45$ G (2H), $a^H = 0.31$ G (3H). So, I concluded that extracellular peroxidase of *P. ostreatus* might abstract an electron from the syringic acid to yield the cation radical, and this

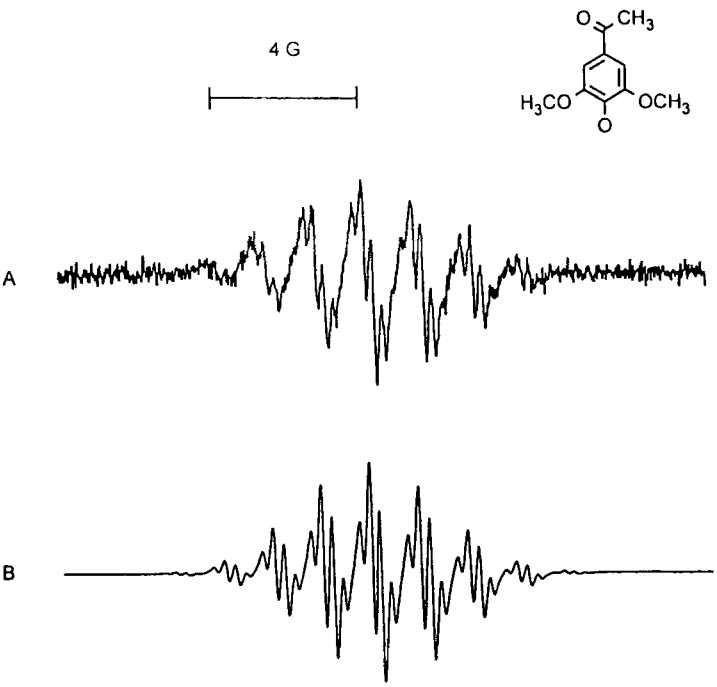

Fig. 2. EPR spectrum of reaction mixture obtained during oxidation of acetosyringone by *P. ostreatus* extracellular peroxidase. (A) shows the EPR spectrum actually obtained, and (B) is a computer simulation.

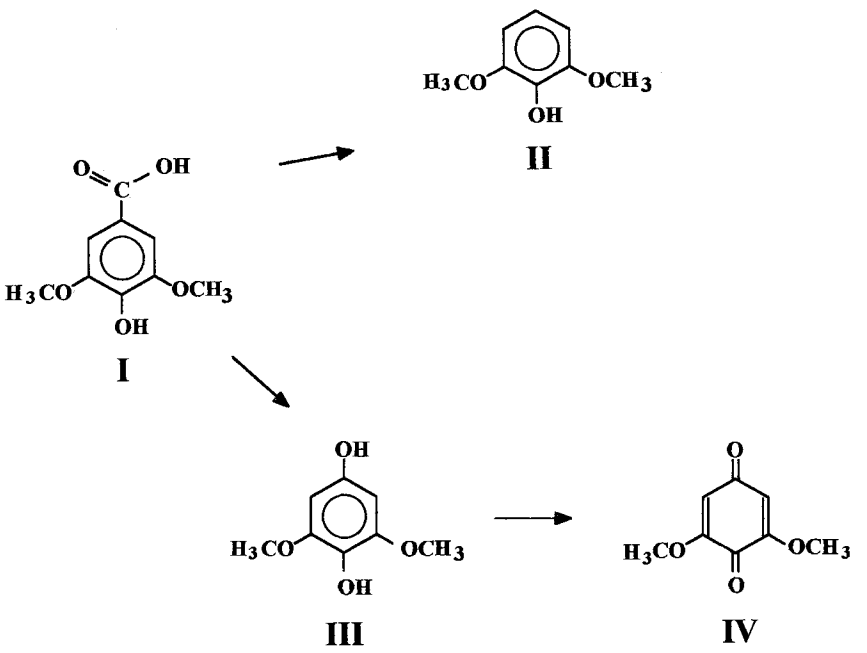

Fig. 3. Proposed sequence of reactions for the degradation of syringic acid by H_2O_2 and extracellular peroxidase of *P. ostreatus*.

radical might produce dimethoxyphenol, dimethoxydihydroxybenzene and dimethoxybenzoquinone. On the basis of these results, I propose the sequences shown in Fig. 3 for the oxidation of syringic acid by an extracellular peroxidase of *P. ostreatus*.

Catabolism of methoxylated lignin monomers normally proceeds via demethylation to protocatechuic acid followed by cleavage of the aromatic ring. White-rot ligninolytic fungus *Phanerochaete chrysosporium* Burdsall oxidized syringic acid to 5-hydroxyvanillic acid, which is metabolized via gallic acid (Higuchi, 1986). Iyayi and Dart (1982) also proposed catabolism of syringic acid via gallic acid by the fungus *Schizophyllum commune* Fr. but they did not find methylgallic acid as an intermediate. They suggested that both methyl groups were removed simultaneously. On the other hand, Leonowicz et al. (1984) showed that extracellular laccases from *Rhizoctonia* sp. and *Trametes versicolor* (L.: Fr.) Pilát were able to form hydroquinone from syringic acid. Formation of the same hydroquinone from syringic acid had been shown to occur in higher plant cells (Bolkart and Zenk, 1968). In higher plants, the methoxylated phenols methoxyhydroquinone and dimethoxyhydroquinone are formed through oxidative decarboxylation of the corresponding substituted benzoic acid, vanillic acid and syringic acid.

The extracellular peroxidase of *P. ostreatus* had decarboxylase activity and oxidized syringic acid to dimethoxyphenol, dimethoxyhydroquinone and dimethoxybenzoquinone, as LiP of *P. chrysosporium* and higher plants. This is probably the first report of production of 2,6-dimethoxyphenol by a fungal extracellular peroxidase. It was reported that 2,6-dimethoxyphenol acted as good substrate for fungal laccase (Bollag et al., 1979). So, I concluded that extracellular peroxidase of *P. ostreatus* might attack phenolic fragments in lignin and act as a synergistic degradation system for lignin with other ligninolytic enzyme systems.

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