Aromatic ring cleavage of β-*O*-4 lignin substructure model dimers by lignin peroxidase of *Phanerochaete chrysosporium*

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Extracellular lignin peroxidase (ligninase) from *Phanerochaete chrysosporium* catalyzed aromatic ring cleavage of β-*O*-4 lignin substructure model dimers to give three esters of arylglycerol, cyclic carbonate, formate and methyl oxalate. H_2O_2 was required for the activity of the enzyme.

Aromatic ring cleavage *Lignin peroxidase* *β-O-4 Lignin substructure* *Cyclic carbonate* *Formate* *Methyl oxalate*

1. INTRODUCTION

The white-rot fungi *Phanerochaete chrysosporium* and *Coriolus versicolor* cause various oxidative degradations of lignin and lignin substructure model compounds [1–9]. Some of the reactions were found to be catalyzed by an extracellular lignin-degrading enzyme (lignin peroxidase, ligninase) from *P. chrysosporium* [10–17]. However, it is not yet certain whether aromatic ring cleavages of lignin and lignin substructure models are also catalyzed by the same lignin peroxidase, although Leisola et al. [18] recently reported aromatic ring cleavage of veratryl alcohol by the extracellular enzyme system from *P. chrysosporium*.

The present investigation reports for the first time evidence that β-*O*-4 lignin substructure models are degraded in the presence of the lignin peroxidase of *P. chrysosporium*, yielding aromatic ring cleavage products.

2. MATERIALS AND METHODS

2.1. *Preparation of substrates and authentic compounds*

The following compounds were prepared previously: 4-ethoxy-3-methoxyphenylglycerol-β-(2,6-dimethoxyphenyl) ether (II) [5], 4-ethoxy-3-methoxyphenylglycerol (IV) [7] and its acetate (IV-Ac) [7], β,γ-cyclic carbonate (V) [4] and its acetate (V-Ac) [4], acetate of γ-formate (VI-Ac) [8], 4-ethoxy-3-methoxybenzaldehyde (VIII) [6], 1-(4-ethoxy-3-methoxyphenyl)-2,3-dihydroxypropane-1-one (IX) [7].

4-Ethoxy(3-[$^2H_3$]methoxy)phenylglycerol-β-guaiacyl ether (I-D) was prepared by the method of Nakatsubo et al. [19] from 4-ethoxy-(3-[$^2H_3$]methoxy)benzaldehyde (VIII-D) prepared as given below. MS ((I-D-Ac), acetate of (I-D)), *m/z* (%): 435 (M^+, 12.9), 375(6.4), 315(5.7), 286(7.1), 253(9.3), 252(4.8), 226(8.0), 211(7.1), 210(30.5), 209(41.7), 184(100). 4-Ethoxy-(3-[$^2H_3$]methoxy)-phenylglycerol-β-(2,6-dimethoxyphenyl) ether (II-D) was prepared in the same way for the preparation of (II) [5] from 4-ethoxy(3-[$^2H_3$]methoxy)-acetophenone which was synthesized from 4-ethoxy-3-hydroxybenzaldehyde (Aldrich) as follows. (1) C^2H_3I (Merck, ^2H: min. 99%)/K_2CO_3 in DMF, room temperature, giving (VIII-D); (2) methyl magnesium bromide (Tokyo Kasei, Japan) in THF, room temperature; (3) Jones reagent in acetone, 0°C. MS (II-D-Ac) *m/z* (%): 465(M^+, 5.2), 345(8.9), 316(10.8), 253(18.9), 210(52.5),

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209(51.3), 184(31.3), 154(100). 4-Ethoxy-3-methoxyphenylglycerol-β-phenyl ether (III), 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-3-hydroxypropane-1-one (X), and 1-(4-ethoxy-3-methoxyphenyl)-2-phenoxy-3-hydroxypropane-1-one (XI) were prepared as in the preparation of (I) [9]. (III-Ac) ^{1}H-NMR ($CDCl_3$) δ(ppm): 1.45(3H $\times$2,t,O-C-CH_3,J=7.0); 1.99, 2.03, 2.03, 2.07(3H$\times$4,s,OAc); 3.85, 3.85(3H$\times$2,s,OCH_3); 4.0–4.2(2H$\times$2,γ-H), 4.08(2H$\times$2,q,-O-CH_2-C, J=7.0); 4.72(1H$\times$2,m,β-H); 5.96(1H,d,α-H, J=5); 6.02(1H,d,α-H,J=6); 6.8–7.1, 7.2–7.4 (8H,m,aromatic). MS m/z (%): 402(M^+, 8.4), 342(11.8), 282(10.2), 253(11.9), 223(15.2), 181(100). (X-Ac) ^{1}H-NMR ($CDCl_3$) δ(ppm): 1.50(3H,t,-O-C-CH_3,J=7.0), 2.05(3H,s,-OAc), 3.77 and 3.92(3H$\times$2,s,-$OCH_3\times$2), 4.17(2H,q,-O-CH_2-C,J=7.0), 4.50(1H,dd,γ-H), 4.68(1H,dd,γ-H), 5.62(1H,dd,β-H), 6.8–7.0(5H,m,aromatic), 7.66(1H,d,J=2.0), 7.81(1H,dd,J=2.1,8.5). MS m/z (%): 328(M^+-$C_2H_4O_2$,24.3), 179(100), 177(24.8), 151(63.9). (XI-Ac) ^{1}H-NMR δ(ppm): 1.50(3H,t,-C-CH_3,J=7.0), 2.07(3H,s,OAc), 3.90(3H,s,OCH_3), 4.18(2H,q,-O-CH_2-,J=7.0), 4.51(1H,dd,γ-H,J=7.3,11.8), 4.68(1H,dd,γ-H,J=3.7,11.8), 5.63(1H,dd,β-H,J=3.7,7.3), 6.8–7.0 and 7.2–7.3(6H,m,aromatic), 7.62(1H,d, aromatic,J=2.0), 7.82(1H,dd,aromatic,J=2.1, 8.5). MS m/z (%): 298(M^+-$C_2H_4O_2$, 45.5), 179(92.5), 177(48.5), 151(100). Diethyl ether of (II), (II-Et), was prepared by ethylation of (II) (ethyl iodide/NaH in DMF, room temperature). ^{1}H-NMR δ(ppm): 1.06(3H,t,-C-CH_3,J=7.0), 1.12(3H,t,-C-CH_3,J=7.0), 1.46(3H,t,Ph-O-C-CH_3,J=7.0), 3.18(1H,dd,γ-H), 3.3–3.5(4H,m,-CH_2-C$\times$2), 3.65(1H,dd,γ-H), 3.74(6H,s,-OCH_3 $\times$2), 3.86(3H,s,-OCH_3), 4.10(2H,q,Ph-O-CH_2-), 4.34(1H,m,β-H), 4.67(1H,d,α-H), 6.51(2H,d,aromatic,J=8.3), 6.8–7.1(4H,m,aromatic). MS m/z (%): 434(M^+, 0.8), 280(4.8), 235(6.7), 209(100), 181(11.3), 165(17.1), 153(10.8). 4-Ethoxy-3-methoxyphenylglycerol-α,β-cyclic carbonate (V′) was synthesized as a by-product in the synthesis of (V) [4]: cyclic carbonate of (IX) was reduced with $NaBH_4$ to yield (V′) as well as (V). (V′-Ac) ^{1}H-NMR ($CDCl_3$) δ(ppm): 1.48(3H,t,-O-C-CH_3,J= 7.0), 2.15(3H,s,-O-Ac), 3.90(3H,s,-OCH_3), 4.11(2H,q,-O-CH_2-C,J=6.9), 4.32(1H,dd,γ-H), 4.45(1H,dd,γ-H), 4.72(1H,m,β-H), 5.33(1H,d,α-H), 6.9(3H,m,aromatic). MS m/z (%): 310(M^+, 61.4), 206(41.6), 181(12.0), 179(14.6), 178(37.1), 177(18.9), 165(13.8), 151(100).

1-(4-Ethoxy-3-methoxyphenyl)-1,3-diethoxypropyl methyl oxalate (VII-Et) was prepared from 4-ethoxy-3-methoxyacetophenone prepared previously [9]. (1) $CuBr_2$ in ethyl acetate, reflux; (2) HCOONa in DMF, 60°C; (3) saturated solution of $NaHCO_3$ in H_2O in DMF and CH_3OH, room temperature; (4) ethyl vinyl ether/dl-10-camphorsulfonic acid in CH_2Cl_2, 0°C; (5) paraformaldehyde/K_2CO_3 in DMSO, room temperature; (6) $NaBH_4$ in CH_3OH, 0°C; (7) ethyl bromide/NaH in DMF; (8) 1 N HCl in acetone, room temperature; (9) oxalyl chloride/CH_3OH in pyridine, room temperature. ^{1}H-NMR ($CDCl_3$) δ(ppm): about 1.15(3H$\times$2,CH_3-C-O-), 1.48(3H,t,CH_3-C-O-Ph), 3.3–3.5(6H,m,C-CH_2-O-$\times$2 and γ-H), 3.88(3H,s,-O-CH_3), 4.10(2H,q,C-CH_2-O-Ph), 4.51(1H,d,α-H), 5.3(1H,m,β-H), about 6.9(3H, m,aromatic). MS m/z (%): 384(M^+, 2.6), 209(100), 181(14.3), 153(7.4). (IX-Ac) was prepared by acetylation of (IX) [7] (acetic anhydride/pyridine). MS m/z (%): 324(M^+, 6.9), 264(15.5), 222(7.6), 191(33.4), 179(100), 151(70.0), 123(16.3).

2.2. *Enzyme production and purification*

Lignin peroxidase (ligninase) was prepared by the modified method of Tien and Kirk [12,20] from a culture filtrate of *P. chrysosporium* Burds. (ME-446). The enzyme protein was concentrated by ultrafiltration from the culture filtrate (88 h cultures). The concentrated crude protein was applied to a DEAE-Bio-Gel A and eluted by use of a gradient of NaCl (0–0.25 M) in 10 mM sodium succinate buffer at pH 5.5. The elution profile of the lignin peroxidase showed two peaks of enzyme activity. The major enzyme fractions were collected, concentrated and used for enzyme in the present experiment. Enzyme activity (IU) was assayed by spectrometric quantification of veratraldehyde (ϵ_{310} = 9300 $M^{-1}\cdot cm^{-1}$) formed on oxidation of veratryl alcohol at 36°C [12].

2.3. *Enzymatic reactions*

2.3.1. Product identification

The reaction mixture (3.3 ml) contained 60 μl of 25 mM H_2O_2, 1 μmol model substrate in 30 μl CH_3OH, 15 μl lignin peroxidase (0.4–1 IU), and

3195 μl of 100 mM sodium tartrate buffer (pH 3.0). The enzymatic reaction was started by the addition of lignin peroxidase to the mixture, which was incubated for 30 min at 37°C under air. The reactions were terminated by extraction with ethyl acetate (15 ml × 2). Products were acetylated [acetic anhydride/pyridine, 1:1 (v/v), in ethyl acetate, 15 h, room temperature) and manipulated for gas chromatography-mass spectrometry (GC-MS).

2.3.2. Product quantification

Products (VIII), (IV), (V), (V'), (IX) and (X) were estimated quantitatively by a stable isotope dilution method. Deuterated substrates (I-D) and (II-D) were incubated in the same way for product identification (0.73 IU lignin peroxidase was used for both substrates). Then products were extracted and acetylated as above except that unlabeled internal standards [5 μl dioxane containing 1 μg each of (VIII), (IV), (V), (IX) and (X)] were added into the ethyl acetate in extraction. Acetylated products containing internal standards were analyzed by GC-MS (mass chromatography).

2.4. *Instruments*

^{1}H-NMR spectra were taken with a Varian XL-200 FT-NMR spectrometer (TMS as an internal standard). GC-MS (EI-MS, 70 eV) was done on a Shimadzu GCMS QP-1000, column: 1.5% OV-17 on Chromosorb W AW DMCS (Shinwa Kakou, Japan), glass column 1 m × 0.26 cm (i.d.), column temperature, 170–240°C, 5°C/min or a Shimadzu GCMS QP-1000 equipped with a Shimadzu SPL-G9 split/splitless injection system (splitless mode), column: chemical bonded fused silica capillary column HiCap CBP1 (non-polar methyl silicone polymer, Shimadzu, Japan), 25 m × 0.33 mm (i.d.), column temperature, 170°C (1 min) then 170–240°C (10°C/min).

3. RESULTS

3.1. *Product identification*

GC-MS analysis (fig.1A) of acetate of degradation products of compound (II) showed formation of aromatic ring cleavage products, β,γ-cyclic carbonate (V-Ac) [MS *m/z* (%): 310(M^+, 14.2), 223(3.5), 206(4.4), 181(100), 153(11.6), 151(10.6)], α,β-cyclic carbonate (V'-Ac) [MS *m/z* (%): 310(M^+, 79.7), 206(49.9), 181(17.4), 179(19.6), 178(39.9), 177(29.3), 165(19.9), 151(100)], and formate (VI-Ac) [MS *m/z* (%): 354(M^+, 8.7), 294(4.5), 223(4.6), 206(18.8), 181(100), 153(8.8), 151(8.5)] as well as C_α-C_β cleavage product (VIII) [MS *m/z* (%): 180(M^+, 48.9), 152(44.7), 151(100)], arylglycerol (IV-Ac) [MS *m/z* (%): 368(M^+, 8.0), 308(4.3), 266(1.6), 250(1.8), 248(1.0), 223(5.1), 206(18.8), 181(100)], and (IX-Ac) [MS *m/z* (%): 324(M^+, 8.7), 264(6.4), 222(5.2), 191(12.9), 179(100), 151(54.3), 123(14.1)]. The mass spectra and retention times in GC-MS of the above compounds were identical to those of the synthesized authentic samples. Furthermore a product which has the mass spectrum [MS *m/z* (%): 412(M^+, 12.1), 352(3.4), 223(5.0), 207(14.6), 206(18.8), 181(100)] was detected and finally identified as methyl oxalate of 4-ethoxy-3-methoxyphenylglycerol, (VII-Ac), because compound (VII-Et), diethyl ether analog of (VII-Ac), [MS *m/z* (%): 384(M^+, 5.3), 209(100), 181(14.4), 153(8.3)] was also produced from diethyl ether of (II), (II-Et) (fig.2), and identified by comparison of the mass spectrum and the retention time (capillary GC-MS) with those of the synthesized authentic compound.

When compound (I-D) was degraded by the enzyme system, compounds (IV-D-Ac), (V-D-Ac), (V'-D-Ac), (VII-D-Ac), (VIII-D), (IX-D-Ac), and (X-D-Ac) were detected by GC-MS analysis (fig.1B). (X-D-Ac): MS *m/z* (%): 331(M^+-$C_2H_4O_2$, 20.2), 182(100), 180(25.1), 154(66.0).

Compound (III) having no methoxyl group on the β-aryl group (B-ring, see also fig.2) was also degraded by the ligninase system. However, only (VIII) and (XI-Ac) were identified as degradation products by GC-MS analysis of the acetate of reaction products (fig.1C). (XI-Ac): MS *m/z* (%): 298(M^+-$C_2H_4O_2$, 40.4), 179(81.0), 177(42.0), 151(100). (IV-Ac), (V-Ac), (V'-Ac), (VI-Ac), (VII-Ac), and (IX-Ac) were not detected.

3.2. *Product quantification*

Table 1 summarizes the results of quantitative analysis. The above described cleavage reactions were completely dependent on the presence of H_2O_2.

4. DISCUSSION

We previously reported that the aromatic rings

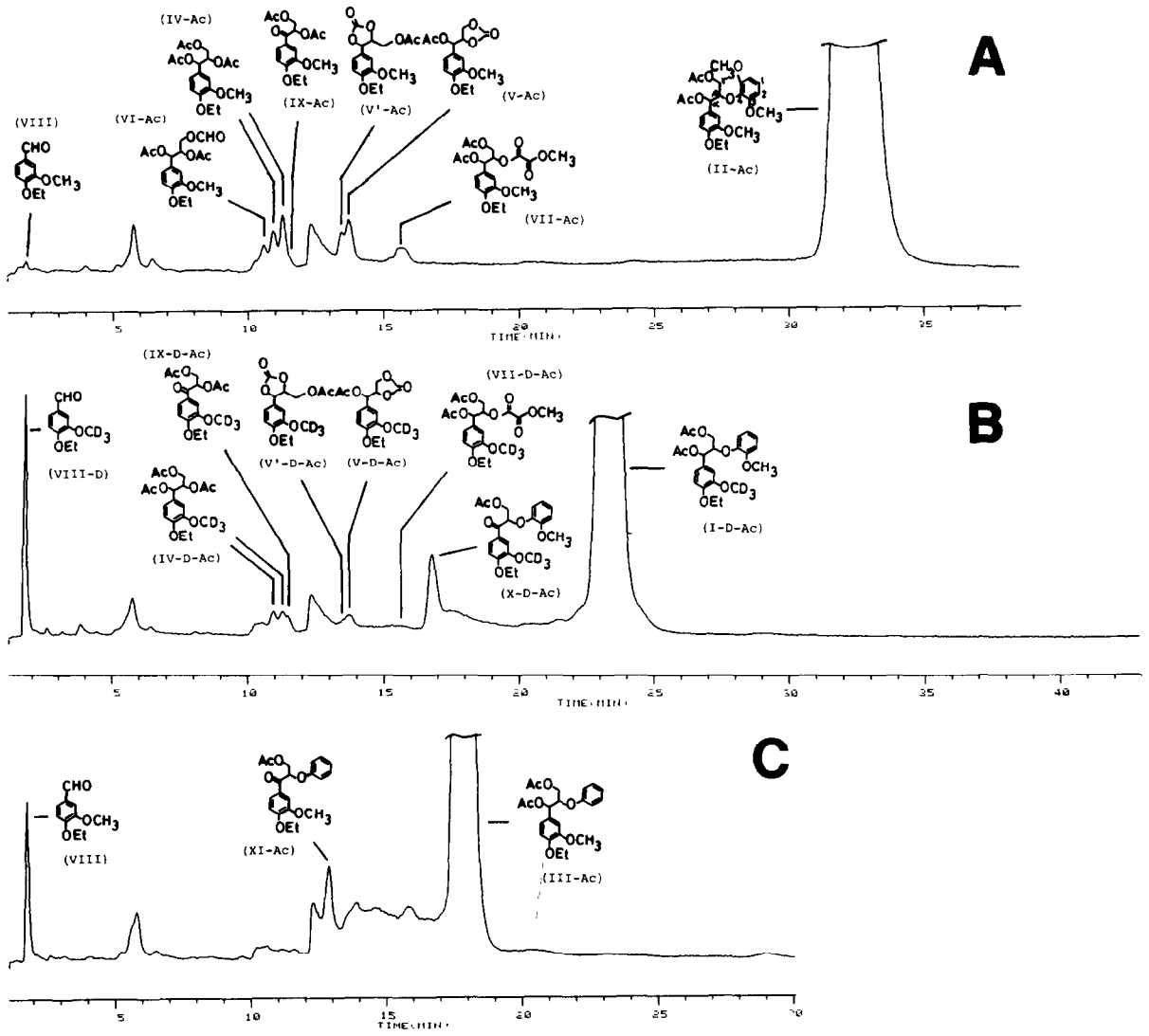

Fig.1. Gas chromatogram (total ion chromatogram in GC-MS analysis) of reaction products. (A) Degradation products from (II), (B) degradation products from (I-D), (C) degradation products from (III). As for (IV-Ac) or (IV-D-Ac), the peaks at 10.9 and 11.1 min were *erythro* and *threo* forms, respectively. The peak at 10.9 min in chromatogram A was a mixture of *erythro* of (IV-Ac) and an isomer (stereo or regio) of (VI-Ac). Column: 1.5% OV-17, 1 m. Other conditions given in the text.

of *β-O-4* lignin substructure model dimers were cleaved by ligninolytic cultures of *P. chrysosporium* and *C. versicolor*. Cyclic carbonate (V) was identified as an aromatic ring cleavage product formed from the substrates 4-ethoxy-3-methoxyphenylglycerol-*β*-guaiacyl ether (I) and (II) by both fungi [4,5]. Formate (VI) was also identified as products of (II) by both fungi ([5]; Umezawa et al., in preparation). Although Leisola et al. [18] recently reported aromatic ring cleavage of a monomeric aromatic compound, veratryl alcohol, by crude extracellular ligninase preparation, this is the first paper on the demonstration that the extracellular lignin peroxidase catalyzed aromatic ring cleavage of non-phenolic *β-O-4* lignin substructure models (I-D), (II) and (II-Et) yielding

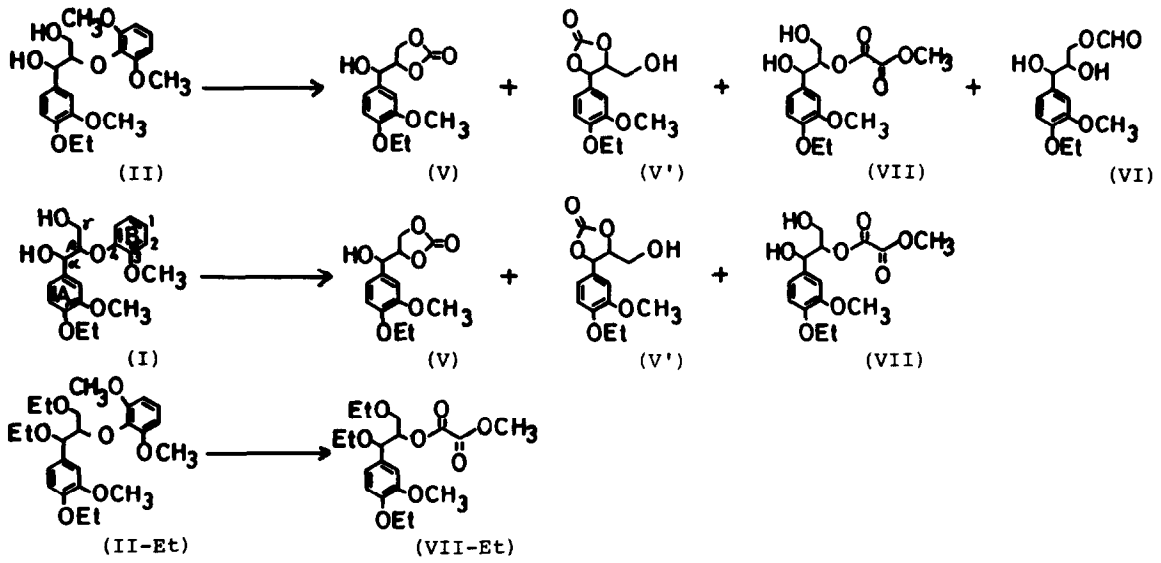

Fig.2. Aromatic ring cleavage products and their substrates. As for (VII), position of oxalyl group was assigned tentatively at the β-position based on the identification of (VII-Et) from (II-Et). Et = CH_2CH_3.

Table 1

Yield of products from substrates (I-D) and (II-D)

Substrates		Products[d] (nmol)					
		(VIII-D)	(IV-D)	(IX-D)	(V-D)	(V'-D)	(X-D)
(I-D)	complete[a]	130	14	8.2	3.3	1.1	15
	$-H_2O_2$[b]	1.3	ND[e]	ND	ND	ND	1.4
	denatured[c]	1.1	ND	ND	ND	ND	ND
(II-D)	complete[a]	0.9	26	0.9	13	11	–
	$-H_2O_2$[b]	0.7	2.0	ND	1.4	0.6	–
	denatured[c]	0.2	ND	ND	ND	ND	–

[a] Complete system consisted of 1 μmol substrate (I-D) or (II-D), H_2O_2, lignin peroxidase, and buffer (see the text)

[b] The same system as the complete system except that 25 mM H_2O_2 (60 μl) was replaced with distilled water (60 μl). The '$-H_2O_2$ system' also exhibited weak activity of oxidation of veratryl alcohol

[c] The same system as the complete system except that lignin peroxidase was replaced with the enzyme denatured on boiling at 100°C for 5 min

[d] Chemical structures are shown in fig.1

[e] ND, not detected

cyclic carbonates, formate and methyl oxalate (fig.2) in the presence of H_2O_2. In the accompanying paper, we show that the carboxyl carbons of the products are derived from the β-aryl group (B-ring) with ^{13}C tracer experiments. Since lignin peroxidase produces aryl cation radicals from methoxylated aromatic substrates [15,17], the aromatic ring cleavage probably proceeds via the

aryl cation radicals.

Lignin peroxidase is now known to catalyze C_α–C_β cleavage of β-*O*-4 and β-1 lignin substructure models and lignin [10–14,17] and O–C_4 cleavage of the β-*O*-4 bond to give an arylglycerol such as (IV) [11,13,14,17]. Recently, Kirk et al. [17] reported that the major consequence of ligninase (lignin peroxidase) catalyzed oxidation of veratrylglycerol-β-guaiacyl ether analogous to (I) is C_α–C_β cleavage [17]. Our quantitative results indicated that a major product was (VIII-D) in the degradation of β-guaiacyl ether (I-D) by the lignin peroxidase, whereas β-(2,6-dimethoxyphenyl) ether (II) was degraded by preferential attack on higher alkoxylated B-ring yielding no significant amount of (VIII), but giving mainly aromatic ring cleavage products and O–C_4 cleavage product (IV). Therefore, the aromatic ring cleavage was one of the main reactions catalyzed by the lignin peroxidase. It is interesting and noteworthy that aromatic ring cleavage of lignin substructure models does not need conventional dioxygenases, although the possible involvement of other enzymes including dioxygenases in biodegradation of lignin is still open.

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