Volume 236, number 2, 309-311 FEB 06222 August 1988

Aromatic ring cleavage of 4,6-di(*tert*-butyl)guaiacol, a phenolic lignin model compound, by laccase of *Coriolus versicolor*

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Received 27 June 1988

It was found that 2,4-di(*tert*-butyl)-4-(methoxycarbonylmethyl)-2-buten-4-olide (**II**) was formed as an aromatic ring cleavage product of a phenolic lignin model compound, 4,6-di(*tert*-butyl)guaiacol (**I**), by laccase of *Coriolus versicolor*. Based on isotopic experiments with $^{18}O_2$ and $H_2{}^{18}O$, the mechanism of formation of **II** from **I** is discussed.

Laccase; Aromatic ring cleavage; Lignin model compound; Phenoxy radical; (*Coriolus versicolor*)

1. INTRODUCTION

We [1,2] previously found that laccase from *Coriolus versicolor* catalyzed not only alkyl-aryl cleavage and Cα oxidation but also Cα-Cβ cleavage of the side chain of β-1 lignin substructure model compounds. Here, we examined the possibility of aromatic ring cleavage of the phenolic lignin model compound, 4,6-di(*t*-butyl)guaiacol (**I**), by laccase from *C. versicolor*. The substrate **I** was found to be degraded by laccase to form an aromatic ring cleavage product, muconolactone derivative **II** (chemical structures shown in fig.1). The following tracer experiments with $H_2{}^{18}O$ and $^{18}O_2$ demonstrated the incorporation of ^{18}O from $^{18}O_2$ into the product **II**.

2. MATERIALS AND METHODS

2.1. *Syntheses of substrate and authentic compound*

4,6-Di(*t*-butyl)guaiacol (**I**) was prepared using the method of Ley and Müller [3]. However, the product contained the isomer, 3,5-di(*t*-butyl)guaiacol, of which the chemical properties are identical with those of **I** (R_f value on TLC, retention time in GC, ^{1}H-NMR and mass spectra). When the product containing both isomers was treated with acetic anhydride and pyridine at room temperature for 24 h, one isomer [3,5-di(*t*-butyl)guaiacol] was acetylated, but the other was not. The non-acetylated form was selected as the target compound (**I**), since the phenolic hydroxyl group of **I** is not susceptible to acetylation with acetic anhydride owing to steric hindrance by the neighboring bulkyl *t*-butyl group.

Aromatic ring cleavage product **II** was synthesized from 4-(*t*-butyl)pyrocatechol (Nakarai Chemicals) via the following steps: (i) introduction of the *t*-butyl group into pyrocatechol with *t*-butanol and H_2SO_4 in acetic acid at room temperature [4] [3,5-di(*t*-butyl)pyrocatechol]; (ii) aromatic ring cleavage of 3,5-di(*t*-butyl)pyrocatechol to **III** with ferric acetylacetonate and approx. 8% peracetic acid [5] in acetic acid at room temperature (modified method of Pandell [6]), **III** being recrystallized from petroleum ether; and (iii) methylation of **III** with CH_2N_2 in diethyl ether at room temperature. ^{1}H-NMR ($CDCl_3$) δ (ppm): 0.98(9H,s,*t*-butyl), 1.24(9H,s,*t*-butyl), 2.80(1H,dd,J = 13.8 Hz, >C-CH_2-COO-), 2.96 (1H,dd,J = 13.8 Hz, >C-CH_2-COO-), 3.59 (3H,s,-OCH_3), 6.97(1H,s,>C=CH-). ^{13}C-NMR ($CDCl_3$) δ (ppm): 25.2(q), 28.0(q), 31.5, 37.6, 37.8(t), 51.8(q), 88.4, 143.5, 146.0(d), 169.7, 171.2. EIMS *m/z* (intensity, %): 268(M^+, missing), 213(12), 212($M^+ - C_4H_8$, 100), 198(11), 197(95), 153(64), 137(15), 109(13). CIMS (intensity, %): 270(16), 269(MH^+, 100). IR (KBr) $\nu_{C=O}$ 1746, 1733 cm^{-1}, $\nu_{C=C}$ 1639 cm^{-1}.

Deuterated **II** (**II**-D) was prepared from **III** by treatment with $C^2H_3O^2H$ (99.5%, CEA) in the presence of trifluoroacetic anhydride at 35°C [CIMS (intensity, %): 272(MH^+, 100), 269(4.5)].

2.2. *Enzyme preparation*

Crude laccase from *C. versicolor* was prepared by the method of Fåhraeus and Reinhammer [7]. Xylidine was added to the

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

4-day-old culture as an inducer of laccase. After 8 days, cultures (3 l) were filtered and concentrated to about 100 ml using a Millipore ultrafiltration system (10 kDa pore size). The concentrated solution was saturated with ammonium sulfate and cooled to 4°C overnight. The precipitate was separated by centrifugation (10000 × *g*, 20 min, 4°C), redissolved in 70 ml of 0.1 M phosphate buffer (pH 6.0) and used as a crude laccase solution. This solution showed an activity of 1.2×10^{-4} kat/ml with syringaldazine as substrate [2], but did not oxidize veratryl alcohol to veratraldehyde in the presence of H_2O_2.

2.3. *Enzyme reaction*

Enzyme solution (3 ml) and substrate (2 μmol in 5 μl acetone solution) were placed in a flask and the reaction mixture was incubated for 2 h at 30°C. In a control experiment, enzyme was replaced by 0.1 M phosphate buffer (pH 6.0).

The reaction mixture was then 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 separated by TLC (Kieselgel 60 F_{254}, Merck; solvent, ethyl acetate/hexane, 1:10) to give a fraction of R_f value approximately equal to that of **II**. The fraction was analyzed using a gas chromatograph-mass spectrometer [GC-MS, Shimadzu capillary column Hicap CBP1-W12-100 (methyl silicone), 12 m × 0.53 mm (i.d.), column temperature, 120–140°C, 5°C/min).

The amount of **II** formed was calculated quantitatively by the stable isotope dilution method. 2 μg **II**-D as an internal standard (20 μl dioxane solution) was added to a flask before extraction, and the fraction containing **II** and **II**-D was separated and analyzed by GC-MS (mass chromatography).

2.4. *Isotopic experiments*

2.4.1. Tracer experiment with $^{18}O_2$

The experiment under $^{18}O_2$ (^{18}O: 98.58 atom%, CEA) was performed according to [2]. The reaction was carried out in a total volume of 3 ml, containing 100 μl enzyme, 2 μmol substrate and 0.1 M phosphate buffer (pH 6.0). The reaction mixture was incubated for 3 h at 30°C.

2.4.2. Tracer experiment with $H_2{}^{18}O$

To a flask containing lyophilized enzyme (300 μl), 150 μl of $H_2{}^{18}O$ (^{18}O: 97 atom%, CEA), 150 μl water and 2 μmol substrate were added, and the reaction mixture was incubated for 3 h at 30°C.

2.5. *Instruments*

^1H- and ^{13}C-NMR spectra were recorded on a Varian XL-200 FT-NMR spectrometer (200 MHz). Mass spectra were measured on a Shimadzu GC-MS QP-1000 gas chromatograph-mass spectrometer [EIMS (70 eV) and CIMS (reagent gas, isobutane]. IR spectra were registered using a Hitachi 260-30 infrared spectrophotometer. Enzyme activity was determined with a Hitachi model 200-20 double-beam spectrophotometer.

3. RESULTS

It was confirmed by GC-MS analysis that **II** was formed as an aromatic ring cleavage product by

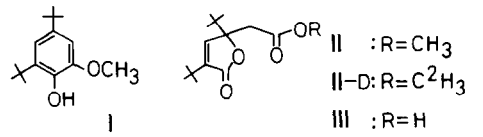

Fig.1. Structural formulae of compounds **I**–**III**.

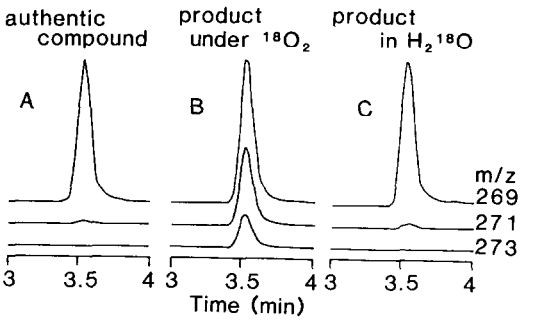

Fig.2. Mass chromatograms of the MH^+ region of muconolactone derivative **II**. (A) Authentic compound, (B) product from substrate **I** under $^{18}O_2$ (^{18}O: 98.58 atom%), (C) product from substrate **I** in $H_2{}^{18}O$ (^{18}O; 48.5 atom%).

laccase from substrate **I**, which was not completely degraded in 2 h. The mass spectra (EI and CI) and retention time of the degradation product **II** were identical with those of the authentic compound. Furthermore, quantification by the stable dilution method showed that 2×10^{-2} μmol (5.4 μg) of **II** was formed as degradation product from 2 μmol (472.7 μg) of **I**, while the amount of **II** produced nonenzymatically was 1.5×10^{-3} μmol (0.4 μg).

Other products were observed by TLC, but their structures were not determined.

Incorporation of ^{18}O from $^{18}O_2$ or $H_2{}^{18}O$ into **II**

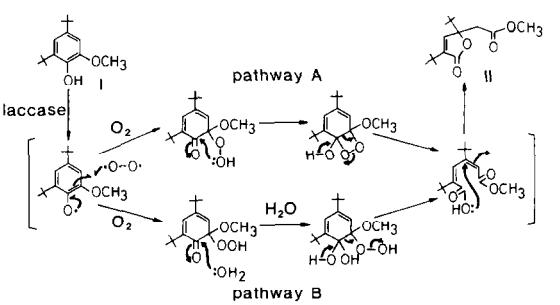

Fig.3. Possible mechanisms of formation of muconolactone (**II**) from substrate (**I**).

was investigated by use of GC-MS. Fig.2 shows mass chromatograms of the MH^+ region of **II**. Analysis showed that $H_2{}^{18}O$ was not incorporated into **II** (fig.2C), while one (22%) or two (11%) atoms of ^{18}O were incorporated into **II** from molecular oxygen (fig.2B).

4. DISCUSSION

Our investigation showed that monomeric lignin degradation phenols, vanillyl alcohol, syringyl alcohol, etc., were mostly converted to polymerized and/or quinone-type compounds by laccase (not shown). It appeared rather difficult to identify the structures of the aromatic ring cleavage products without having synthetic authentic compounds available, even if a small amount of such products could be obtained from these substrates. Therefore, as substrate for laccase we synthesized 4,6-(di(*t*-butyl)guaiacol (**I**), in which the *ortho* and *para* positions for the phenolic hydroxyl group were blocked with bulky *t*-butyl groups to prevent coupling and side chain reactions of phenoxy radical(s), and succeeded for the first time in identifying a ring cleavage product, muconolactone derivative (**II**), by laccase compared with the authentic compound.

Fig.3 depicts two possible mechanisms of formation of **II**. The substrate (**I**) is oxidized by laccase to form the phenoxy radical, which is subsequently attacked by molecular oxygen. The resulting hydroperoxide reacts with nucleophilic oxygen species. Since isotopic experiments showed that H_2O was not incorporated into **II**, formation of **II** via pathway B was ruled out. However, mass spectrometric analysis showed that two ^{18}O atoms from $^{18}O_2$ were incorporated into a part of product **II**. These results are in accordance with the contention that the hydroperoxide group of an intermediary O_2 adduct reacts with the adjacent carbonyl group to form a cyclic peroxide (pathway A), which is converted to a muconate derivative. The muconate compound then undergoes cyclization to yield lactone **II**. Further investigations are now in progress to elucidate the details of the pathways for formation of lactone **II**.

Muconolactone derivative **II** was isolated previously as a product in photosensitized [4] and alkaline-oxygen [8,9] oxidations of the same substrate.

It is known that aromatic ring cleavage of phenolic compounds by microorganisms is generally catalyzed by dioxygenase. However, the present investigation showed that in addition to side chain cleavage of phenolic β-1 lignin substructure compounds [1,2], aromatic ring cleavage of phenolic lignin model compounds is catalyzed by laccase from *C. versicolor*. Although laccase cannot oxidize nonphenolic compounds, the cleavage reactions of phenolic lignin model compounds by laccase proceed via one-electron oxidations as in the oxidation of phenolic and nonphenolic lignin model compounds by lignin peroxidase [10–13]. Thus, it is concluded that on cleavage of the side chain and aromatic ring of lignin, both lignin peroxidase and laccase could be involved as initial enzymes.

Acknowledgements: This research was partly supported by Grants-in-Aid for Scientific Research (nos 60440015 and 62790250) from the Ministry of Education of Japan.

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