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Regiospecific oxygenations during ring cleavage of a secondary metabolite, 3,4-dimethoxybenzyl alcohol catalyzed by lignin peroxidase

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Enzymatic oxidation of veratryl alcohol yielded a new ring cleavage product (δ-lactone) in addition to the two known γ-lactone products. The experiment with ^{18}O-enriched water and dioxygen clearly showed that one oxygen atom each from water and dioxygen is specifically incorporated into the cleavage product at the original C_3 or C_4 position of 3,4-dimethoxybenzyl alcohol. A new type of reaction mechanism proposed for the ring cleavage of this compound is rationally explained in good accord with the one-electron transfer mechanism.

Aromatic ring cleavage; Veratryl alcohol; Ligninase; Oxygenation; Regiospecificity; (White-rot fungus)

1. INTRODUCTION

3,4-Dimethoxybenzyl alcohol (veratryl alcohol) is a secondary metabolite produced by white-rot fungi [1,2], which was first shown to be biosynthesized from L-phenylalanine by a lignin-degrading basidiomycete *Phanerochaete chrysosporium* [3]. Although physiological and biochemical correlations between biosynthesis of this secondary metabolite and biodegradation of lignin [4–6] have been demonstrated, the biological significance of the secondary metabolism of veratryl alcohol has not yet been fully elucidated. In this context, it is noteworthy that lignin peroxidase (LPO) isolated from cultures of the same white-rot fungus [7,8] catalyzes the unique oxidative ring cleavage of monomeric veratryl alcohol [9] and dimeric lignin model compounds [10–12]. Therefore, it is also of great importance to elucidate the mechanism of ring cleavage of the natural non-phenolic lignin model compound in connection with mechanisms for aromatic ring cleavages of lignin polymer [13], which contrasts sharply with the prominent ring opening of catechols catalyzed by dioxygenases [14].

We report here a new type of regiospecific oxygenation mechanism for the LPO-catalyzed ring cleavage of veratryl alcohol, which is distinct from the mechanism previously suggested by Schoemaker et al. [15]. A 6-membered lactone isolated as a new enzymatic cleavage product is also reported.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Veratryl alcohol was purchased from Tokyo Kasei Chemicals and thoroughly purified by thin-layer chromatography with hexane:ethyl acetate (1:1) before use.

^{18}O-enriched dioxygen (99.7 atom%) and water (98.4 atom%) were procured from Amersham and Shoko, respectively.

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2.2. *Ligninase preparation*

LPO or ligninase was prepared by a modified method of Tien and Kirk [7] from a ligninolytic culture of *P. chrysosporium* Burds (ME-446). An extracellular fraction of the culture filtrate (10 l) was concentrated to about 50 ml by use of a Millipore ultrafiltration system. The concentrate (0.64 mg protein/ml) was used as the LPO enzyme solution, which exhibits an activity of 3.75 IU/ml with veratryl alcohol used as substrate when spectrophotometrically assayed [7]. Alternatively, however, an electrophoretically homogeneous enzyme preparation (0.215 mg protein with specific activity of 6.5 IU/mg protein; 42 kDa) was obtained by chromatography on DEAE-Bio-Gel A (1.6 × 30 cm column) with 10 mM succinate buffer (pH 5.5, 0–0.25 M NaCl gradient), and finally on Sephadex G-100.

2.3. *Experiment with ^{18}O-enriched dioxygen and water*

The enzymatic reaction was carried out as follows. The reaction mixture (final volume, 5 ml) containing 50 μmol veratryl alcohol, 12.5 μmol hydrogen peroxide, 4.0 ml of 0.2 M tartrate buffer (pH 3.1) and 0.08 ml crude enzyme preparation was incubated under a [^{18}O]dioxygen atmosphere (99.8 atom%) at room temperature for 30 min.

For the experiments investigating the incorporation of oxygen from water, 1.38 ml [^{18}O]water (98.0 atom%) was diluted to 71.5 atom% by addition of 0.290 ml of 0.4 M tartrate buffer (pH 3.1), other aqueous solutions containing veratryl alcohol (20 μmol), 0.5 M H_2O_2 (10 μl each × 6) and 0.16 ml enzyme.

2.4. *Isolation of ring cleavage product*

The reaction was terminated by extraction with ethyl acetate. The extract was evaporated and the concentrate was submitted to TLC with benzene:ethyl acetate (4:1). The lactone isomers were identified by measurement of spectra with ^1H-NMR and GC-MS spectrometers as described [16].

2.5. *Instruments*

Mass spectra of the compounds were recorded by use of a Shimadzu GC-MS QP-1000 (EI-MS, 70 eV) equipped with a Shimadzu SPL-G9 split/splitless injection system (splitless mode), column: 25 m × 0.33 mm, i.d., chemically bonded-fused silica capillary column HiCap BP1 (non-polar methylsilicone polymer, Shimadzu) at 100–200°C with the program: isothermal at 100°C for the first 1 min and then 10°C/min.

3. RESULTS

3.1. *Product identification*

We confirmed first that oxidation of veratryl alcohol (I) with the purified enzyme preparation yields the previously reported γ-lactone (IIa,IIb) as the ring cleavage products accompanied by the major product veratraldehyde (III). However, alternative oxidation of (I) with the crude enzyme preparation which was used in this case on account of the shortage of purified enzyme gave a 6-membered lactone (IIc) as a new cleavage product. The chemical structure of this δ-lactone was determined by both mass- and ^1H-NMR spectrometry as described in [16,17]. These enzymatic oxidation products of (I) are shown in fig.1.

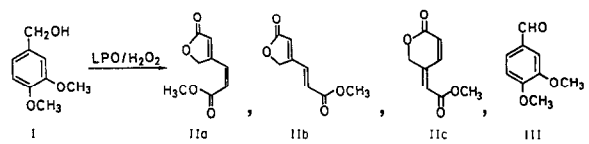

Fig.1. Oxidation of veratryl alcohol (I) catalyzed by the lignin peroxidase (LPO)/H_2O_2 system in the presence of dioxygen.

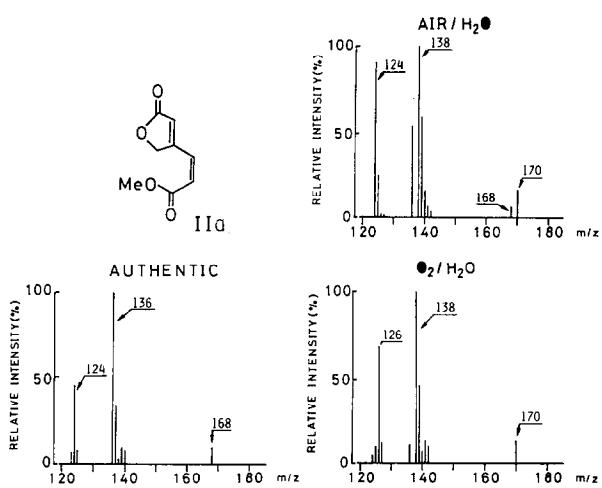

Fig.2. Mass spectra of the 5-membered lactone (IIa) oxygenated with dioxygen and water during the ring cleavage of veratryl alcohol catalyzed by the LPO system. (●) ^{18}O atom.

3.2. *Incorporation of the ^{18}O atom from $H_2{}^{18}O$*

Mass spectrometry (fig.2) clearly indicates that only one ^{18}O atom from $H_2{}^{18}O$ was incorporated into the lactone isomer (IIa), since the molecular ion peak is not detected at *m/z* 172 but *m/z* 170 shifted by 2 mass units from the molecular ion peak (*m/z* 168) of the authentic lactone (IIa). The content of ^{18}O atom in the labeled product (IIa) was calculated to be 71 atom% by measurement of the peak areas of mass chromatograms observed at *m/z* 168 and 170. Thus, the degree of ^{18}O incorporation was determined as nearly 100%. Similarly, 100% ^{18}O incorporation was also determined for the *trans*-lactone isomer (IIb). However, the degree of ^{18}O incorporation for the lactone (IIc) was not determined because of the unavailability of (IIc) in sufficient amounts in this case.

3.3. *Oxygenation with $^{18}O_2$*

Fig.2 also clearly shows that one ^{18}O atom from the labeled dioxygen was incorporated into both lactones (IIa,IIb), since the molecular ion peak is observed exclusively at *m/z* 170. Similarly, the degrees of ^{18}O incorporation into the *cis* (IIa) and *trans* isomers (IIb) were calculated to be 91 and 95%, respectively.

It is noteworthy that the parent ion of the product (IIa) labeled with [^{18}O]water still retains the ^{18}O atom in the fragment at *m/z* 138 which might be formed by loss of methanol but almost completely loses the label at *m/z* 124 possibly due to loss of the labeled CO_2. On the other hand, (IIa) oxygenated with [^{18}O]dioxygen retains the label in significant amounts in both fragments at *m/z* 138 and 126, which suggests that neither the methanol nor CO_2 lost in this case contains the ^{18}O atom.

Control experiments with the lactone (IIa) in $H_2{}^{18}O$ or ^{18}O-enriched buffer (pH 3.0) with an equal amount of the crude enzyme protein showed that the oxygens in IIa do not exchange with water.

4. DISCUSSION

Since the enzymatic mechanisms for the aromatic ring cleavage of lignin have not yet been completely clarified, a mechanistic study of the ring

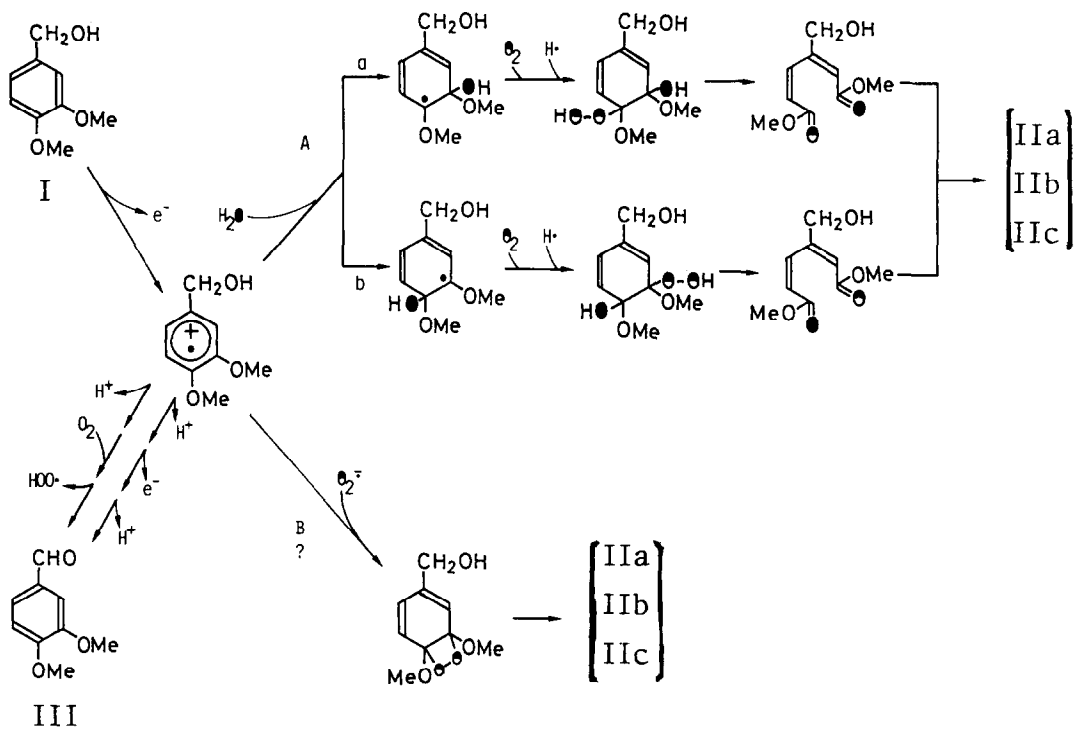

Fig.3. Oxygenation mechanism proposed for the aromatic ring cleavage of veratryl alcohol with LPO system. (●,⊖) ^{18}O label for water and dioxygen, respectively. Chemical structures of IIa, IIb and IIc are the same as indicated in fig.1.

cleavage of the natural secondary metabolite veratryl alcohol is an ideal model in its simplicity not only for a comprehensive understanding of the ring cleavages of complex lignin polymers, but also for providing knowledge of its intrinsic biochemical relevance to lignin degradation manifested by white-rot fungi.

First, as predicted by the biomimetic oxidation of veratryl alcohol with the hemin catalyst [17], the present investigation has successfully shown that the 6-membered lactone (IIc) is, in fact, also produced as a ring cleavage product from the enzymatic reaction mixture in addition to the previously reported 5-membered lactones [9]. The result indicates that *β*-hydroxymethyl *cis-,cis*-muconate dimethyl ester as the possible initial cleavage product is cyclized in two different modes to form such *γ*- and *δ*-lactones concomitant with the liberation of methanol, although methanol was not determined as the reaction product.

Second, the experiment with ^{18}O label provides such striking evidence that the lignin peroxidase exhibits the unprecedented regiospecific monooxygenation with either water or dioxygen at the original C_3 or C_4 position of the 3,4-dimethoxybenzyl alcohol substrate (fig.2). Thus, we propose a new mechanism for the oxygenative ring cleavage of veratryl alcohol catalyzed by LPO (route A in fig.3). According to this mechanism, veratryl alcohol undergoes one-electron transfer with compound I of LPO [18–20], yielding the aryl cation radical and its C_3 or C_4 position is nucleophilically attacked first by water and then by added dioxygen. Finally, a possible hydroxylated cyclohexadienyl hydroperoxide intermediate thus formed undergoes C_3–C_4 bond cleavage concomitantly with O–O bond cleavage, yielding *β*-hydroxymethyl *cis-,cis*-muconate dimethyl ester as a possible initial product, which is cyclized to form *γ*- or *δ*-lactones as described above.

It is not yet conclusive whether the enzyme system chose route a or b during the cleavage reaction, although route Aa might be more plausible than route Ab (fig.3), since the major fragmentations of the parent ion of authentic IIb yielding the daughter ions at *m/z* 124 and 136 (fig.2) are most reasonably interpreted by loss of CO_2 from the lactone ring and methanol from the methyl ester moiety, respectively. Thus, the results indicate that the oxygen atom in the lactone carbonyl is derived from water, i.e. water might attack specifically the C_3 position of the substrate. However, route B in fig.3 involving $O_2^{\cdot-}$ followed by dioxetane intermediate is eliminated although such a ring opening of the aryl cation radical was suggested by Schoemaker et al. [15], since no doubly ^{18}O-labeled products were obtained in our enzyme system. Furthermore, participation of $O_2^{\cdot-}$ might be eliminated, even if it is formed, since it seems to decay spontaneously under this acidic condition [21]. Thus, the ring cleavage of 3,4-dimethoxybenzyl alcohol is distinguished, either from dioxygenative intradiol or extradiol cleavages of catechols catalyzed by the commonly known dioxygenases [14,22,23].

In conclusion, the present findings are consistent, in principle, with results quite recently obtained for the ring cleavage of a dimeric lignin model compound [24] although the regiospecific oxygenation mechanism was not reported. The mechanism proposed here could be applied to the aromatic cleavages of lignin in good accord with the one-electron transfer mechanism proposed for ligninase [18–20] and enzyme model systems [25].

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