Volume 242, number 2, 325-329 FEB 06647 January 1989

Cleavages of aromatic ring and β-*O*-4 bond of synthetic lignin (DHP) by lignin peroxidase

Toshiaki Umezawa and Takayoshi Higuchi

Research Section of Lignin Chemistry, Wood Research Institute, Kyoto University, Uji, Kyoto 611, Japan

Received 20 October 1988

Lignin peroxidase from a white-rot basidiomycete, *Phanerochaete chrysosporium*, catalyzed cleavages of the aromatic ring and the β-*O*-4 bond of a synthetic lignin, a dehydrogenation copolymer (DHP) of coniferyl alcohol and a (β-*O*-4)-(β-β) lignin substructure model trimer.

Aromatic ring cleavage; Lignin β-*O*-4 bond cleavage; Dehydrogenation polymer; Lignin peroxidase

1. INTRODUCTION

In 1983, a lignin degrading enzyme, lignin peroxidase (ligninase) which catalyzes cleavages of the Cα-Cβ bond and the β-*O*-4 bond of β-*O*-4 and β-1 lignin substructure model compounds, was isolated from the culture filtrate of a white-rot basidiomycete *Phanerochaete chrysosporium* [1,2]. Subsequently, we found that the enzyme catalyzed the aromatic ring cleavage of β-*O*-4 lignin substructure model compounds involving compound **(III)** [3–5], and proposed mechanisms for the ring cleavage which involved initial single-electron oxidation of the aromatic ring of the models by the enzyme to produce the corresponding aryl cation radical [6]. Mechanisms involving the aryl cation radicals were also proposed for Cα-Cβ cleavage and the β-*O*-4 bond cleavage of the lignin substructure model dimers by the enzyme [7,8]. Thus, reaction mechanisms for degradation of β-*O*-4 and β-1 lignin substructure model dimers by the enzyme are being clarified rapidly. However, the action of the enzyme on lignin, which is a complex aromatic polymer, is not fully elucidated, although several studies of incubation of lignin preparations with the enzyme were reported [1,9,10]. The elucidation of the ability of the enzyme to degrade polymeric lignin is crucial. This paper discusses cleavages of the aromatic ring and the β-*O*-4 bond of synthetic lignin (dehydrogenation polymer, DHP) by the enzyme.

Correspondence address: T. Umezawa, Research Section of Lignin Chemistry, Wood Research Institute, Kyoto University, Uji, Kyoto 611, Japan

2. MATERIALS AND METHODS

2.1. *Substrates and authentic samples*

The following compounds were prepared previously: 1,3-dihydroxy-1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propane **(III)** [11]; the acetate of 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane-2,3-cyclic carbonate **(IV)**-acetate [11]; the acetate of 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane-1,2-cyclic carbonate **(V)**-acetate [3]; the acetate of 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane-3-formate **(VI)**-acetate [12]; the acetate of 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane **(VII)**-acetate [13] and the acetate of 1-(4-ethoxy-3-methoxyphenyl)-2,3-dihydroxypropane-1-one **(VIII)**-acetate [3].

A (β-*O*-4)-(β-β) lignin substructure model trimer, 4-ethoxy-3-methoxyphenylglycerol-β-syringaresinol ether **(I)**, was prepared from syringaresinol monobenzyl ether [14] via the following steps: (i) 2-bromo-4′-ethoxy-3′-methoxyacetophenone [15]/K_2CO_3 in *N,N*-dimethylformamide (DMF), at room temperature; (ii) paraformaldehyde/K_2CO_3 in dimethyl sulfoxide, at room temperature; (iii) $NaBH_4$ in methyl alcohol/dioxane (1:1, by vol.), at 0°C; (iv) 10% Pd-C/H_2 in methyl alcohol, at room temperature. MS (acetate) *m/z* (%): 768(M^+, 4.1), 460(25.8), 419(19.8), 418(84.6), 309(12.7), 250(14.1), 249(30.9), 208(17.3), 207(100), 206(62.9), 193(10.4),

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

191(11.4), 182(13.0), 181(52.5), 179(10.5), 167(32.8), 161(11.0), 151(10.1). ^{1}H-NMR (acetate) ($CDCl_3$) δ (ppm): 1.45(t,J=7,Ph-O-C-CH_3); 1.94, 1.95, 1.98, 2.03 and 2.13(s, alcoholic OAc); 2.34(s, phenolic OAc); 3.00–3.15(m); 3.77, 3.80, 3.84 and 3.85 (s, OCH_3); 3.9–4.0(m); 4.07(q, J=7, Ph-O-CH_2-); 4.24–4.39(m); 4.50–4.60(m); 4.73(d, J=3); 4.78(d, J=4); 6.02(d, J=5); 6.10(d, J=7); 6.51, 6.54 and 6.59(s, aromatic); 6.78–6.95(m, aromatic). Coniferyl alcohol (**II**) was prepared by the method of Freudenberg and Hübner [16]. A dehydrogenation copolymer (DHP) of the trimer (**I**) and coniferyl alcohol (**II**) was prepared as follows. Compound (**I**) (0.0389 mmol, 25 mg) and compound (**II**) (0.167 mmol, 30 mg) were dissolved into 50 ml phosphate buffer (pH 6.5, 0.1 M). The solution and 50 ml of 7.4 mM H_2O_2 in the same buffer solution were added dropwise over a period of 10 h into the same buffer (50 ml) containing 0.16 mg of horseradish peroxidase (Sigma Type II), which was stirred with a magnetic stirrer at room temperature. After the addition was completed, the stirring was continued for an additional 8 h at room temperature. Then, the precipitates formed were collected by centrifugation (2851 × g, 20 min), washed twice with distilled water (25 and 35 ml), and dried under high vacuum (yield 14.5 mg). The ^{1}H-NMR spectrum of the copolymer thus prepared was similar to that of the dehydrogenation polymer prepared from (**II**), except that the former has the extra peaks which were assigned to the moiety derived from (**I**) (fig.1). 8 mg DHP thus prepared was dissolved into 2 ml DMF and 2 ml ether solution of diazoethane, prepared by the method of preparation of diazopropane [17], was added. After the solution was stirred at room temperature for 12.5 h, excess reagent and the solvent were evaporated off to give the ethylated DHP. The ethylated DHP was separated twice by gel filtration (conditions: the same as described in fig.2) in order to remove the low molecular mass fraction. The high molecular mass (void) fraction thus obtained (fig.2) was submitted to enzymatic degradation. The molecular mass calibrated with polystyrenes was estimated to be more than 2200 Da, showing its polymeric nature.

2.2. *Enzyme*

Lignin peroxidase of *P. chrysosporium* Burds. (ME-446) which was prepared and assayed as described in [3], except that purification with DEAE-Bio-Gel A was not performed, was a generous gift from Nagase Biochemicals Ltd. (Fukuchiyama, Kyoto). In some cases, the concentrated extracellular fluid of the fungus, which was prepared by a modified method reported previously [18] (Kurosaka, Hattori, Shimada and Higuchi, manuscript in preparation), was used as lignin peroxidase preparation.

2.3. *Enzymatic reaction*

To a flask containing 5 ml of 100 mM sodium tartrate buffer (pH 3.0) placed in an ultrasonic bath, 0.33 mg substrate (high molecular mass fraction of the ethylated DHP) dissolved in 30 μl DMF was added with a small glass pipette. The flask was taken out of the bath immediately after the addition of the substrate to give a uniform suspension. To the suspension were added 0.3 ml of 25 mM H_2O_2 and then 1.75 IU lignin peroxidase, and the reaction mixture was shaken at 20°C for 1 h. Lignin peroxidase (1.75 IU) and 0.3 ml of 25 mM H_2O_2 were again added 30 min after the start of the reaction. After the incubation of 1 h, the reaction mixture was extracted with ethyl acetate (50 ml), and the ethyl acetate solution was washed with a saturated NaCl solution, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue of the evaporation was acetylated (acetic anhydride/pyridine/ethyl acetate, 3:3:10, by vol., for 10 h). The acetylated products were submitted to TLC (Kieselgel 60 F_{254}, Merck, developing solvent: ethyl acetate/*n*-hexane, 1:1, by vol.) and the R_f value of the fraction which was approximately equal to those of (**IV**)-acetate, (**V**)-acetate and (**VII**)-acetate was obtained. The fraction was analyzed by GC-MS.

2.4. *Instruments*

GC-MS (EI-MS, 70eV) was carried out on a Shimadzu GCMS QP-1000 equipped with a Shimadzu SPL-G9 split/splitless injection system (splitless mode); column: Shimadzu Hi-cap CBP1-S25-050, bonded phase, methyl silicone fused silica capillary column, 0.33 mm I.D., 25 m; column temperature: 170(1 min)⟶240°C, 10°C/min. ^{1}H-NMR was recorded with a Varian XL-200 FT NMR spectrometer (TMS as an internal standard).

3. RESULTS

GC-MS analysis of the acetate of the degradation products of DHP by lignin peroxidase showed the presence of the following products (fig.3): the acetate of β,γ-cyclic carbonate (**IV**)-acetate [MS m/z (%): 310(4.4), 250(5.0), 223(2.6), 206(11.1), 181(100), 153(11.2), 151(10.6); retention time: 9.2 min]; the acetate of γ-formate (**VI**)-acetate [MS m/z (%): 354(5.0), 294(3.1), 223(3.7), 206(28.2), 181(100), 153(10.2), 151(13.2); retention time: 7.6 min]; the acetate of α-carbonylarylglycerol (**VIII**)-acetate [MS m/z: 324(2.5), 264(5.2), 222(5.0), 191(10.9), 179(100), 151(53.6), 123(11.5); retention time: 8.1 min]. Mass spectra and retention times of the degradation products were identical to those of the authentic samples: (**IV**)-acetate [MS m/z (%): 310(M^+,9.1), 250(8.6), 223(5.1), 206(9.1), 181(100), 153(14.2), 151(14.8); retention time: 9.1 min]; (**VI**)-acetate [MS m/z (%): 354(M^+,2.9), 294(3.7), 223(4.1), 206(31.4), 181(100), 153(12.6), 151(14.6); retention time: 7.5 min]; (**VIII**)-acetate [MS m/z (%): 324(M^+,3.3), 264(8.1), 222(6.2), 191(23.0), 179(100), 151(71.0), 123(17.3); retention time: 8.1 min]. Furthermore, mass chromatographic analysis of the acetate of the degradation products suggested the presence of the acetate of α,β-cyclic carbonate (**V**)-acetate and the acetate of arylglycerol (**VII**)-acetate. Retention times of parent and base ions of both the products in mass chromatography [(**V**)-acetate: 9.5 min, m/z 310 and 151; (**VII**)-acetate: 8.1 min, m/z 368 and 181]

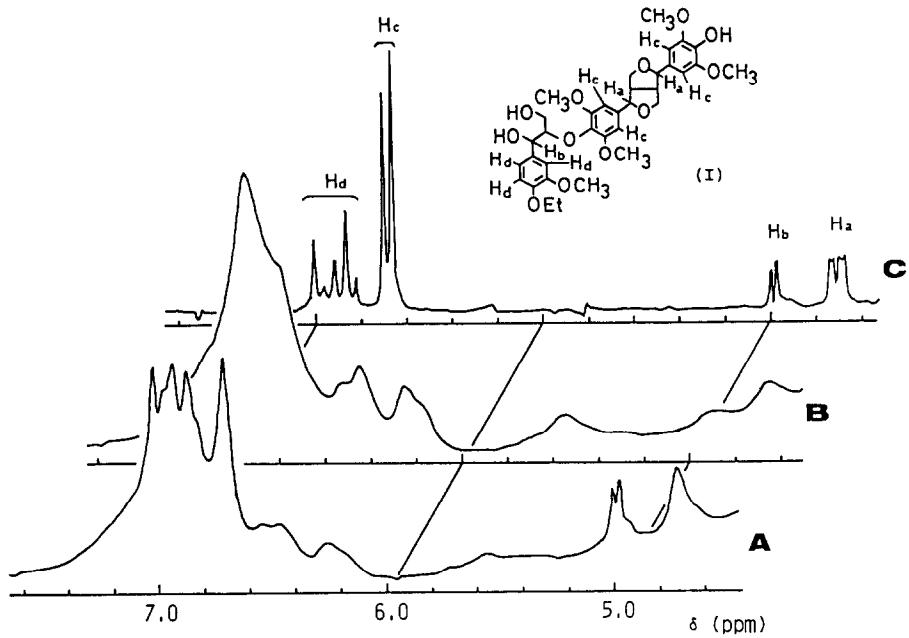

Fig.1. ^{1}H-NMR spectra of dehydrogenation polymers and compound (I). (A) Dehydrogenation copolymer of (I) and (II); (B) dehydrogenation polymer of (II); (C) compound (I). Solvent: acetone-d_6/D_2O = 8:2, by volume. The additional peaks (4.75, 5.0, 6.7, 6.9 and 7.0 ppm) in spectrum A in comparison with B are ascribed to benzyl and aromatic protons of the moieties derived from (I), which correspond to Ha-Hd in C.

were identical to those of authentic samples [(V)-acetate: 9.5 min, *m/z* 310(M^+), 151(base); (VII)-acetate: 8.1 min, *m/z* 368(M^+), 181(base)].

Control reaction systems with denatured enzyme (boiled at 100°C for 5 min) and the reaction mixture without hydrogen peroxide did not mediate the formation of these products.

It might be possible that the substrate (DHP) is partly degraded non-enzymatically during the incubation with the enzyme to produce a low molecular mass fraction. However, the possible enzymatic formation of degradation products [compounds (IV), (V), (VII) and (VIII)] from the low molecular mass fraction was eliminated as follows. DHP was incubated with the buffer at 20°C for 1 h and then the whole reaction mixture was lyophilized. The residue of lyophilization was subjected to gel filtration. The void fraction (Fr-V) and the fraction (Fr-L) elution volume, which was 25–32 ml under the same condition as in fig.2, were collected. After removal of the solvent, the Fr-L was incubated with lignin peroxidase. The products of incubation were analyzed by GC-MS as described above, but none of the degradation

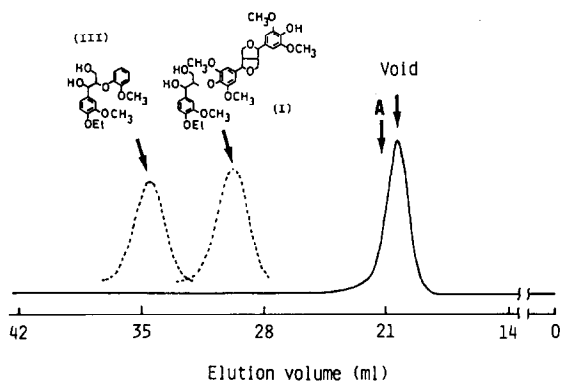

Fig.2. Gel filtration of dehydrogenation copolymer of arylglycerol-β-syringaresinol ether (I) and coniferyl alcohol (II). (Solid line) Dehydrogenation copolymer of (I) and (II) after removal of the low molecular mass fraction. The column was calibrated with (β-*O*-4)-(β-β) lignin model trimer (I) (molecular mass 642 Da); β-*O*-4 lignin model dimer (III) (molecular mass 348 Da) and polystyrenes (Chemco Scientific Co., Ltd.) of molecular mass 9000 Da, 4000 Da (void), 2200 Da (indicated by A). Thus, molecular mass of the DHP calibrated with polystyrenes was estimated as >2200 Da. Column: Sephadex LH-20, 1.1 × 48 cm. Eluent: DMF, 13.5–14.4 ml/h. Detector: refractive index detector RI-2 (Japan Analytical Industry Co., Ltd). Et = CH_2CH_3.

products [(IV)-acetate, (V)-acetate, (VI)-acetate, (VII)-acetate and (VIII)-acetate] were detected. It has generally been recognized that molecular association can markedly affect the observed elution profiles of lignin preparations separated on Sephadex columns in organic media. This effect can be suppressed by using 0.1 M LiCl in DMF as an eluent [19]. The Fr-V was, therefore, chromatographed on LH-20 in 0.1 M LiCl/DMF, but it was eluted at the void position. The fraction next to the void and thereafter (low molecular mass fraction) in the LiCl system was collected, although negligible, and incubated with lignin peroxidase after removal of LiCl by using LH-20 in DMF. The reaction product was analysed by GC-MS as above, but none of the degradation products were detected.

4. DISCUSSION

Lignin peroxidase of *Phanerochaete chrysosporium* was found to catalyze cleavages of the side chain (Cα-Cβ cleavage and β-*O*-4 bond cleavage) [1,2,7,8] and the aromatic ring [3–6,18,20–23] of low molecular mass lignin substructure model compounds and a monomeric aromatic compound, veratryl alcohol. However, the action of the enzyme on polymeric lignin has not been fully elucidated, although Tien and Kirk [1] showed the formation of Cα-Cβ cleavage products in the degradation of methylated spruce and birch lignins by the enzyme. In the present investigation, the aromatic ring cleavage and the β-*O*-4 bond cleavage of polymeric lignin (synthetic lignin, DHP) by the enzyme were demonstrated for the first time (fig.3).

The present results showed that lignin peroxidase degraded DHP to give the β,γ-cyclic carbonate of arylglycerol (**IV**), the formate of arylglycerol (**VI**), and α-carbonylarylglycerol (**VIII**). The formation of α,β-cyclic carbonate (**V**) and arylglycerol (**VII**) was suggested by mass chromatography. These results demonstrated that lignin peroxidase cleaved the aromatic ring of DHP as well as its β-*O*-4 bond, since these compounds were identified previously as aromatic ring cleavage products [(**IV**), (**V**) and (**VI**)] and β-*O*-4 bond cleavage products [(**VII**) and (**VIII**)] of β-*O*-4 lignin substructure model dimers [such as (**III**)] and a (β-*O*-4)-(β-*O*-4) lignin substructure model trimer by the enzyme [3,4,23].

Previously, we proposed the mechanism for aromatic ring cleavage of β-*O*-4 lignin substructure models by the enzyme, which involves an initial single-electron oxidation of the aromatic ring by the enzyme, followed by reactions with H_2O and O_2 [6]. Furthermore, our previous experiments showed that demethylation (or demethoxylation) was not a prerequisite for the aromatic ring cleavage by the enzyme [4]. Since the present study showed that lignin peroxidase catalyzed the aromatic ring cleavage of the synthetic lignin, the mechanism involving a single-electron oxidation and no demeth(ox)ylation proposed previously for the ring cleavage of β-*O*-4 lignin model dimers by the enzyme [4,6] is probably the case with aromatic ring cleavage of the polymeric lignin by the enzyme. The mechanism is completely different from the hypothetical mechanism for aromatic ring cleavage of lignin by *P. chrysosporium* proposed by Chen et al. [24], which involves demethylation prior to aromatic ring cleavage.

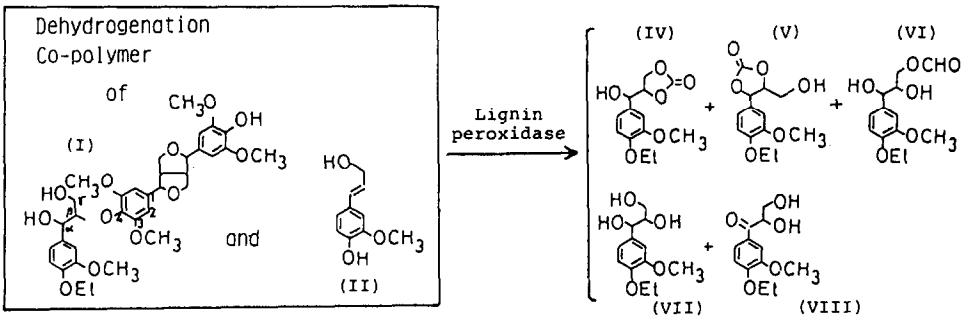

Fig.3. Dehydrogenation copolymer of arylglycerol-β-syringaresinol ether (**I**) and coniferyl alcohol (**II**), and products of its degradation by lignin peroxidase. Et = CH_2CH_3.

Acknowledgement: This research was partly supported by a Grant-in-Aid for Scientific Research (nos 60440015, 62760135, 63760137) from the Ministry of Education of Japan.

REFERENCES

[1] Tien, M. and Kirk, T.K. (1983) Science 221, 661–663.
[2] Glenn, J.K., Morgan, M.A., Mayfield, M.B., Kuwahara, M. and Gold, M.H. (1983) Biochem. Biophys. Res. Commun. 114, 1077–1083.
[3] Umezawa, T., Shimada, M., Higuchi, T. and Kusai, K. (1986) FEBS Lett. 205, 287–292.
[4] Umezawa, T. and Higuchi, T. (1986) FEBS Lett. 205, 293–298.
[5] Umezawa, T. and Higuchi, T. (1987) Agric. Biol. Chem. 51, 2281–2284.
[6] Umezawa, T. and Higuchi, T. (1987) FEBS Lett. 218, 255–260.
[7] Kirk, T.K., Tien, M., Kersten, P.J., Mozuch, M.D. and Kalyanaraman, B. (1986) Biochem. J. 236, 279–287.
[8] Miki, K., Renganathan, V. and Gold, M.H. (1986) Biochemistry 25, 4790–4796.
[9] Haemmerli, S.D., Leisola, M.S.A. and Fiechter, A. (1986) FEMS Microbiol. Lett. 35, 33–36.
[10] Odier, E., Mozuch, M., Kalyanaraman, B. and Kirk, T.K. (1987) in: Lignin Enzymic and Microbial Degradation (Odier, E. ed.) pp.131–136, INRA, Paris.
[11] Umezawa, T. and Higuchi, T. (1985) FEBS Lett. 182, 257–259.
[12] Kawai, S., Umezawa, T. and Higuchi, T. (1985) Agric. Biol. Chem. 49, 2325–2330.
[13] Umezawa, T. and Higuchi, T. (1984) Agric. Biol. Chem. 48, 1917–1921.
[14] Kamaya, Y., Nakatsubo, F. and Higuchi, T. (1980) Mokuzai Gakkaishi 26, 471–475.
[15] Umezawa, T., Higuchi, T. and Nakatsubo, F. (1983) Agric. Biol. Chem. 47, 2945–2948.
[16] Freudenberg, K. and Hübner, H.H. (1952) Ber. 85, 1181–1191.
[17] Dyer, J.R., Randall, R.B., jr and Deutsch, H.M. (1964) J. Org. Chem. 29, 3423–3424.
[18] Shimada, M., Hattori, T., Umezawa, T., Higuchi, T. and Uzura, K. (1987) FEBS Lett. 221, 327–331.
[19] Connors, W.J., Sarkanen, S. and McCarthy, J.L. (1980) Holzforschung 34, 80–85.
[20] Miki, K,. Renganathan, V., Mayfield, M.B. and Gold, M.H. (1987) FEBS Lett. 210, 199–203.
[21] Leisola, M.S.A., Schmidt, B., Thanei-Wyss, U. and Fiechter, A. (1985) FEBS Lett. 189, 267–270.
[22] Haemmerli, S.D., Schoemaker, H.E., Schmidt, H.W.H. and Leisola, M.S.A. (1987) FEBS Lett. 220, 149–154.
[23] Umezawa, T. and Higuchi, T. (1988) Mokuzai Gakkaishi, in press.
[24] Chen, C.-L., Chang, H.-M. and Kirk, T.K. (1983) J. Wood Chem. Technol. 3, 35–57.