Aromatic ring cleavage of a non-phenolic β-*O*-4 lignin model dimer by laccase of *Trametes versicolor* in the presence of 1-hydroxybenzotriazole

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Abstract The novel cleavage products, 2,3-dihydroxy-1-(4-ethoxy-3-methoxyphenyl)-1-formyloxypropane (II) and 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane-2,3-cyclic carbonate (III) were identified as products of a non-phenolic β-*O*-4 lignin model dimer, 1,3-dihydroxy-2-(2,6-dimethoxylphenoxy)-1-(4-ethoxy-3-methoxyphenyl)propane (I), by a *Trametes versicolor* laccase in the presence of 1-hydroxybenzotriazole (1-HBT). An isotopic experiment with a ^{13}C-labeled lignin model dimer, 1,3-dihydroxy-2-(2,6-[U-ring-^{13}C]dimethoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl)propane (I-^{13}C) indicated that the formyl and carbonate carbons of products II and III were derived from the β-phenoxy group of β-*O*-4 lignin model dimer I as aromatic ring cleavage fragments. These results show that the laccase-1-HBT couple could catalyze the aromatic ring cleavage of non-phenolic β-*O*-4 lignin model dimer in addition to the β-ether cleavage, Cα-Cβ cleavage, and Cα-oxidation.

Key words: Laccase; 1-Hydroxybenzotriazole; Lignin; Non-phenolic β-*O*-4 lignin model dimer; Aromatic ring cleavage

1. Introduction

Laccase (EC 1.10.3.2) is widely found in white-rot fungi, and is known to cause Bavendamm's reaction, which has been used to investigate the lignin-degrading abilities of fungi [1]. To determine the possibility of the degradation of non-phenolic moieties of lignin by laccase in the presence of an appropriate compound, coupled degradative systems of laccase with various compounds such as 2,2′-azobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 1-hydroxybenzotriazole (1-HBT), were proposed [2,3]. Furthermore, 3-hydroxyanthranilic acid (3-HAA) which was found in the culture of *Pycnoporous cinnabarinus* [4] was reported as a natural mediator. However, there has been little discussion of the reaction mechanisms for underlying the cleavage of lignin model compounds with a non-phenolic β-*O*-4 lignin substructure by laccase-mediator couples.

In a previous paper [5], we reported that *Trametes versicolor* laccase catalyzed not only Cα-Cβ cleavage and Cα-oxidation but also the β-ether cleavage of a non-phenolic β-*O*-4 lignin model dimer, 1,3-dihydroxy-2-(2,6-dimethoxyl-4-formylphenoxy)-1-(4-ethoxy-3-methoxyphenyl)propane, in the presence of 1-HBT. The oxidation reactions with the non-phenolic β-*O*-4 lignin model dimer were very similar to those with lignin peroxidase except for an aromatic ring cleavage.

Here, we examined the possibility of an aromatic ring cleavage of a non-phenolic β-*O*-4 lignin model dimer, 1,3-dihydroxy-2-(2,6-dimethoxylphenoxy)-1-(4-ethoxy-3-methoxyphenyl)propane (I), by a *T. versicolor* laccase-1-HBT couple. The dimer I was degraded by this system to form the aromatic ring cleavage products, 2,3-dihydroxy-1-(4-ethoxy-3-methoxyphenyl)-1-formyloxypropane (II) and 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane-2,3-cyclic carbonate (III).

2. Materials and methods

2.1. Laccase preparation

A crude laccase from *T. versicolor* IFO 30340 was obtained by the method of Fåhraeus and Reinhammar [6]. The crude laccase preparation (18 ml) was partially purified by DEAE-Biogel A [5]. The column (1×10 cm) was equilibrated with 5 mM phosphate buffer, pH 6.0, and the solution was loaded and eluted with a NaCl gradient (0–400 mM, total volume 800 ml). The elution profile indicated the presence of two laccases. The latter fraction was used as the laccase in this experiment without further purification. The enzyme activity was determined spectroscopically by measuring the absorption at 525 nm using syringaldazine [7]. No lignin peroxidase activity was found in the enzyme preparation [8].

2.2. Substrate, authentic compounds and reagents

1,3-Dihydroxy-2-(2,6-dimethoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl)propane (I) was synthesized by the modified method of Kawai et al. [5] from acetovanillone (Nakarai Tesque). The substrate was purified by preparative thin-layer chromatography (TLC) before use in the experiments.

The same preparation as previously described was used for 1,3-dihydroxy-2-(2,6-[U-ring-^{13}C]dimethoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl)propane (I-^{13}C) [9], the diacetate of 2,3-dihydroxy-1-(4-ethoxy-3-methoxyphenyl)-1-formyloxypropane (II-Ac) [10], 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane-2,3-cyclic carbonate (III) [11], 2,6-dimethoxy-*p*-benzoquinone (IV) [9], 4-ethoxy-3-methoxybenzoic acid (V) [5], 1-(4-ethoxy-3-methoxyphenyl)-3-hydroxypropanone (VI) [12], 2,3-dihydroxy-1-(4-ethoxy-3-methoxyphenyl)-propanone (VII) [9], and 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane (VIII) [10].

1-Hydroxybenzotriazole (1-HBT) was purchased from Nakarai Tesque and purified by preparative TLC before use. All other chemicals were reagent grade or better.

2.3. Enzyme reaction of I

To a flask containing 5 ml of 0.2 M acetate buffer (pH 4.0), we added the laccase (500 nkat), substrate I (2.6 μmol in 50 μl of DMF solution) and 1-HBT (15 μmol in 50 μl of DMF solution). The reaction mixture was incubated at 28°C for 60 min while being stirred. In the control experiments, the laccase or 1-HBT was omitted in the reaction mixture.

The flask was then partitioned into 50 ml of ethyl acetate and 30 ml of water. The organic layer was washed with saturated NaCl solution, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was acetylated with acetic anhydride and pyridine (1:1, v/v). The acetylated extract was further trimethylsilylated with TMSI-H (hexamethyldisilazane and trimethylchlorosilane in pyridine (2:1:10, v/v)) (GL Sciences) and analyzed by GC-MS using a Shimadzu GC-MS QP 5000 gas chromatograph-mass spectrometer (EI, 70 eV) with a DB-1 column (J and W Scientific), 0.25 mm

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(i.d.) ×30 m×1 μm (film). The column temperature was 150–280°C, changing at 5°C/min.

The degradation products were identified by direct comparison of the mass spectra and retention times with those of the authentic compounds.

2.4. Enzyme reaction of I-^{13}C

To a flask containing 1 ml of 0.2 M acetate buffer (pH 4.0), we added the laccase (100 nkat), substrate I-^{13}C (0.6 μmol in 10 μl of DMF solution) and 1-HBT (3 μmol in 10 μl of DMF solution). The reaction mixture was incubated at 28°C for 60 min while being stirred.

The flask was then partitioned into 20 ml of ethyl acetate and 10 ml of water. The organic layer was washed with saturated NaCl solution, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was acetylated and analyzed by GC-MS, as described above.

3. Results

Lignin model dimer I was oxidized by the *T. versicolor* laccase in the presence of 1-HBT. GC-MS analyses (Fig. 1) showed that the diacetate of 2,3-dihydroxy-1-(4-ethoxy-3-methoxyphenyl)-1-formyloxypropane (II-Ac) [MS *m/z* (%): 354 (M^+, 7.6), 294 (4.0), 223 (6.3), 207 (8.6), 206 (24), 182 (10), 181 (100), 153 (12), 125 (12)] and the acetate of 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane-2,3-cyclic carbonate (III-Ac) [MS *m/z* (%): 310 (M^+, 8.5), 223 (5.3), 206 (3.2), 182 (11), 181 (100), 153 (15), 151 (8.2), 125 (18)] were formed as aromatic ring cleavage products by laccase-1-HBT couple from the dimer I. In addition to these products, the following were confirmed as oxidation products of the dimer I: 2,6-dimethoxy-*p*-benzoquinone (IV) [MS *m/z* (%): 168 (M^+, 18), 138 (5.6), 125 (7.2), 112 (10), 97 (7.3), 80 (24), 69 (100)], trimethylsilyl ether of 4-ethoxy-3-methoxybenzoic acid (V-TMS) [MS *m/z* (%): 268 (M^+, 74), 253 (96), 225 (65), 210 (100), 181 (68), 179 (53), 151 (80), 123 (28)], the acetate of 1-(4-ethoxy-3-methoxyphenyl)-3-hydroxypropanone (VI-Ac) [MS *m/z* (%): 266 (M^+, 14), 207 (7.6), 206 (26), 179 (57), 152 (9.4), 151 (100), 123 (18)], the diacetate of 2,3-dihydroxy-1-(4-ethoxy-3-methoxyphenyl)propanone (VII-Ac) [MS *m/z* (%): 324 (M^+, 4.6), 264 (2.2), 191 (4.0), 180 (15), 179 (100), 151 (63), 123 (14)], and the triacetate of 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane (VIII-Ac) [MS *m/z* (%): 368 (M^+, 5.3), 308 (5.4), 223 (6.0), 207 (10), 206 (27), 182 (11), 181 (100), 153 (7.9), 125 (10)]. The mass spectra and retention times of these products were identical with those of the authentic compounds. The dimer I was not oxidized when 1-HBT or laccase was omitted. Semi-quantitative analysis with the diacetate of 3-(4-hydroxy-3-methoxyphenyl)propanol as an internal standard [12] showed that 30.6% of substrate I was oxidized, and, that 3.7 nmol (0.14%) of II, 33 nmol (1.3%) of III, 74 nmol (2.8%) of IV, 153 nmol (5.8%) of V, 34 nmol (1.5%) of VI, and ca. 40 nmol of VII+VIII were produced by the laccase-1-HBT couple under this condition.

In an isotopic experiment with ^{13}C-labeled substrate I-^{13}C under the same conditions, the diacetate of the γ-formyl compound (II-Ac) and the acetate of the cyclic carbonate (III-Ac) were identified. Mass spectra of II-Ac and III-Ac were shown in Fig. 2. Molecular ion peaks of the mass spectra of II-Ac and III-Ac increased by one mass unit in comparison with those of the spectra of unlabeled authentic compounds. The results clearly indicated that the formyl carbon of II and the carbonate carbon of III contained ^{13}C derived from substrate I-^{13}C.

4. Discussion

We recently showed that the *T. versicolor* laccase catalyzed β-ether cleavage, Cα-Cβ cleavage, and Cα-oxidation of non-phenolic β-*O*-4 lignin model dimer, and effectively depolymerized synthetic guaiacyl lignin with a normal phenolic content in the presence of 1-HBT [5]. This paper reports that the lac-

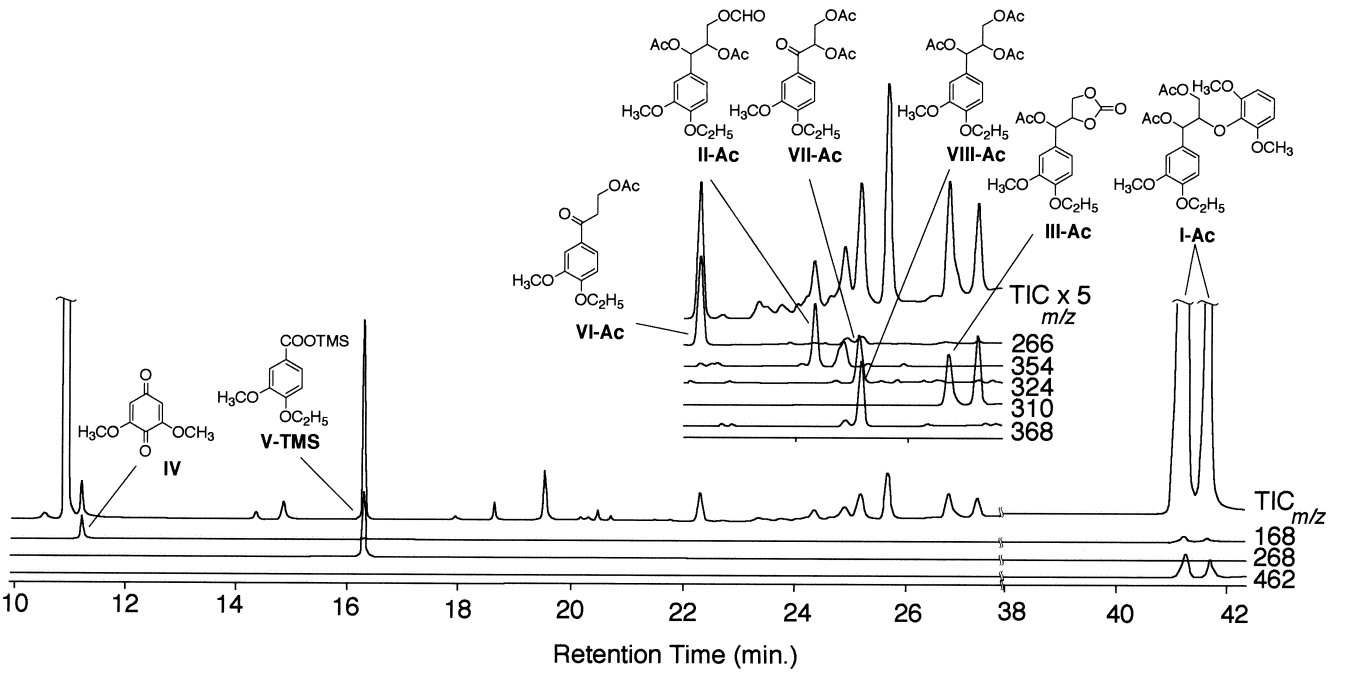

Fig. 1. Total ion chromatogram and single-ion chromatographic traces of the reaction residue of lignin model dimer I treated with *T. versicolor* laccase in the presence of 1-hydroxybenzotriazol. The products were acetylated and trimethylsylilated. A product at 10.8 min is trimethylsilylated 1-hydroxybenzotriazole.

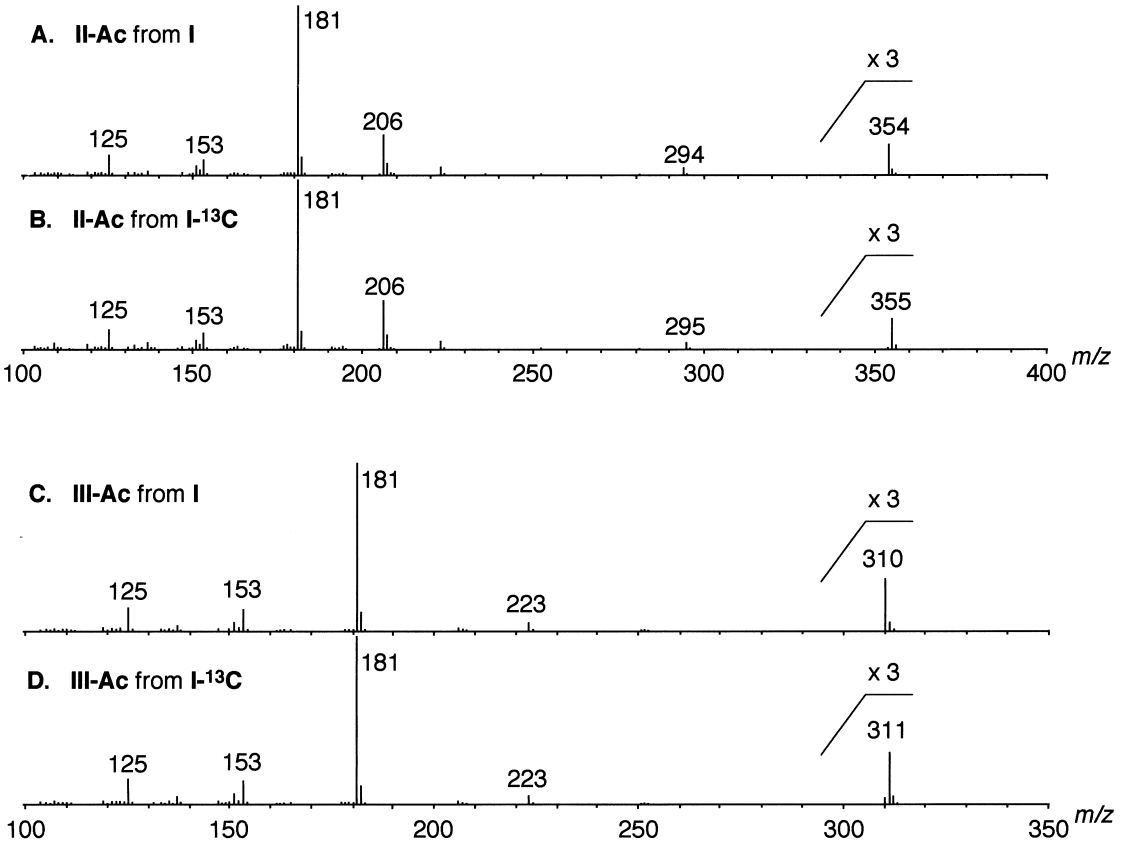

Fig. 2. Mass spectra of the degradation products II-Ac and III-Ac from lignin model dimers I (A and C) and I-^{13}C (B and D) by the laccase-1-HBT couple.

case-1-HBT couple catalyzed the formation of the γ-formyl compound (II) and the cyclic carbonate (III) from non-phenolic β-*O*-4 lignin model dimer I and that the products II and III were aromatic ring cleavage products based on the tracer experiments with ^{13}C-labeled substrate I-^{13}C. Although we [13] previously reported the aromatic ring cleavage of a phenolic lignin model compound, 4,6-di-*tert*-butyl-2-methoxyphenol, by *T. versicolor* laccase, this is the first report on the aromatic ring cleavage of non-phenolic lignin model dimer by laccase in the presence of an appropriate compound. It is both noteworthy and interesting that the laccase-1-HBT couple catalyzes both the cleavages of side chains and the aromatic rings of non-phenolic lignin model dimers.

We examined the oxidation of lignin model dimer I by *T. versicolor* laccase in the presence of a natural product, 3-HAA [4], under our experimental condition, but we could not find any degradation products (data not shown). However, the present results with synthetic compound, 1-HBT, presumed that laccase coupled with some natural product(s) could oxidize the non-phenolic moiety of lignin macromolecule in nature.

At present there is no evidence for the mechanisms underlying the aromatic ring cleavage of non-phenolic β-*O*-4 lignin model dimer by the laccase-1-HBT couple. However, the degradation products of lignin model dimer I formed by this system were almost the same as those formed by lignin peroxidase [12,14–16]. Lignin peroxidase produces aryl cation radicals from methoxylated aromatic substrates [17–19], and the mechanisms for aromatic ring cleavage via the aryl cation radicals were proposed [20–23]. These results strongly suggest that the β-aryl cation radical intermediate of lignin model dimer I (Fig. 3) is involved in the formation of the products II and III from lignin model dimer I by the laccase-1-HBT couple.

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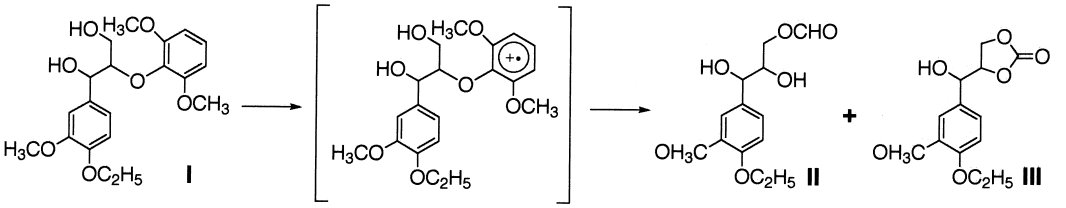

Fig. 3. Aromatic ring cleavage products, II and III, and a possible intermediate formed from lignin model dimer I by the laccase-1-HBT couple.

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