

Original

Aromatic Ring Opening of Lignin Monomeric Model Compounds by Lignin Peroxidase^{*1}

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Introduction

During photosynthesis 10^{11} – 10^{12} tons of terrestrial biomass is estimated to be produced every year^{1,2)} and 15–36% of this lignocellulosic biomass of gymnosperms and angiosperms is lignin^{3,4)}.

Lignin occurs in cell walls of true vascular plants, ferns and club mosses but not in those of mosses, algae and microorganisms^{4,5)}. Lignin is a phenylpropanoid structural polymer which binds the fiber cell walls together, and gives the plants ri-

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gidity. Lignin also decreases water permeation across the cell walls of xylem tissue makes the wood more resistant to attack by microorganisms^{3, 6-8)}.

In the late 1960s the chemical structure of lignin became clear⁹⁻¹²⁾. Lignin is a three-dimensional aromatic polymer formed by the dehydrogenative polymerization of monolignoles such as *p*-coumaryl, coniferyl and sinapyl alcohols by peroxidase. Therefore, in contrast to cellulose lignin contains various ether and carbon-carbon linkages such as arylglycerol- β -aryl ether (β -O-4), phenylcoumaran (β -5), biphenyl (5-5'), 1,2-diarylpropane-1,3-diol (β -1), non cyclic benzyl aryl ether (α -O-4), pinoresinol (β - β'), diphenyl ether (5-O-4)¹²⁾.

Lignin has no stereoregularity in its structure and is resistant to microbial attack. However, lignin is ultimately degraded to humus, water and carbon dioxide by microorganisms¹³⁾.

Lignin biodegradation research is important for several reasons. Next to cellulose, lignin is the most abundant renewable materials. For the biotechnical production of useful products from lignin and the improvement of pulping and bleaching processes in the pulp and paper industry, a detail knowledge of the mechanisms of biological lignin degradation is necessary. Furthermore, on the pure scientific point of view, elucidation of the lignin biodegradation mechanisms is essential for understanding the earth's carbon cycle.

Lignin is degraded by various microorganisms, such as Actinomycetes, Fungi imperfecti, Ascomycetes and Basidiomycetes. On the basis of the type of decay, lignin degrading fungi can be classified as white-rot, brown-rot and soft-rot fungi. White-rot fungi (*Phanerochaete chrysosporium* (= *Sporotrichum pulverulentum*), *Coriolus* (*Polyporus*, *Trametes*, *Polystictus*) *versicolor*, *Phlebia radiata*, *Lentinula* (= *Lentinus*) *edodes* (*Shiitake*)) are the best lignin degraders and have been used for lignin biodegradation research.

Until the early 1980, microbial lignin degradation study had focused on at least two important objectives: 1. Analysis of the decayed lignin isolated from decayed wood. 2. Establishment of the optimal parameters for ligninolytic culture of fungi.

As for 1, many investigators studied on analysis of decayed lignin isolated from decayed wood and the results were discussed and reviewed¹⁴⁾. At least the three modes of degradative reactions were suggested to occur in the decomposition of lignin macromolecules by white-rot fungi: (i) Oxidative cleavage of side chains between α - and β - carbons leading to the formation of aromatic acids, (ii) cleavage of β -aryl ether bonds and modification of side chain structures, and (iii) degradation of aromatic nuclei through oxidative ring opening.

As for 2, Kirk *et al.*¹⁵⁾ established the optimal culture parameters for *P. chrysosporium* with nitrogen-limiting media at high O₂ partial pressure. Stationary cultures are required for expression of the ligninolytic activity. Furthermore, lignin is degraded

only during secondary metabolism which is triggered mainly by nitrogen starvation¹⁶⁾ concomitant with production of 3,4-dimethoxybenzyl alcohol [veratryl alcohol (I)] as a secondary metabolite¹⁷⁾. Later Jefferis *et al.*¹⁸⁾ and Buswell *et al.*¹⁹⁾ found that secondary metabolism can also be triggered by carbohydrate or sulfur starvation.

Lignin degradation mechanisms by *P. chrysosporium* in ligninolytic culture were elucidated mainly by Higuchi and co-workers to demonstrate Ca-C β cleavage and β -O-4 cleavage as the major degradation modes²⁰⁾. In these studies instead of a complex lignin polymer, several types of lignin substructure model compounds, especially β -O-4 type, were synthesized²¹⁾ and used to elucidate their degradation mechanisms.

In 1983, a novel enzyme which is capable of oxidizing non-phenolic lignin and lignin models was isolated from ligninolytic cultures of the white-rot fungus, *P. chrysosporium*^{22, 23)}. The discovery of the lignin degrading enzyme (now named Lignin peroxidase) provoked a world wide research effort and competition.

Research effort was focused on at least two aims: 1. characterization of lignin peroxidase, and 2. rationalization of the fungal lignin degradation modes by the activity of lignin peroxidase.

As for 1, the extracellular lignin peroxidase has been purified and characterized^{24, 25)}. It has a molecular weight of 41000–42000 dalton and an isoelectric point of 3.5. The enzyme is a glycoprotein that contains about 15% carbohydrate (or 11% according to Paszczynski *et al.*²⁶⁾) and a heme prosthetic group of iron protoporphyrin IX. The native enzyme gives a solet band at 407 nm. The enzyme was first described as a unique H₂O₂-requiring oxygenase^{24, 25)}. However, Renganathan and Gold²⁷⁾ showed the five redox state of lignin peroxidase similar to those of horse radish peroxidase by electronic absorption spectroscopy. Hence the enzyme was defined to be not oxygenase but peroxidase.

As for 2, Kersten *et al.*²⁸⁾ showed by ESR spectroscopy that 1,4-dimethoxybenzene was converted to *p*-benzoquinone and methanol via an aryl cation radical and proposed that Ca-C β cleavage and all other reactions catalyzed by lignin peroxidase proceed via aryl cation radicals.

Indeed, some of the reactions such as Ca-C β and β -O-4 bond cleavage were found to be catalyzed by an extracellular lignin-degrading enzyme (lignin peroxidase) of *P. chrysosporium*^{29–34)}. However, it was not elucidated yet whether aromatic ring opening of lignin is also catalyzed by the same lignin peroxidase, although earlier studies of the polymeric lignin degradation suggested the involvement of ring opening reactions¹⁴⁾.

In 1985, when the present author started this research, Umezawa and Higuchi³⁷⁾ reported for the first time aromatic ring opening reaction of β -O-4 lignin substructure models in ligninolytic culture of *P. chrysosporium*. Furthermore Leisola *et al.*³⁸⁾ reported

for the first time the aromatic ring opening reaction of veratryl alcohol (I) by lignin peroxidase. The present author took a great interest in such type of aromatic ring opening reaction because of the following three reasons: 1. Veratryl alcohol (I) is synthesized from phenylalanine via 3,4-dimethoxycinnamyl alcohol and veratrylglycerol by *P. chrysosporium*³⁹⁾ and is metabolized to CO₂¹⁷⁾. Therefore, aromatic ring opening reaction is most important in its metabolism by *P. chrysosporium*. 2. Although aromatic ring opening of 1,2,4-trihydroxybenzene⁴⁰⁾ by a dioxygenase of *Sporotrichum pulverulentum* (= *Phanerochaete chrysosporium*) had been reported, the aromatic ring opening reaction catalyzed by extracellular enzyme (lignin peroxidase) was reported for the first time. 3. Veratryl alcohol (I) had been used as the simplest lignin model compound and the ring opening reaction of veratryl alcohol (I) has a relevancy to aromatic ring opening reaction of lignin polymer.

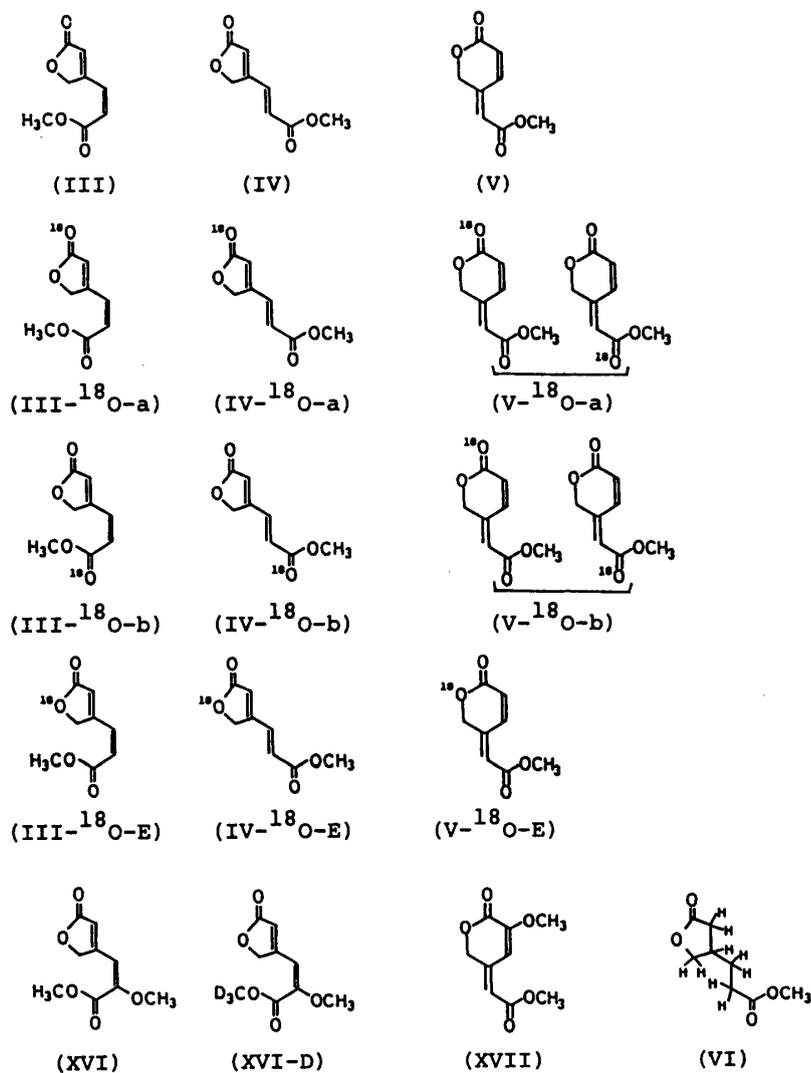


Fig. 1. Structural formulae of the compounds.

HATTORI: Aromatic Ring Opening of Veratryl Alcohol

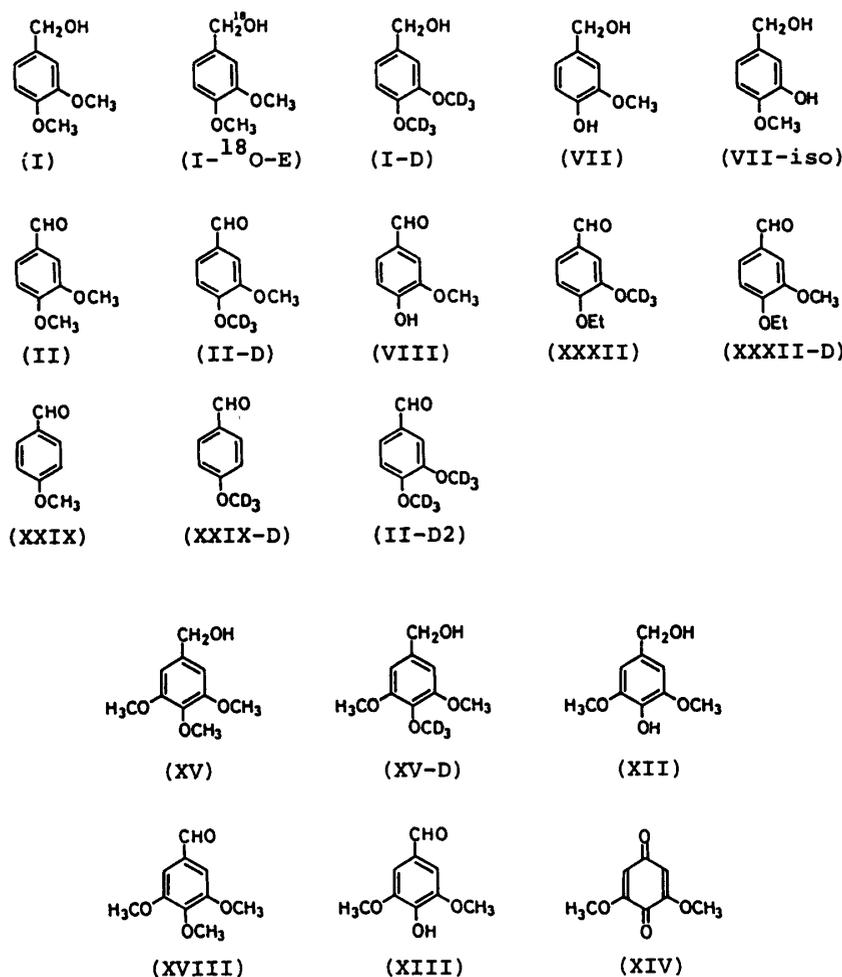


Fig. 1. (cont'd)

The present author, first of all, investigated the aromatic ring opening reaction of veratryl alcohol (I). Chemical structures of substrates, degradation products, etc. are shown in Fig. 1. The symbol "Ac" in the registry number of a compound represents acetate, (I-Ac): acetate of (I). A new ring opening product (*Z*)-6-Oxo-2H-pyran-3(6H)-ylideneacetic acid methyl ester [δ -lactone (V)] was found to be formed in addition to the two known (*Z*)-5-Oxo-2H-furan-3(5H)-ylpropenoic acid methyl ester [*cis*- γ -lactone (III)] and (*E*)-5-Oxo-2H-furan-3(5H)-ylpropenoic acid methyl ester [*trans*- γ -lactone (IV)]. The experiment with ¹⁸O-enriched water and dioxygen clearly showed that one oxygen atom each from water and dioxygen was regiospecifically incorporated into the cleavage product (*cis*- γ -lactone, III) at the original C₃ and C₄ positions of veratryl alcohol (I), respectively⁴¹. This reaction mechanism for the ring opening of this compound is rationally explained in good accordance with the one-electron transfer mechanism^{30, 42}. (Chapter 1, Section 1.1).

In the section 1.2, "Biomimetic approach for lignin degradation" was described.

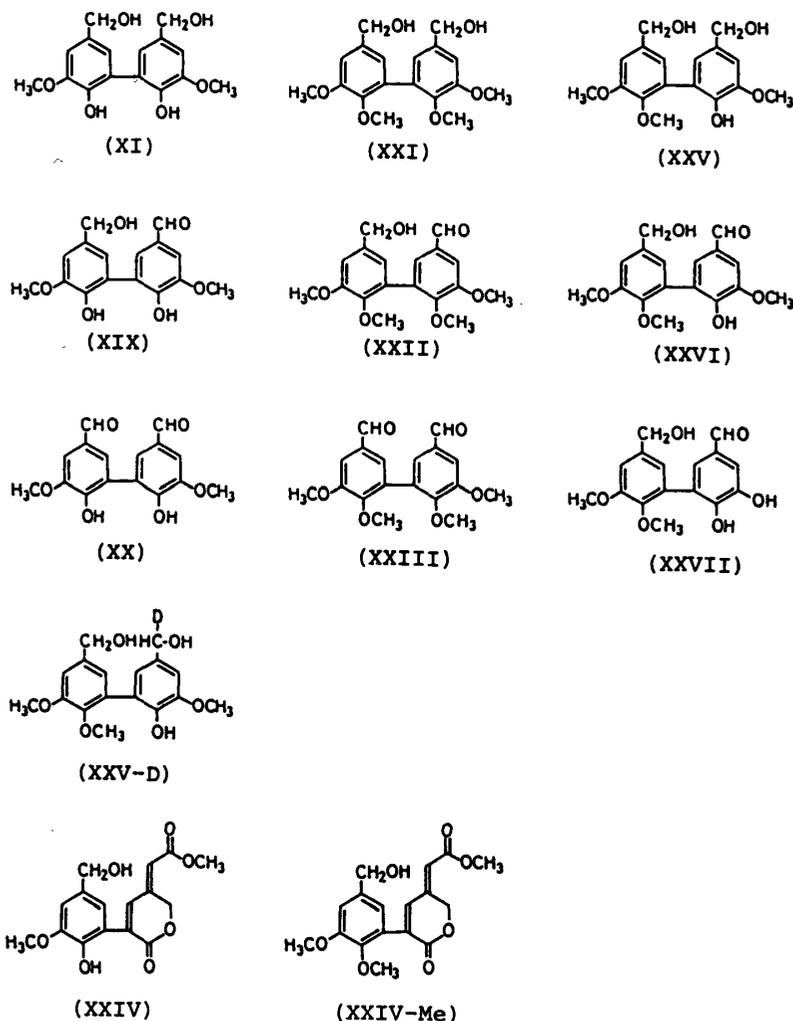


Fig. 1. (cont'd)

“Biomimetic chemistry” is related to fundamental and applied research fields in life sciences. The biomimetic chemistry has mainly two purposes. The first one is to develop new materials and systems which not only mimic the biological function but also serve for practical uses in industry. Robots and biomimetic catalysts are examples of human beings and enzymes, respectively. The second purpose is to understand or to explain the complex functions of biosystems by use of their biomimetic systems. Lignin peroxidase is a heme protein containing protoporphyrin IX. Such porphyrin have therefore been tested for their ability to replace lignin peroxidase.

Shimada *et al.*⁴³⁾ found that β -1 model compound was successfully degraded by tetraphenylporphyrinatoiron (III) chloride with *tert*-butylhydroperoxide [*t*-BHP]. Habe *et al.*^{29, 44)} found that hemin, which is a natural iron porphyrin, catalyzed Ca-C β cleavage of 1,2-bis(4-methoxyphenyl)propane-1,3-diol (XXVIII). However aromatic ring opening reaction of nonphenolic compound by the catalysts had not been reported.

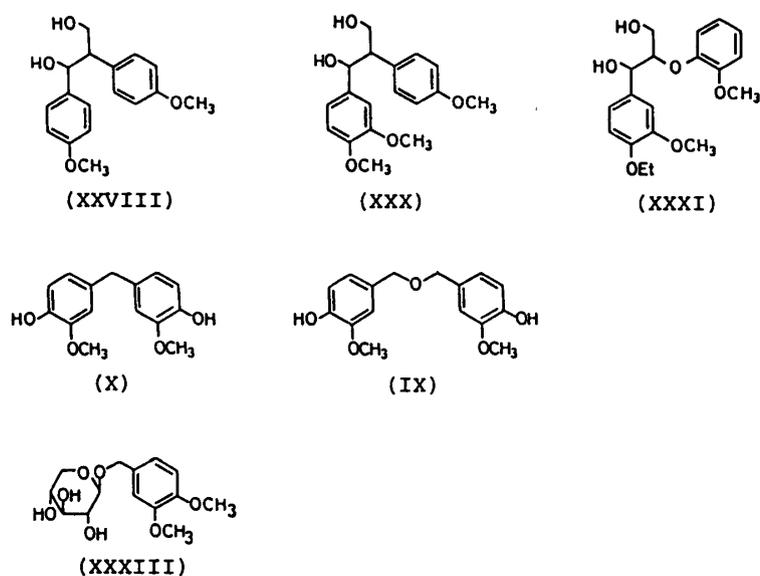


Fig. 1. (cont'd)

The present author investigated the aromatic ring opening of veratryl alcohol (I) by hemin. Three aromatic ring opening products (III, IV and V) were formed and tracer experiment with ^{18}O atom showed that the regiospecific oxygenation to yield aromatic ring opening products by lignin peroxidase was not governed by protein moiety of lignin peroxidase⁴⁵.

In chapter 2 phenolic ring opening reaction by lignin peroxidase was described (section 2.1) by use of 4-hydroxy-3-methoxybenzyl alcohol [vanillyl alcohol (VII)]. As described in chapter 1, lignin peroxidase was found to catalyze nonphenolic aromatic ring opening reaction of veratryl alcohol (I). However, phenolic guaiacyl units which not only present in protolignin¹²) but also newly formed after the $\text{C}\alpha\text{-C}\beta$ bond cleavage followed by ether bond cleavage of the lignin substrate⁴⁶) were reported to polymerize by lignin peroxidase⁴⁷⁻⁴⁹). Therefore it is of great importance to examine whether the phenolic moieties of the guaiacyl units in lignin undergo only condensation but not other reactions such as ring opening reaction.

The enzymatic degradation of vanillyl alcohol (VII) gave polymerized products such as dehydrodivanillyl alcohol (XI) as major product accompanied with a small amount of aromatic ring opening product (V)⁵⁰). Therefore it is also examined whether or not the aromatic ring opening reaction of dehydrodivanillyl alcohol (XI) and other biphenyl compounds proceed. The results showed that although nonphenolic biphenyl compound (dehydrodiveratryl alcohol XXI) was degraded to give aromatic ring opening product, no ring opening product from phenolic ring of biphenyl moiety was detected⁵¹). Hence it was suggested that lignin peroxidase predominantly degrades nonphenolic aromatic ring rather than phenolic ring. Furthermore if poly-

merization to give biphenyl moiety does not proceed during wood decay process, the phenolic ring opening reaction may occur in lignin biodegradation.

In chapter 3 the present author focused on the role of veratryl alcohol (I) during wood decay process in relation to its metabolism described in chapter 1. Veratryl alcohol (I) has been reported to induce lignin peroxidase⁵²⁾ to protect lignin peroxidase from inactivation^{47, 53, 54)} and to be a radical mediator in the degradation of the lignin substrate which is poorer substrate than veratryl alcohol (I)⁵⁵⁾. Furthermore veratryl alcohol (I) was found to be converted to veratryl β -D-xyloside (XXXIII) in the presence of holocellulose and xylan in the ligninolytic culture of *P. chrysosporium*⁵⁶⁾. However no experimental evidence has been reported whether veratryl alcohol (I) mediates C α -C β bond cleavage of lignin substructure model compounds. Furthermore present author suspected that veratryl β -D-xyloside (XXXIII) might be a radical mediator as veratryl alcohol (I). The effects of veratryl alcohol (I) and veratryl β -D-xyloside (XXXIII) in the enzymatic degradation system of β -1 and β -O-4 substructure model compounds were compared. It was found that the addition of a catalytic amount of veratryl alcohol (I) or its xyloside (XXXIII) enhanced the C α -C β bond cleavage reaction of β -1 but not β -O-4 substructure model compound^{57, 58)}.

Chapter 1

Mechanisms for Aromatic Ring Opening of 3,4-Dimethoxybenzyl Alcohol by Lignin Peroxidase and its Model Catalyst

Section 1.1

Aromatic Ring Opening of Veratryl Alcohol by Lignin Peroxidase

1.1.1 Introduction

Leisola *et al.*³⁸⁾ first reported aromatic ring opening reaction of veratryl alcohol (I) by lignin peroxidase, although the structure of the aromatic ring opening products proposed were tentative. After that, the aromatic ring opening reaction of β -O-4 lignin substructure model dimer by lignin peroxidase was also reported^{31, 59)}. It is of great importance to elucidate the mechanism of ring opening of veratryl alcohol (I) by the following two reasons. First, the elucidation of reaction mechanism for ring opening of veratryl alcohol (I) is connected with not only natural metabolism of veratryl alcohol (I) but also the mechanism for aromatic ring opening of lignin polymer. Second, the aromatic ring opening reaction by lignin peroxidase contrasts sharply with the prominent ring opening of catechol catalyzed by dioxygenase⁶⁰⁾.

In section 1.1 is described a new type of regiospecific oxygenation mechanism for ring opening of veratryl alcohol (I) catalyzed by the lignin peroxidase, which is distinct from the mechanisms previously suggested by Schoemaker *et al.*⁶¹⁾.

1.1.2 Experimental

Preparation of lignin peroxidase

The electrophoretically homogeneous lignin peroxidase prepared from a ligninolytic culture of *P. chrysosporium* Burds (ME-446) by the modified method of Tien and Kirk²⁵⁾ was kindly provided by Nagase Biochemicals. Lignin peroxidase activity was determined spectrophotometrically by measuring the absorption at 310 nm by use of veratryl alcohol (I) as substrate ($\epsilon=9300 \text{ M}^{-1}\text{cm}^{-1}$ at 310 nm for 3,4-dimethoxybenzaldehyde [veratraldehyde (II)]²⁴⁾. One kat of enzyme activity was defined as the amount of enzyme protein which produced 1 mol of veratraldehyde (II) per second from veratryl alcohol (I) at 20°C.

Degradation of veratryl alcohol (I) catalyzed by lignin peroxidase

To a reaction mixture (final volume, 10 ml) containing veratryl alcohol (I, 100 μmol), lignin peroxidase (0.7 μkat) and 9.3 ml of 0.2 M tartrate buffer (pH 3.0), 5 μmol H_2O_2 was added 15 times at 5 min intervals at room temperature under air. After 75 min from the first addition of H_2O_2 , the reaction products containing ring opening products (*cis*- γ -lactone (III), *trans*- γ -lactone (IV) and δ -lactone (V)) were extracted with ethyl acetate and submitted to GC-MS. Enzymatic reaction was repeated three times in order to obtain enough amount of products for $^1\text{H-NMR}$.

Identification of the *cis*- γ -lactone (III) and *trans*- γ -lactone (IV)

In order to determine the structure of *cis*- γ -lactone (III) and *trans*- γ -lactone (IV), about 250 μg of each γ -lactone was hydrogenated by $\text{H}_2/\text{Pd-C}$ in methanol at room temperature for 10 min. The reduced products (3-carboxyethyl- γ -butyrolactone methyl ester, VI) were analyzed by $^1\text{H-NMR}$ and GC-MS.

Tracer experiment with $^{18}\text{O}_2$

The reaction mixture (10 ml) containing veratryl alcohol (I, 100 μmol) and 9.3 ml of 0.2 M tartrate buffer (pH 3.0) was evacuated and flushed with argon gas. The exchange of gas phase was repeated 5 times, in order to remove free dioxygen. After the final evacuation, $^{18}\text{O}_2$ was introduced into the evacuated reaction vessel by use of a syringe and lignin peroxidase (0.7 μkat) was injected with another syringe. The reaction was initiated by addition of 5 μmol H_2O_2 and the same amount of H_2O_2 was further added 14 times at 5 min intervals at room temperature. After 90 min, the reaction products were extracted with ethyl acetate. The ring opening products separated by TLC were analyzed by GC-MS.

Tracer experiment with H_2^{18}O

To a reaction mixture containing veratryl alcohol (I, 20 μmol), lignin peroxidase (0.23 μkat) and 900 μl of H_2^{18}O , 200 μl of 0.4 M tartrate buffer (pH 3.0), 1.25 μmol H_2O_2 was added 10 times at 3 min intervals at room temperature under air. After 60 min from the first addition of H_2O_2 the reaction products were extracted with ethyl acetate. The ring opening products separated by TLC were individually analyzed

by GC-MS.

Preparation of lactones (III-¹⁸O-E, IV-¹⁸O-E) labeled with ¹⁸O at the ethereal oxygens of lactone ring moiety

To a reaction mixture containing veratraldehyde (II, 90 μmol), 200 μl of H_2^{18}O (¹⁸O: 98.4 atom%) and 5 drops of anhydrous THF, one drop of 1N-HCl was added. The reaction mixture was stirred at 40°C under N_2 . After 2.5 h the reaction solution was cooled to 0°C in an ice bath and 1 ml of anhydrous THF, the 280 μmol NaBH_4 were added. After 10 min, 3,4-dimethoxybenzyl-[¹⁸O]-alcohol (I-¹⁸O-E) was extracted with ethyl acetate and purified by TLC. To a reaction mixture containing 3,4-dimethoxybenzyl-[¹⁸O]-alcohol (I-¹⁸O-E, 21 μmol), 1.5 ml of 0.2 M tartrate buffer (pH 3.0) and lignin peroxidase (0.23 μkat), 1.25 μmol H_2O_2 was added 20 times at 3 min intervals at room temperature. The reaction products containing γ -lactones (V-¹⁸O-E and III-¹⁸O-E, IV-¹⁸O-E, respectively) were extracted with ethyl acetate and separated by TLC and analyzed by GC-MS.

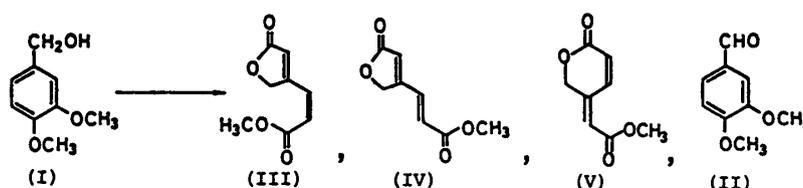


Fig. 2. Enzymatic oxidation of substrate (I) and its products (II, III, IV and V).

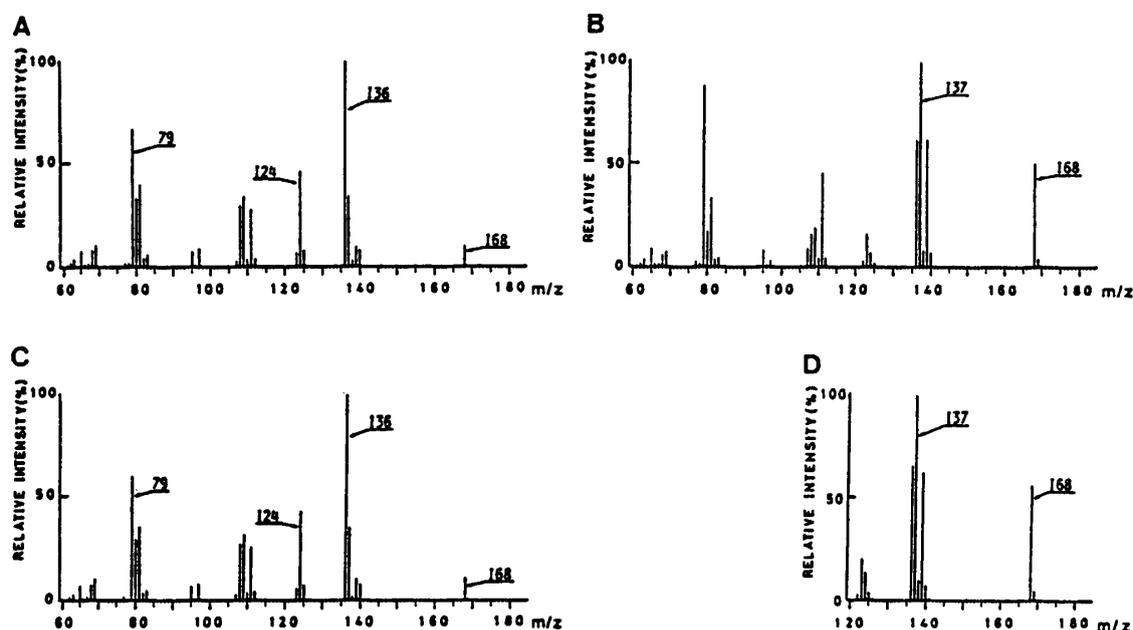


Fig. 3. Mass spectra of the products (III and IV) obtained from enzyme system. A, authentic *cis*- γ -lactone (III); B, authentic *trans*- γ -lactone (IV); C, *cis*- γ -lactone (III) obtained from enzyme system; D, *trans*- γ -lactone (IV) obtained from enzyme system.

1.1.3 Results

Product identification

The enzymatic degradation of veratryl alcohol (I) yielded *cis*- γ -lactone (III), *trans*- γ -lactone (IV), and δ -lactone (V) as the aromatic ring opening products accompanied by veratraldehyde (II) as the major product (Fig. 2). The control experiment omitting the enzyme or H₂O₂ did not yield such ring opening products. Mass-spectra of the products (III, IV) were identical with those of authentic lactones (Fig. 3).

The mass-spectra of the reduced compounds of (III) and (IV) (i.e. (VI)) gave the same fragmentation pattern of EI-MS as shown in Fig. 4, although they did not give the molecular ion peak at *m/z* 172. Therefore (III) and (IV) are isomers with each other. The chemical structure of (VI) was determined by comparison of its

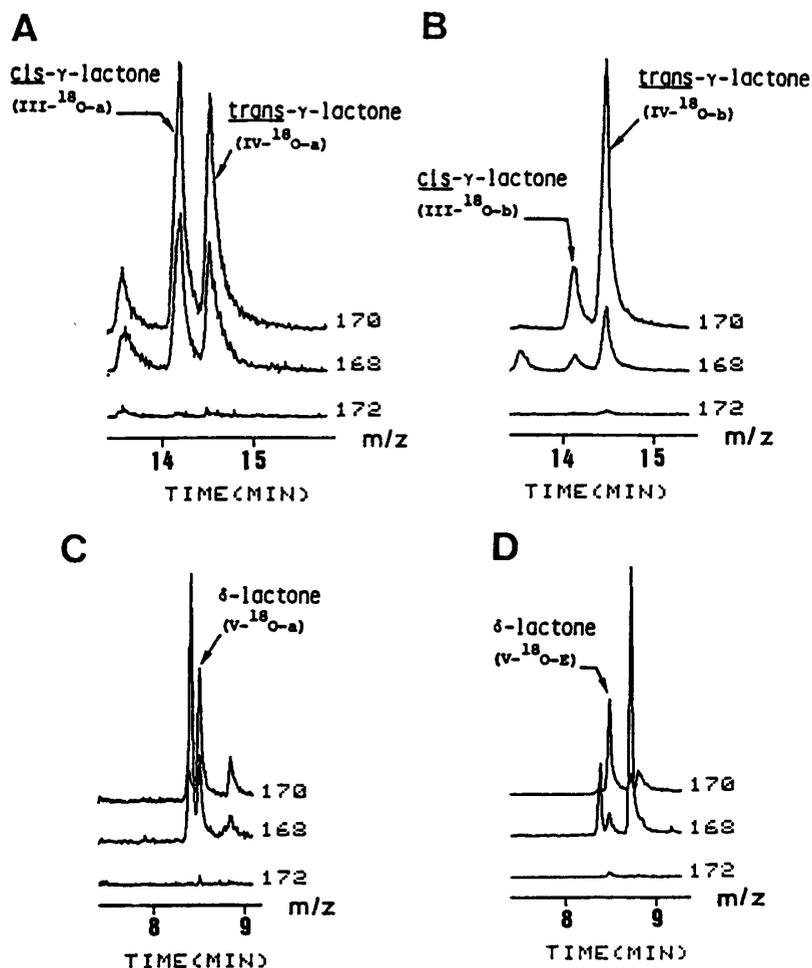


Fig. 6. Mass chromatograms of the fragment ions for *m/z* 168, 170 and 172 of the γ -lactones (III and IV) and δ -lactone (V).

A, γ -lactones oxygenated with ¹⁸O from H₂¹⁸O (III-¹⁸O-a and IV-¹⁸O-a);
 B, γ -lactones oxygenated with ¹⁸O from ¹⁸O₂ (III-¹⁸O-b and IV-¹⁸O-b);
 C, δ -lactone oxygenated with ¹⁸O from H₂¹⁸O (V-¹⁸O-a); D, δ -lactone oxygenated with ¹⁸O from ¹⁸O₂ (V-¹⁸O-b).

$^1\text{H-NMR}$ spectrum (Fig. 5) with that of authentic (VI) reported by Ceder and Hanson⁶²). The chemical structure of the δ -lactone (V) was determined by comparison of its $^1\text{H-NMR}$ and mass spectra with those of δ -lactone (V) obtained in the biomimetic experiment (section 1.2).

Incorporation of the ^{18}O atom from $^{18}\text{O}_2$ and H_2^{18}O

Fig. 6 shows the ion peak at m/z 170 which was formed by incorporation of only one ^{18}O atom. Incorporated oxygen of lactones did not exchange with water. The relative intensity of the ion peak at m/z 172 was negligible. Thus the result clearly shows that the only one ^{18}O atom each from either $^{18}\text{O}_2$ and H_2^{18}O was incorporated into the lactones (III, IV and V). The contents of ^{18}O -incorporated were determined

Table 1. Incorporation of ^{18}O from H_2^{18}O and $^{18}\text{O}_2$ into lactones in enzyme system.

Reaction system	^{18}O -Incorporation (%)		
	<i>cis</i> - γ -lactone	<i>trans</i> - γ -lactone	δ -lactone
with H_2^{18}O	100	100	100
with $^{18}\text{O}_2$	86	90	84

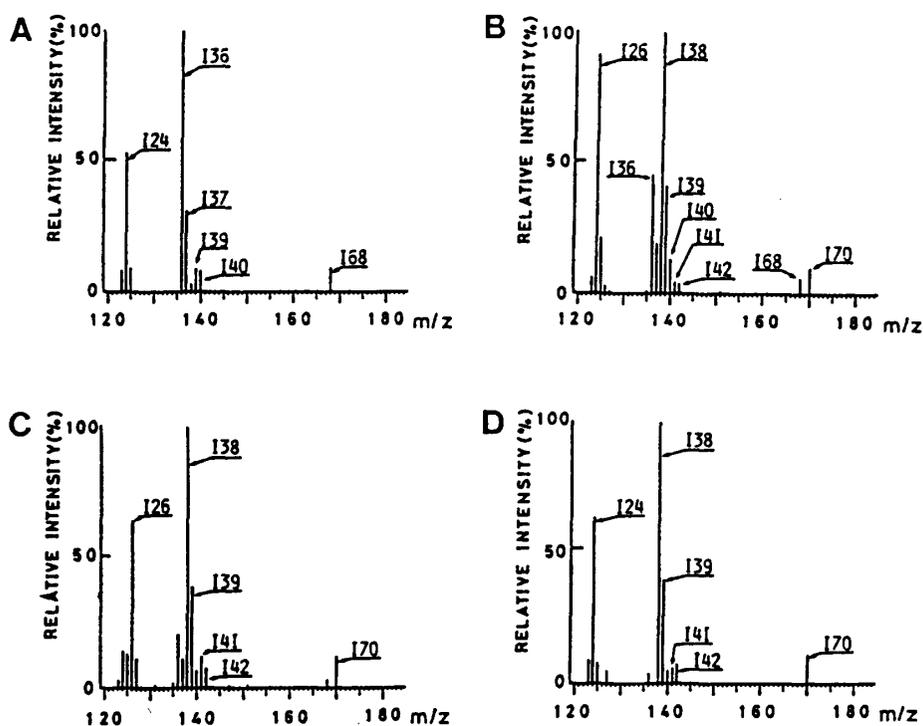


Fig. 7. Mass spectra of the *cis*- γ -lactone species formed in enzyme system. A, normal *cis*- γ -lactone (III); B, *cis*- γ -lactone oxygenated with ^{18}O from H_2^{18}O (III- ^{18}O -a); C, *cis*- γ -lactone oxygenated with ^{18}O from $^{18}\text{O}_2$ (III- ^{18}O -b); D, *cis*- γ -lactone labeled with ^{18}O at the ethereal oxygen of lactone ring moiety (III- ^{18}O -E).

by account of the mass-chromatographic peak areas at m/z 168 and 170. The incorporation degree was calculated by the following equation and summarized in Table 1.

$$\frac{^{18}\text{O} \text{ content of products}}{\text{original } ^{18}\text{O} \text{ content of water dioxxygen (\%) or labeled substrates}} \times 100 = ^{18}\text{O}\text{-incorporation (\%)}$$

The comparison of the ratio of the ion peak at m/z 124 to that at m/z 126 *cis*- γ -Lactone (III)

The mass spectra of normal *cis*- γ -lactone (III), *cis*- γ -lactone enzymatically oxygenated with H_2^{18}O (III- ^{18}O -a), or with $^{18}\text{O}_2$ (III- ^{18}O -b), *cis*- γ -lactone labeled with

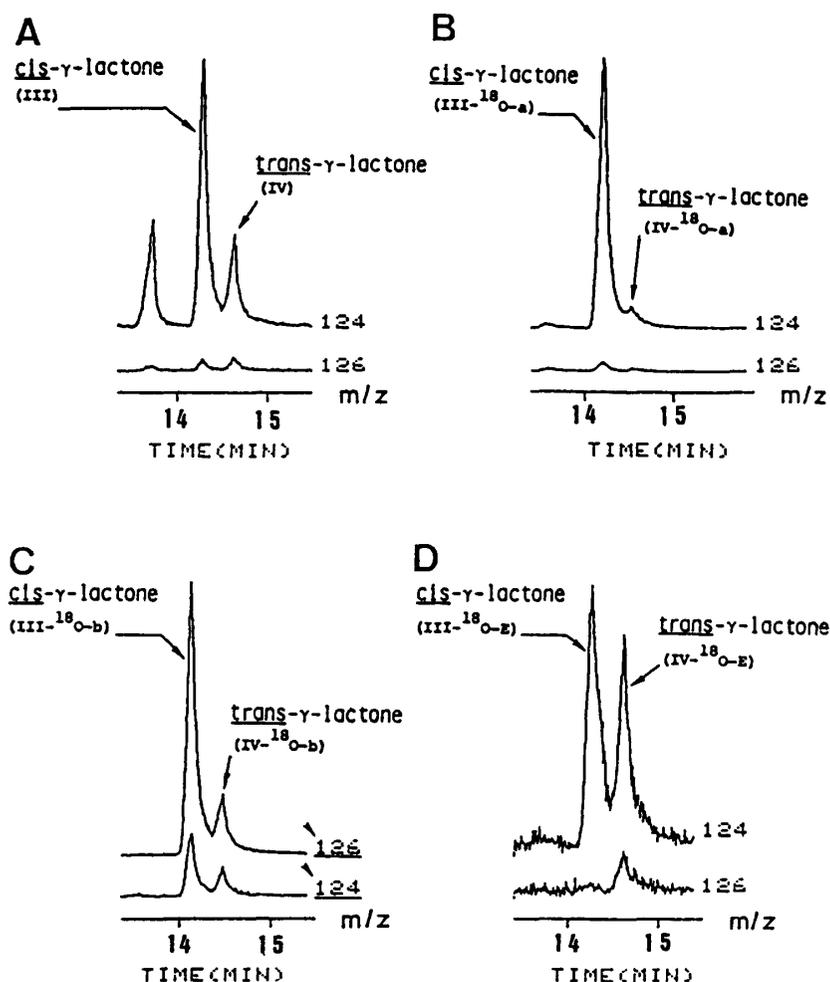


Fig. 8. Mass chromatograms of fragment ions for m/z 124 and 126 of the γ -lactone species.

A, normal γ -lactones (III and IV); B, γ -lactones oxygenated with ^{18}O from H_2^{18}O (III- ^{18}O -a and IV- ^{18}O -a); C, γ -lactones oxygenated with $^{18}\text{O}_2$ (III- ^{18}O -b and IV- ^{18}O -b); D, γ -lactones labeled with ^{18}O at the ethereal oxygen of lactone ring moiety (III- ^{18}O -E and IV- ^{18}O -E).

Table 2. Ratio of the relative intensities of the peaks at m/z 126 to that at m/z 124 in enzyme system.

Lactone species	m/z 126 / m/z 124	
	Ratio	Relative value
III	0.032	1
III- ^{18}O -a	0.035	1
III- ^{18}O -b	5.2	163
III- ^{18}O -E	0.023	0.7

^{18}O atom at the ethereal oxygen of lactone moiety (96 atom %) (III- ^{18}O -E) are shown in Fig. 7. Alternatively, Fig. 8 shows mass-chromatograms of the ions at m/z 124 and 126 of eight different species of *cis*- and *trans*- γ -lactones (III to IV- ^{18}O -E) differently labeled with ^{18}O . The ratios of the ion peak area at m/z 126 to that at m/z 124 of these 4 *cis*- γ -lactone species are summarized in Table 2.

The result in Fig. 8 shows that no significant ion peak at m/z 126 was observed for all the lactones (III, III- ^{18}O -a and III- ^{18}O -E) except for (III- ^{18}O -b). The ratio of the ion peak area at m/z 126 to that at m/z 124 are 0.032, 0.035 and 0.23 for (III),

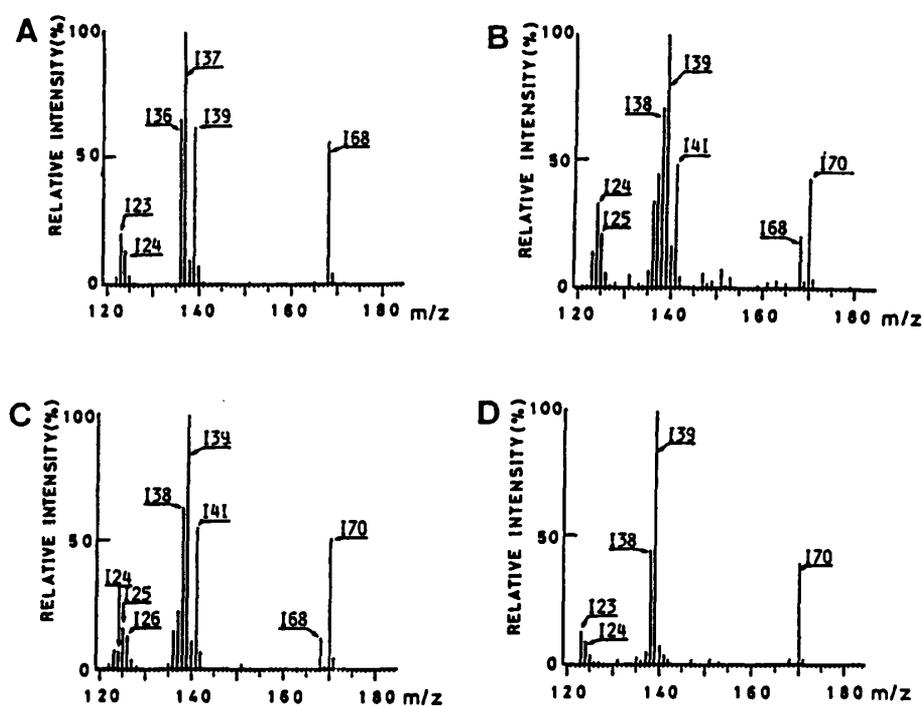


Fig. 9. Mass spectra of the *trans*- γ -lactone species formed in enzyme system. A, normal *trans*- γ -lactone (IV); B, *trans*- γ -lactone oxygenated with ^{18}O from H_2^{18}O (IV- ^{18}O -a); C, *trans*- γ -lactone oxygenated with ^{18}O from $^{18}\text{O}_2$ (IV- ^{18}O -b); D, *trans*- γ -lactone labeled with ^{18}O at the ethereal oxygen of lactone ring moiety (IV- ^{18}O -E).

(III- ^{18}O -a) and (III- ^{18}O -E), respectively, whereas the ratio of (III- ^{18}O -b) was 5.2 or 163-fold greater than that for (III). The contrasted spectra clearly indicate that the ^{18}O from H_2O and O_2 was introduced into the opposite position of the benzene ring.

trans- γ -Lactone (IV)

Figure 9 shows the mass-spectra of normal *trans*- γ -lactone (IV), ^{18}O -incorporated *trans*- γ -lactone from H_2^{18}O (IV- ^{18}O -a) and $^{18}\text{O}_2$ (IV- ^{18}O -b), *trans*- γ -lactone labeled

Table 3. Ratio of the relative intensities of the peaks at m/z 126 to that at m/z 124 in enzyme system.

Lactone species	m/z 126 / m/z 124	
	Ratio	Relative value
IV	0.17	1
IV- ^{18}O -a	*0	—
IV- ^{18}O -b	1.73	10
IV- ^{18}O -E	0.16	0.9

* The accurate value could not be calculated.

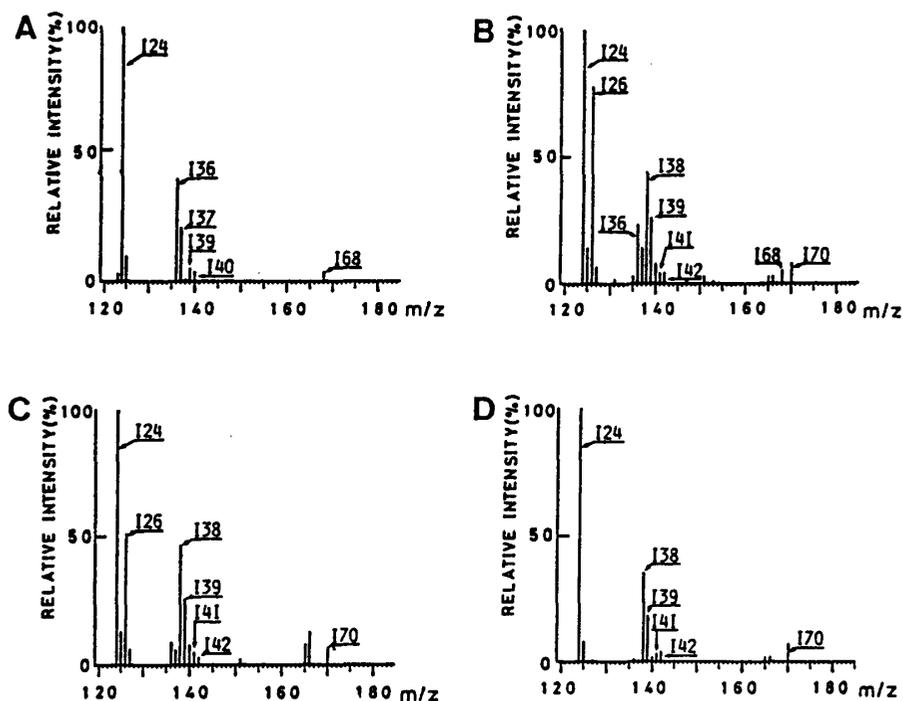


Fig. 10. Mass spectra of the δ -lactone species formed in enzyme system. A, normal δ -lactone (V); B, δ -lactone oxygenated with ^{18}O from H_2^{18}O (V- ^{18}O -a); C, δ -lactone oxygenated with ^{18}O from $^{18}\text{O}_2$ (V- ^{18}O -b); D, δ -lactone labeled with ^{18}O at the etheral oxygen of lactone ring moiety.

with ^{18}O at the ethereal oxygen of lactone moiety (96 atom %) (IV- ^{18}O -E).

The ratios of the peak area for m/z 126 to that for m/z 124 shown in mass-chromatograms (Fig. 8) were calculated in the same way as described for *cis*- γ -lactones of (III)-(III- ^{18}O -a) (Table 3). Table 3 shows that (IV- ^{18}O -E) (labeled with ^{18}O atom at the ethereal oxygen of lactone moiety) gave nearly the same ratio of the peak area for m/z 126 to that for m/z 124 as in normal *trans*- γ -lactone (IV) (0.17 and 0.16 for (IV) and (IV- ^{18}O -E), respectively as given in Table 3). Similarly, (IV- ^{18}O -a) (oxygenated with H_2^{18}O) gave greater fragment ion peak at m/z 124 than that at m/z 126. However, the ratio of the relative intensities of two peaks could not be measured accurately due to the low intensities.

On the contrary, (IV- ^{18}O -b) (oxygenated with $^{18}\text{O}_2$) gave the 10-fold greater ratio than those of (IV) and (IV- ^{18}O -E). The result indicates that oxygenation pattern with dioxygen is completely different from that with water.

δ -Lactone (V)

The mass spectra of normal δ -lactone (V), δ -lactone oxygenated H_2^{18}O (V- ^{18}O -a), δ -lactone oxygenated $^{18}\text{O}_2$ (V- ^{18}O -b), δ -lactone labeled with ^{18}O at the ethereal oxygen of lactone moiety (96 atom %) (V- ^{18}O -E) are shown in Fig. 10.

The peaks at m/z 136, 137, 139 and 140 observed for (V) were considered to correspond to the peaks at m/z 138, 139, 141 and 142, respectively in the ^{18}O -labeled δ -lactones (V- ^{18}O -a, V- ^{18}O -b, and V- ^{18}O -E). However, all these lactones gave the base peak at m/z 124, whereas only (V- ^{18}O -a) and (V- ^{18}O -b) gave the ion peak at m/z 126, during their fragmentations. The mass-chromatograms of δ -lactone species

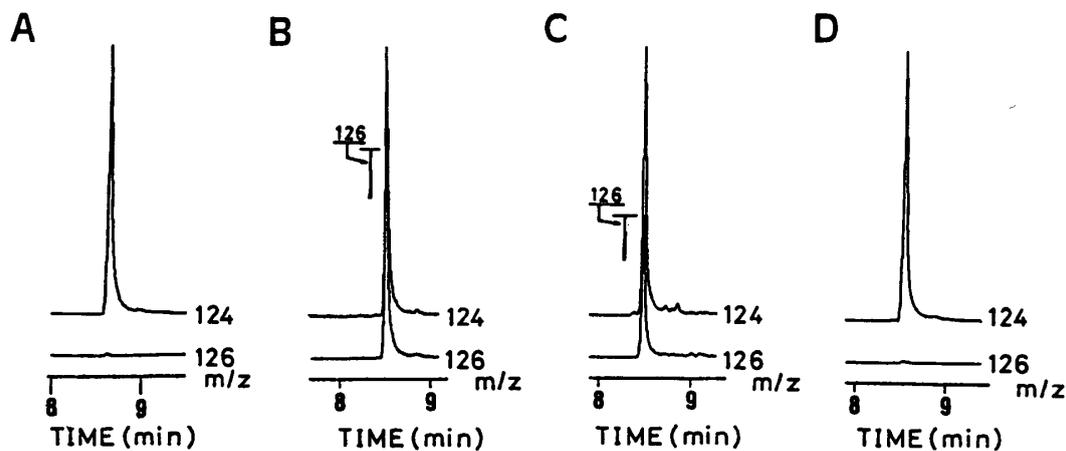


Fig. 11. Mass chromatograms of the fragment ion for m/z 124 and 126 of the δ -lactone species.

A, normal δ -lactone (V); B, δ -lactone oxygenated with ^{18}O from H_2^{18}O (V- ^{18}O -a); C, δ -lactone oxygenated with $^{18}\text{O}_2$ (V- ^{18}O -b); D, δ -lactone labeled with ^{18}O at the ethereal oxygen of lactone ring moiety (V- ^{18}O -E).

Table 4. Ratio of the relative intensities of the peaks at m/z 126 to that at m/z 124 in enzyme system.

Lactone species	m/z 126 / m/z 124	
	Ratio	Relative value
V	0.015	1
V- ¹⁸ O-a	0.73	52
V- ¹⁸ O-b	0.56	37
V- ¹⁸ O-E	0.014	1

(V, V-¹⁸O-a, V-¹⁸O-b, and V-¹⁸O-E) and their ratios of the peaks for m/z 126 to that for m/z 124 are shown in Fig. 11 and Table 4, respectively. Table 4 shows that the ratio (0.014) obtained for (V-¹⁸O-E) is almost equal to that (0.015) obtained for (V). On the contrary, the ratio of (V-¹⁸O-a) (0.73) and (V-¹⁸O-b) (0.56) are sharply different from those of (V) and (V-¹⁸O-E) in mass-chromatograms.

1.1.4 Discussion

The reaction mechanisms for the enzymatic formation of the ring opening products, (III, IV and V), from veratryl alcohol (I) is discussed on the basis of the mass-spectrometric analyses as follows: 1. the ring opening of the substrate and recyclization of the initial ring opening products yielding (III), (IV), and (V). 2. the role of H₂O and O₂ in the ring opening reaction, and 3. the interpretation of ¹⁸O atom incorporation from H₂¹⁸O and ¹⁸O₂ into the lactones (III, IV and V), 4. the unique regiospecific oxygenation with water and dioxygen in the ring opening reaction.

The aromatic ring opening of the veratryl alcohol (I)

The chemical structures of (III) and (IV), in addition to a new ring opening product (V) were established⁴¹⁾. A common reaction intermediate for those lactones might be produced. Although 3-hydroxymethyl-(*cis, cis*)-muconate dimethyl ester is suspected to be the initial ring opening product from (I), such a dimethyl ester could not be isolated from the reaction mixture. Probably because, the dimethyl ester is rapidly converted to (III) and (V) by cyclization of the alcoholic hydroxyl group with C₃ or with C₄ atom, respectively by liberation of methanol. However, Umezawa and Higuchi⁶³⁾ successfully determined the formation of methylmuconate of 1,3-diethoxy-1-(4-ethoxy-3-methoxyphenyl)-2-propanol from 1-(4-ethoxy-3-methoxyphenyl)-1,3-diethoxy-2-(2-methoxyphenoxy)-propane in the lignin peroxidase-catalyzed reaction. Furthermore, Schmidt *et al.*⁶⁴⁾ reported aromatic ring opening reaction of 3,4-dimethoxybenzyl methyl ether to yield 3-(methoxymethyl)-(*cis, cis*)-muconate dimethyl ester, therefore 3-hydroxymethyl-(*cis, cis*)-muconate dimethyl ester might be a reasonable intermediate, in this enzymatic reaction system.

The role of H₂O and O₂ in aromatic ring opening of veratryl alcohol (I)

The tracer experiments with ¹⁸O₂ and H₂¹⁸O showed that one ¹⁸O atom each from water and dioxygen was incorporated almost quantitatively into the ring opening products.

The involvement of water in the oxygenative ring opening of the aromatic compound is in sharp contrast to the dioxygenase-catalyzed ring opening of catechol substrates in which water is not introduced into the ring opening products with exception of only a few case⁶⁵⁾. Since lignin peroxidase is known to catalyze the generation of aryl cation radicals from methoxylated aromatic substrates²⁸⁾, it is suggested that this lignin peroxidase-catalyzed aromatic ring opening of veratryl alcohol (I) also proceeds via the aryl cation radical intermediate, which is attacked first by water nucleophilically at C₃ or C₄ of the original benzene ring, followed by radical addition of dioxygen. The possible cyclodienyl hydroperoxide intermediate thus formed prior to the ring opening may undergoes C₃-C₄ bond cleavage, yielding the postulated 3-hydroxymethyl-(*cis, cis*)-muconate dimethyl ester and water (Fig. 12 route A). As for the role of dioxygen in aromatic ring opening of veratryl alcohol (I), Schoemaker *et al.*⁶¹⁾ suggested that two oxygen atoms from O₂ might be incorporated into the products via O⁻ followed by a dioxetane intermediate, yielding the same ring opening products i.e. *cis*- γ -lactone (III) and *trans*- γ -lactone (IV) dioxygenated with only dioxygen molecule (Fig. 12 route B). In contrast, it is shown that

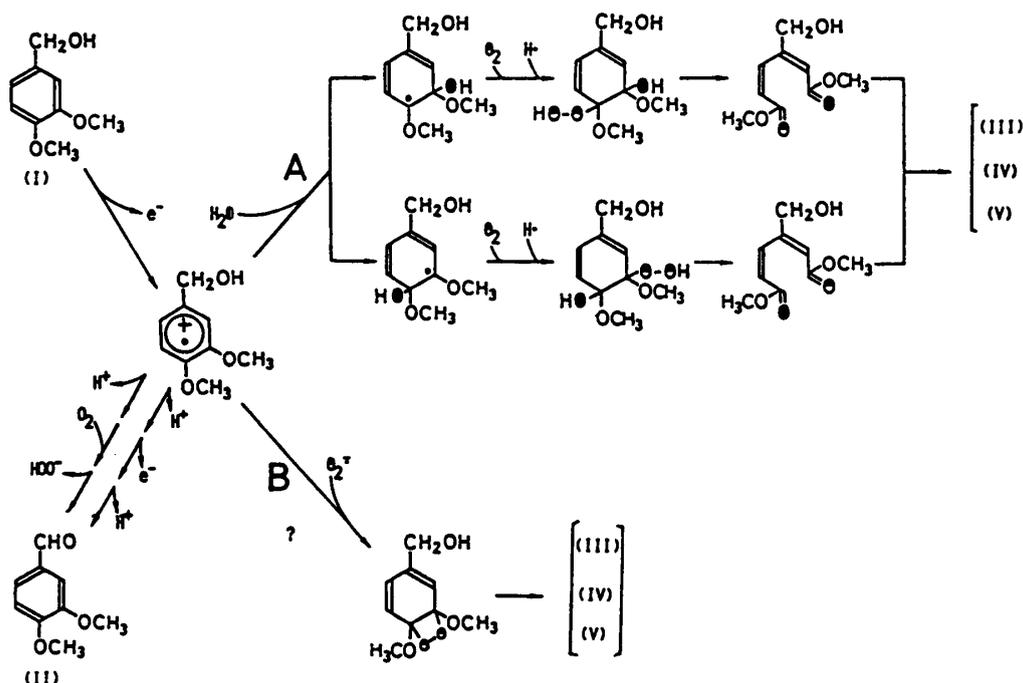


Fig. 12. Proposed mechanisms for the aromatic ring opening reaction of veratryl alcohol (I) with incorporation of water and monooxygen.

the present author has shown that such a ring opening reaction does not occur in this enzymatic reaction, since only one ^{18}O atom was incorporated from $^{18}\text{O}_2$ as described above. Furthermore, Umezawa and Higuchi⁶⁶⁾ reported that ^{18}O -labeling investigations for the formation of methyl *cis*-, *cis*-muconate of 1,3-diethoxy-1-(4-ethoxy-3-methoxyphenyl)-1,3-diethoxy-2-(2-methoxyphenoxy)-propane, have conclusively eliminated the possibility of dioxetane intermediate during the ring opening of the dimeric lignin substructure model compound.

Alternatively, Haemmerli *et al.*⁶⁷⁾ proposed that not dioxygen but hydroperoxy radical produced from dioxygen attacks to benzene ring to give aromatic ring opening products. However, there is no clear experimental evidence that hydroperoxy radical is incorporated except for dioxygen.

The regiospecific oxygenations with water and dioxygen in the ring opening reaction

Mass spectra shown in Fig. 7 indicate that one ^{18}O atom each from water and

Table 5. Major peaks in *cis*- γ -lactone species.

unlabeled	^{18}O -labeled lactone		
	III- ^{18}O -a	III- ^{18}O -b	III- ^{18}O -E
124	124	126	124
136	138	138	138
137	139	139	139
139	141	141	141
140	142	142	142
168	170	170	170

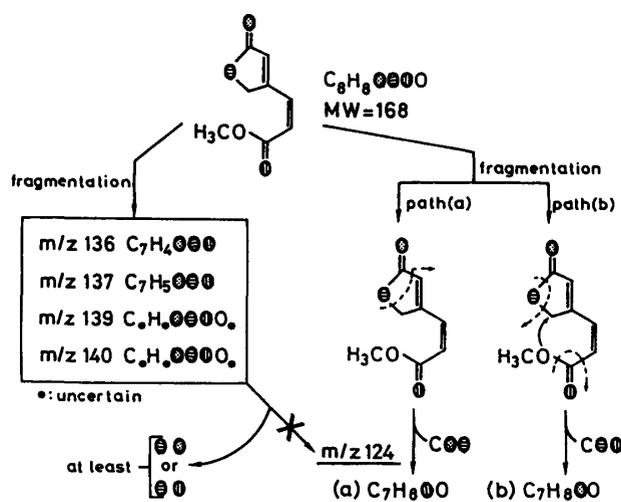


Fig. 13. Possible fragmentation pathways of *cis*- γ -lactone (III) in regard to the loss or retention of the oxygen atoms.

dioxygen was incorporated regiospecifically into (III). The interpretation of each mass spectrum is discussed focused on the formation of *cis*- γ -lactone (III) and π -lactone (V), since the present author could not obtain clear mass spectra for *trans*- γ -lactone (IV).

cis- γ -Lactone (III)

The fragment ions that are shifted by two mass units caused by incorporation of ^{18}O atom into (III- ^{18}O -a), (III- ^{18}O -b) and (III- ^{18}O -E) are given in Table 5. Their possible fragmentation pathways are illustrated with particular attention to the loss or retention of the oxygen atoms from the molecular to the fragment (Fig. 13).

Table 5 shows the following three features of fragmentations.

1. Since (III- ^{18}O -a), (III- ^{18}O -b), and (III- ^{18}O -E) containing ^{18}O yield the base peak at m/z 138, which corresponds to the peak at m/z 136 for (III), the fragment ion m/z 136 for (III) (MW=168) is probably formed by a loss of methanol (MW=32) from the methyl ester moiety (Fig. 14a).

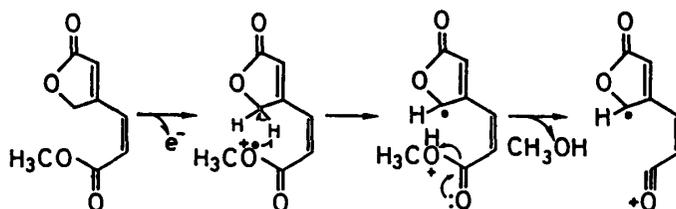
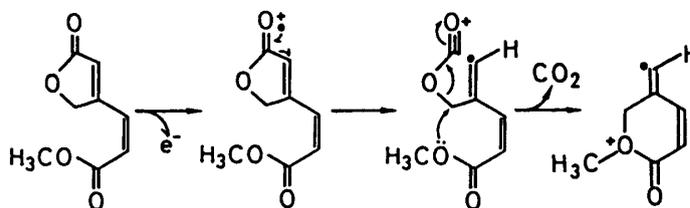


Fig. 14a. Possible fragmentation to yield the fragment ion at m/z 136 of *cis*- γ -lactone (III).

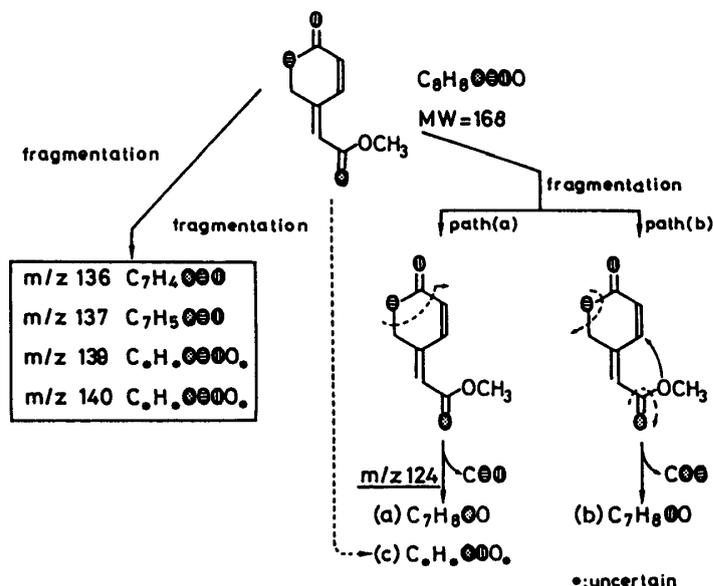
2. (III- ^{18}O -a) and (III- ^{18}O -E) yield the same ion peak at m/z 124 as for (III), but (III- ^{18}O -b) yields m/z 126 (Table 5). The fragment ion m/z 124 could be produced by loss of a certain fragment with the ethereal oxygen and one of the two carbonyl oxygens from (III), (III- ^{18}O -a) and (III- ^{18}O -E).

3. Alternatively, there might be other fragmentation pathways to produce the ion at m/z 124 derived from m/z 136, 137, 139 and 140, that contain two carbonyl oxygens and one ethereal oxygen as shown in Fig. 13, regardless of their peak intensities. However, these pathways can be eliminated, because there is no logical reason accounting for evolution of 12, 13, 15, and 16 mass units obtained by subtraction of 124 from the four masses, respectively.

So, there is no doubt that m/z 124 is formed from molecular ion (M^+ 168) by loss of two oxygens containing ethereal oxygen of lactone ring moiety (32 masses) and one carbon (12 masses). Thus, it is concluded that ^{18}O atom was introduced from H_2^{18}O into the carbonyl oxygen of lactone moiety exclusively and lost as CO^{18}O during fragmentation (Fig. 14b), whereas ^{18}O atom from $^{18}\text{O}_2$ was introduced into the car-

Fig. 14b. Possible fragmentation to yield the fragment ion at m/z 124.Table 6. Major peaks in δ -lactone species

unlabeled	^{18}O -labeled lactone		
	V	V- ^{18}O -a	V- ^{18}O -b
124	126	126	124
125	127	127	125
136	138	138	138
137	139	139	139
139	141	141	141
140	142	142	142
168	170	170	170

Fig. 15. Possible fragmentation pathways of δ -lactone (V) in regard to the loss or retention of the oxygen atoms.

bonyl oxygen of methyl ester moiety.

δ -Lactone (V)

The ions that are shifted by two mass units by ^{18}O atom incorporation for (V- ^{18}O -a), (V- ^{18}O -b) and (V- ^{18}O -E) are listed in Table 6, and possible a fragmentation pathway is illustrated in Fig. 15.

Table 6 shows following features for the fragmentation.

1. The fragment ions at m/z 136, 137, 139 and 140 contain two carbonyl oxygens of both lactone moiety and methyl ester moiety, and ethereal oxygen of lactone ring moiety (Fig. 15). Because (V- ^{18}O -a), (V- ^{18}O -b), and (V- ^{18}O -E) yield the fragment ion peaks at m/z 138, 139, 141 and 142 due to the ^{18}O atom label (Table 6).

2. The fragment ion at m/z 124 does not contain ethereal oxygen of lactone moiety. Because, (V- ^{18}O -E) does not yield the fragment ion peak at m/z 126.

As for the ion species at m/z 124 (V), there are following two possibilities.

A) There are two kinds of species of the fragment ion at m/z 124 for (V). One species is that contains one carbonyl oxygen ((a) and (b) in Fig. 15) and the other is that contains two carbonyl oxygens (c) in (Fig. 15).

B) The fragment ion at m/z 124 contains one carbonyl oxygen ((a) and (b) in Fig. 15).

In the case of A, it is impossible to explain the occurrence of the regiospecificity for ^{18}O atom-incorporation from H_2^{18}O and $^{18}\text{O}_2$ on the basis of the mass spectra of δ -lactone. In the case of B, if fragment ion peak at m/z 124 is formed by the removal of CO_2 from lactone ring moiety (path a) as discussed for the *cis*- γ -lactone, ^{18}O atom from H_2^{18}O and dioxygen could be incorporated into δ -lactone at random. Random oxygenation is not agreement with the reaction mechanism to form γ -lactone via the same reaction intermediate, 3-hydroxymethyl-(*cis, cis*)- muconate dimethyl ester, because it is clear that the carbonyl oxygens of this reaction intermediate is labeled with ^{18}O atom regiospecifically.

There are two possible answer for this question.

First, the regiospecific attack by water and dioxygen at veratryl alcohol (I) does not substantially occur, and (III) is formed in only the case that water attacks to the C_3 carbon and dioxygen attacks to the C_4 carbon. But (V) can be formed in both cases.

Second, the regiospecific attack of water and dioxygen to veratryl alcohol (I) occurs and both (III) and (V) are formed with regiospecific incorporation of ^{18}O atom. However, there is another pathway to form (V) without regiospecific incorporation of ^{18}O atom.

At present there is no clear evidence to support the above postulation, since the detail of this reaction has not yet been fully established. Most plausible reaction mechanism of aromatic ring opening of veratryl alcohol (I) is proposed in Fig. 16. It is the first time that the regiospecific ^{18}O atom incorporation was observed in the enzymatic reaction with lignin peroxidase. However, it is unlikely that the regiospecificities observed are governed by the protein moiety of lignin peroxidase as described in the section 1.2.

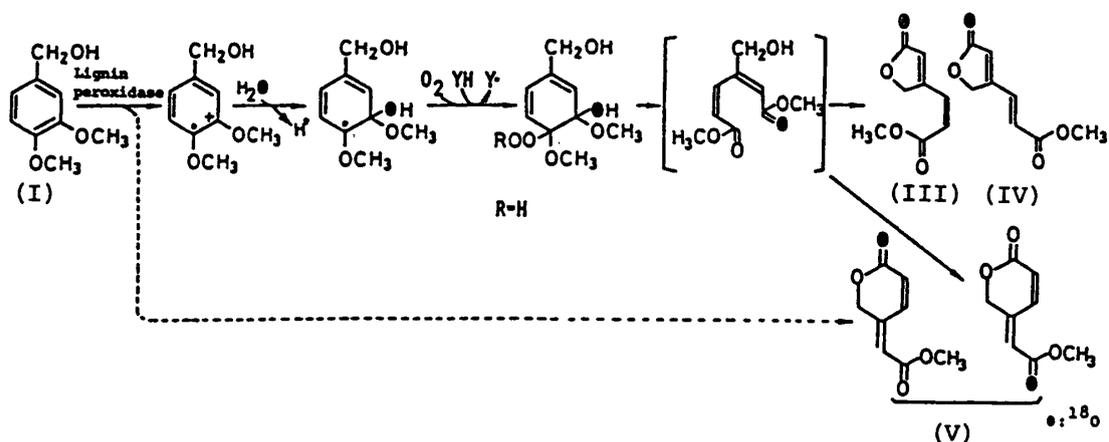


Fig. 16. Mechanisms for the aromatic ring opening of veratryl alcohol (I) to yield lactones in enzyme system.

Section 1.2

Aromatic Ring Opening of Veratryl Alcohol by Hemin Catalyst

1.2.1 Introduction

Lignin peroxidase has been characterized as hemoprotein with a protoporphyrin IX at the active center^{24, 25}). Then the current widespread interest has been focused on designing of the lignin peroxidase mimicing catalyst or systems for delignification of wood based materials since Shimada *et al.*⁴³) reported the first example of biomimetic approach to lignin degradation.

The hemin catalyst was found to mimic the catalysis of lignin peroxidase for the C α -C β bond cleavage of the side chain of lignin model compounds^{29, 44}). The reaction mechanism for the C-C bond cleavage for lignin substructure model compounds by lignin peroxidase has been proposed to proceed via cation radical intermediate initially formed by one electron oxidation by an active oxygen species of hemin (corresponding to compound I of peroxidase)²⁸). However, it is not elucidated whether hemin is able to catalyze the nonphenolic aromatic ring opening of lignin. Furthermore, in contrast to the biomimetic aromatic ring opening of catechol substrates⁶⁸) no examples for the unique ring opening of such nonphenolic substrate in the biomimetic porphyrin systems has yet been reported.

In section 1.2 is described the unique regiospecific ¹⁸O atom incorporation from H₂¹⁸O and ¹⁸O in one of the ring opening products formed. It was clearly demonstrated that the protein moiety of lignin peroxidase is not involved in essentially such a regiospecific oxygenation described in section 1.1⁴⁵).

1.2.2 Experimental

Degradation of veratryl alcohol (I) catalyzed by hemin

Normally, the reaction mixture contained veratryl alcohol (I, 10 μ mol), hemin (1 μ mol) take out from 50 mM solution of DMSO, *t*-BHP (50 μ mol) and 1 ml of water.

Chloroform was also used for the non-aqueous reaction system. The reaction was initiated by the addition of *t*-BHP, which was stirred under air for 2 h at room temperature. The reaction products extracted with ethyl acetate was analyzed by HPLC or submitted to TLC and aromatic ring opening products were identified by GC-MS. The structure of δ -lactone (V) obtained by a large scale experiment was confirmed by $^1\text{H-NMR}$ spectrometry.

Tracer experiment with H_2^{18}O

To a reaction mixture containing veratryl alcohol (I, 20 μmol), hemin (2 μmol) taken out from 50 mM solution of DMSO, 290 μl of distilled water and 1500 μl of H_2^{18}O (98.4 atom %), 5 μmol *t*-BHP was added twice at 15 min intervals at temperature under air. After 30 min from the first addition of *t*-BHP, the products were extracted with ethyl acetate as described above. The aromatic ring opening products (III- ^{18}O -a, IV- ^{18}O -a, V- ^{18}O -a) were isolated by TLC and analyzed by GC-MS.

Tracer experiment with $^{18}\text{O}_2$

The reaction mixture containing veratryl alcohol (I, 50 μmol), hemin (5 μmol) in DMSO solution, *t*-BHP (25 μmol) and 4.9 ml of water was evacuated and flushed with argon. The procedure was repeated 6 times. After removal of argon by the last evacuation, $^{18}\text{O}_2$ was introduced into the evacuated vessel by use of a syringe and the reaction was initiated by addition of 12.5 μmol *t*-BHP twice at 15 min intervals with stirring at room temperature. After 30 min, the reaction products were ex-

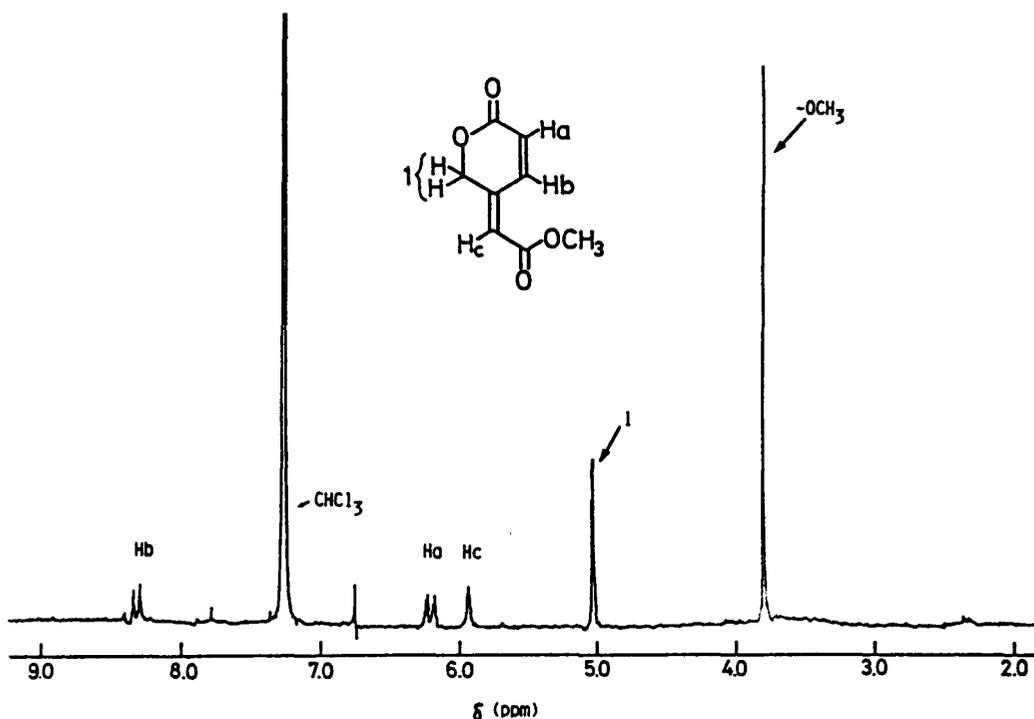


Fig. 17. $^1\text{H-NMR}$ spectrum of δ -lactone (V).

tracted with ethyl acetate in the same manner. The aromatic ring opening products were separated by TLC and γ -lactone (V- ^{18}O -b) was analyzed by GC-MS. The *cis*- γ -lactone (III- ^{18}O -b) and *trans*- γ -lactone (IV- ^{18}O -b) were further separated by TLC for GC-MS analysis.

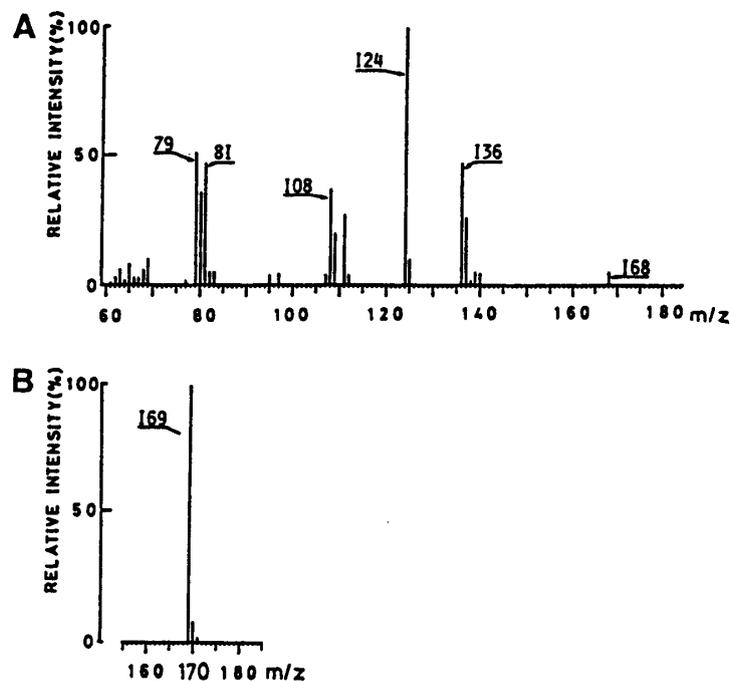


Fig. 18. Mass spectra of the δ -lactone (V).
 A, EI-Mass spectrum; B, CI-Mass spectrum.

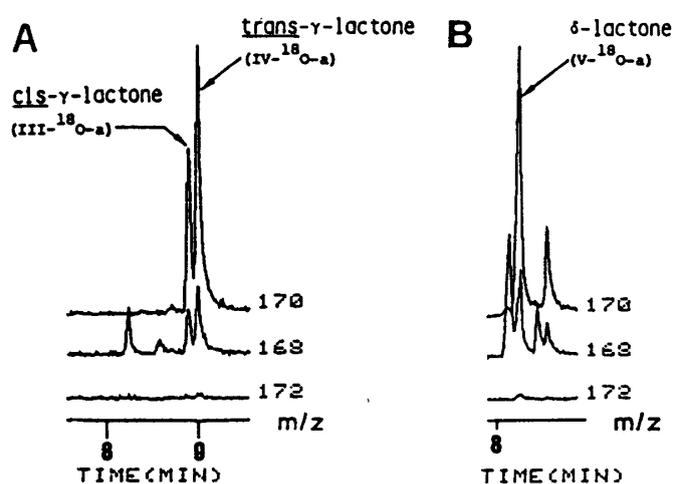


Fig. 19. Mass chromatograms of fragment ions for m/z 168, 170 and 172 of the γ -lactones and δ -lactone formed in catalytic system with hemin.
 A, γ -lactones oxygenated with ^{18}O from H_2^{18}O (III- ^{18}O -a and IV- ^{18}O -a);
 B, δ -lactone oxygenated with ^{18}O from H_2^{18}O (V- ^{18}O -a).

Degradation of veratryl alcohol (I) catalyzed by hemin under argon

The anaerobic reaction was carried out as described above except for using argon gas. The concentrate was purified to obtain aromatic ring opening products and analyzed by GC-MS as described in the tracer experiment with $H_2^{18}O$.

1.2.3 Results**Product identification**

Aromatic ring opening products (*cis*- γ -lactone (III), *trans*- γ -lactone (IV) and δ -lactone (V)) were obtained from the aqueous reaction system, although veratral-

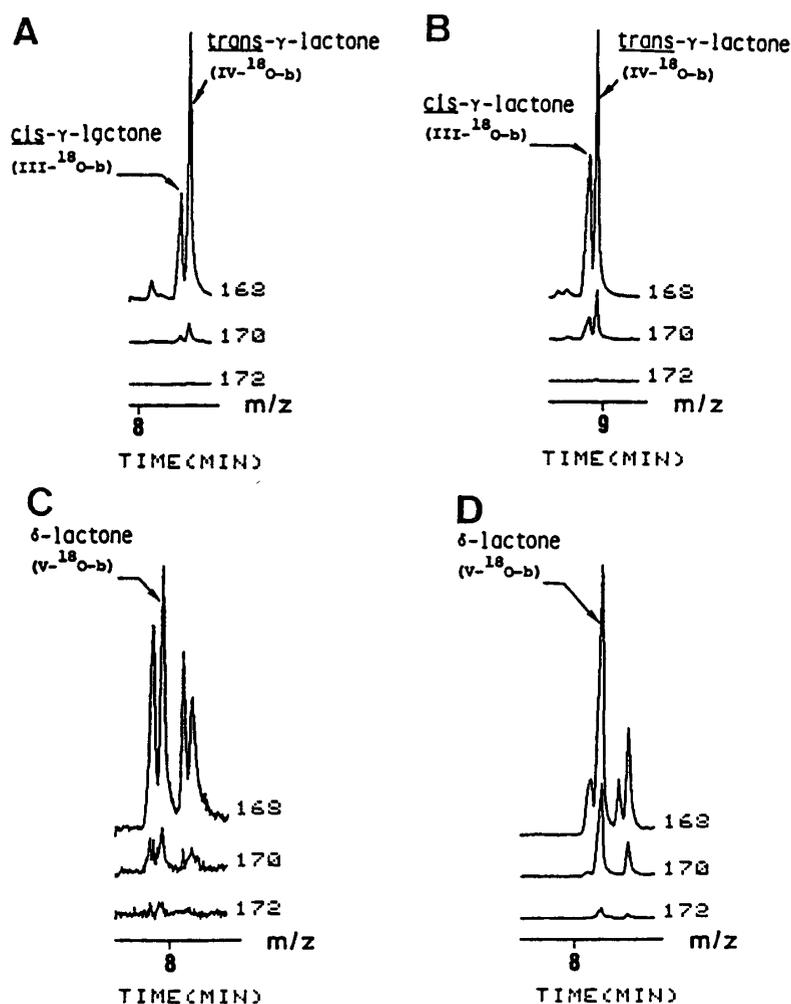


Fig. 20. Mass chromatograms of fragment ions for m/z 168, 170 and 172 of the γ -lactones and δ -lactone.

A, *cis*- and *trans*- γ -lactones with 8% (III- ^{18}O -b) and 7% (IV- ^{18}O -b) ^{18}O -incorporations from $^{18}O_2$, respectively; B, *cis*- and *trans*- γ -lactones with 13% (III- ^{18}O -b) and 14% (IV- ^{18}O -b) ^{18}O -incorporations from $^{18}O_2$, respectively; C, δ -lactone with 13% ^{18}O -incorporation from $^{18}O_2$ (V- ^{18}O -b); D, the δ -lactone with 22% ^{18}O -incorporation from $^{18}O_2$ (V- ^{18}O -b).

dehyde (II) was the major product. The δ -lactone (V) was identified not only by comparison of its $^1\text{H-NMR}$ spectra with the reported one⁶⁹⁾ but also by analysis of its mass spectrum. $^1\text{H-NMR}$ (CDCl_3) δ (ppm) of (V): 3.78 (3H, s, $\text{CH}_3\text{-OOC-}$), 5.02 (2H, d, $J=1.5\text{Hz-}2\text{Hz}$, $\text{-COO-CH}_2\text{-}$), 5.93 (1H, m, $\text{CH}_3\text{-OOC-CH=C-}$), 6.20 (1H, dd, $J=10\text{Hz}$, $1.5\text{Hz-}2\text{Hz}$, -CH=CH-COO-), 8.33 (1H, d, $J=10\text{Hz}$, -HC=CH-COO-), (Fig. 17), EI-MS m/z (%): 168 (M⁺, 5.8), 137 (26.8), 136 (48.0), 124 (100), 111 (28.1), 109 (20.2), 180 (38.0), 81 (47.9), 80 (36.6), 79 (51.7). CI-MS m/z (%): 169 (MH^+ , 100) (Fig. 18).

Incorporation of the ^{18}O atom from H_2^{18}O and $^{18}\text{O}_2$

The mass chromatograms of the fragment ions at m/z 168, 170 and 172 are shown in Fig. 19. The data clearly indicate that only one ^{18}O atom from H_2^{18}O was incorporated into all the lactones, since no significant ion peak at m/z 172 was formed but the strong peaks at m/z 170 were detected for III- ^{18}O -a, IV- ^{18}O -a and V- ^{18}O -a.

Figure 20 shows mass-chromatograms of the lactones, which were obtained from the reaction system with $^{18}\text{O}_2$ gas. All the lactones yielded significant ion peak at m/z 170 but the ion peak at m/z 172 was negligible. The contents of ^{18}O -incorporations from H_2^{18}O and $^{18}\text{O}_2$ determined are given in Table 7. The results show that ^{18}O atoms were quantitatively incorporated from H_2^{18}O into all the lactones, but the incorporations of ^{18}O atom from $^{18}\text{O}_2$ were much less than those obtained with H_2^{18}O and also less than those observed for the enzyme system with $^{18}\text{O}_2$. All the lactones were also formed under dioxygen-free argon gas as shown in Figs. 21a and 21b.

Table 7. Incorporation of ^{18}O from H_2^{18}O and $^{18}\text{O}_2$ into lactones in catalytic system with hemin

Reaction system	^{18}O -Incorporation (%)		
	<i>cis</i> - γ -lactone	<i>trans</i> - γ -lactone	δ -lactone
with H_2^{18}O	100	100	97
with $^{18}\text{O}_2$	13	14	22
	8	7	13

The comparison of the peak ratio of m/z 126 and 124

cis- γ -Lactone (III)

Figure 22 shows the mass-spectra of the normal *cis*- γ -lactone (III), *cis*- γ -lactone oxygenated with H_2^{18}O (III- ^{18}O -a), *cis*- γ -lactone labeled with ^{18}O atom at the ethereal oxygen of lactone moiety (III- ^{18}O -E). The mass spectrum for (III- ^{18}O -a) is completely identical with that of (III- ^{18}O -a) found in the enzyme system. Figure 23 shows the mass-chromatograms of (III), (III- ^{18}O -a), (III- ^{18}O -b) and (III- ^{18}O -E) for m/z 124 and 126, and the ratios of 126/124 are summarized in Table 8. The results show that (III- ^{18}O -a) and (III- ^{18}O -E) gave a strong ion peak at m/z 124 but much lower

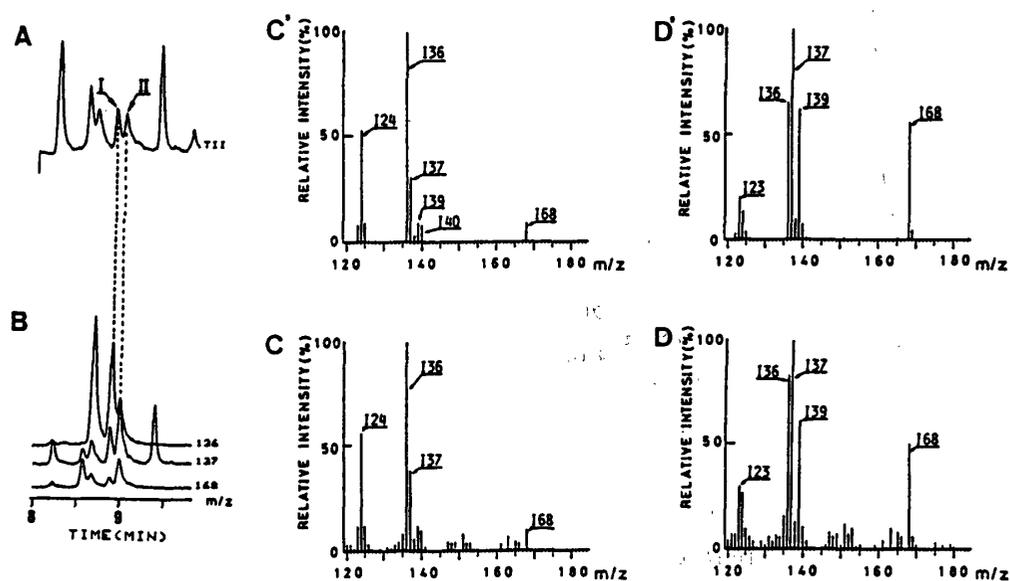


Fig. 21a. Formation of the *cis*- γ -lactone (III) and *trans*- γ -lactone (IV) under anaerobic condition.

A, total ion chromatogram; B, mass chromatograms of the ions at m/z 136, 137 and 168; C, mass spectrum of the peak I, (C', authentic *cis*- γ -lactone); D, mass spectrum of the peak II, (D', authentic *trans*- γ -lactone).

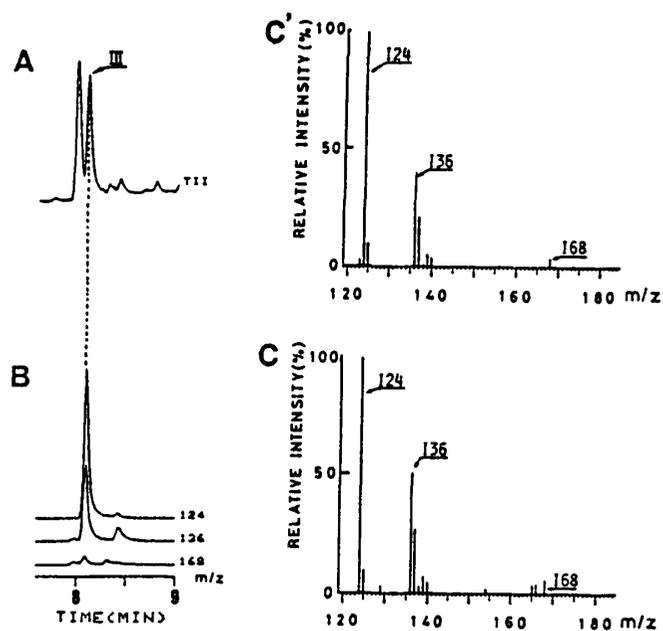


Fig. 21b. Formation of the δ -lactone (V) under anaerobic condition.

A, total ion chromatogram; B, mass chromatograms of the ions at m/z 124, 136 and 168; C, mass spectra of the peak III. (C', authentic δ -lactone).

ones for m/z 126. The calculated peak ratios (126/124) are 0.04, 0.23 and 0.32 for (III- ^{18}O -a), (III- ^{18}O -E) and (III), respectively (Table 8). On the other hand, (III- ^{18}O -b) shows much stronger ion peak for m/z 126, yielding the greater 126/124 ratio (0.065), which is about two to three times greater than the ratios for (III), (III- ^{18}O -a) and (III- ^{18}O -E) (see Table I). Furthermore, as the incorporation of ^{18}O atom from $^{18}\text{O}_2$ increased, the relative intensity of the ion at m/z 126 increased (Fig. 23), and the 126/124 ratio increased (Table 8).

trans- γ -Lactone (IV)

Figure 24 shows the mass spectra of the normal *trans*- γ -lactone (IV), and the *trans*- γ -lactone (VI- ^{18}O -a) oxygenated with H_2^{18}O , *trans*- γ -lactone (IV- ^{18}O -E) labeled with ^{18}O atom at the ethereal oxygen of lactone moiety. Figure 25 shows the mass-chromatograms at m/z 126 and 124 for (IV), (IV- ^{18}O -a), (IV- ^{18}O -b) and (IV- ^{18}O -E). The ratios of ion peaks at m/z 126 and m/z 124 are given in Table 9.

The results show that the ratio m/z 126 / 124 (0.16) for (IV- ^{18}O -E) is almost equal to that for (IV) (0.17). On the contrary, the 126 / 124 ratio (0.22) for (IV- ^{18}O -b) is slightly higher than that for (IV), although no significant differences in those ratios between (IV- ^{18}O -b) and (IV) were observed, because of the low ^{18}O atom incorporation.

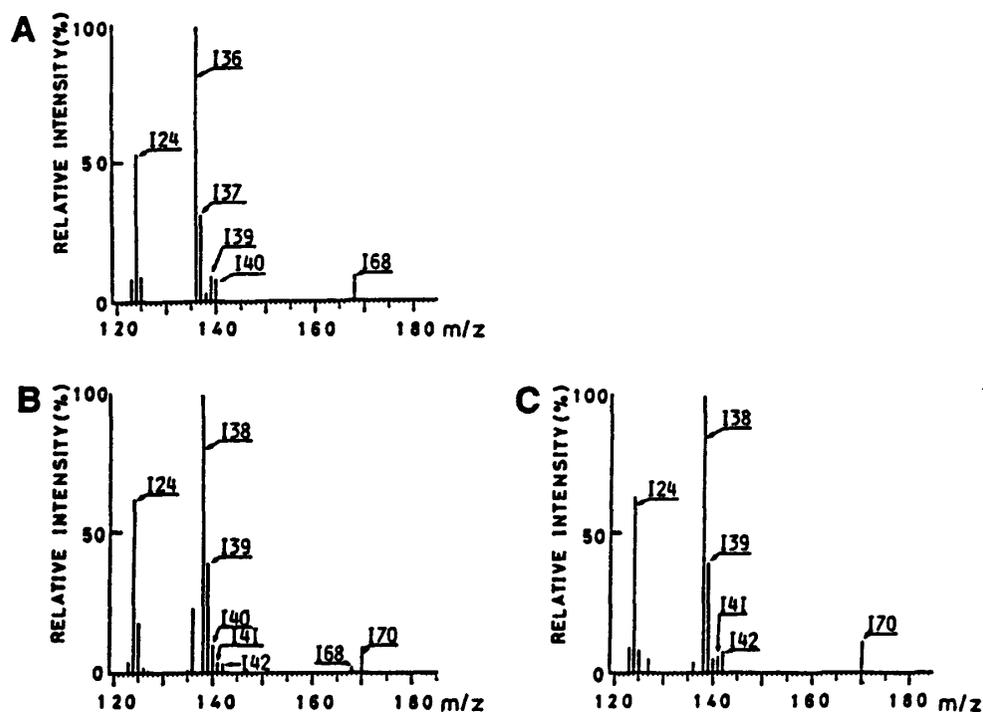


Fig. 22. Mass spectra of the *cis*- γ -lactone species formed in catalytic system with hemin.

A, normal *cis*- γ -lactone (III); B, *cis*- γ -lactone oxygenated with ^{18}O from H_2^{18}O (III- ^{18}O -a); C, *cis*- γ -lactone labeled with ^{18}O at the ethereal oxygen of lactone ring moiety (III- ^{18}O -E).

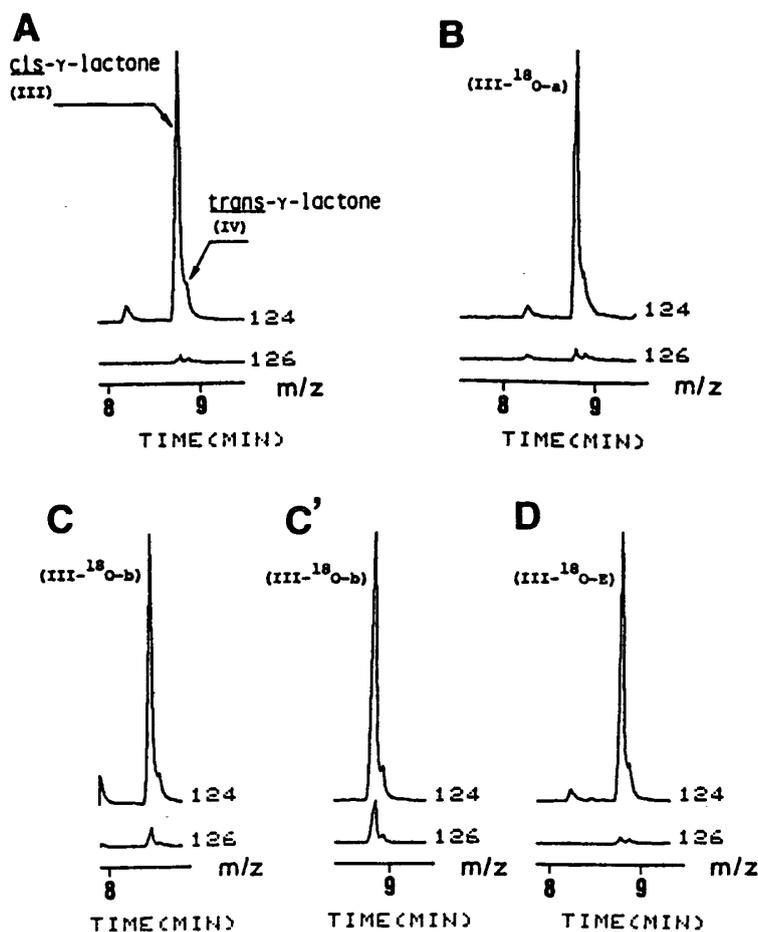


Fig. 23. Mass chromatograms of fragment ions for m/z 124 and 126 of the *cis*- γ -lactone species.

A, *cis*- γ -lactone (III); B, *cis*- γ -lactone oxygenated with ^{18}O from H_2^{18}O ($\text{III-}^{18}\text{O-a}$); C, *cis*- γ -lactone with 8% ^{18}O -incorporation from $^{18}\text{O}_2$ ($\text{III-}^{18}\text{O-b}$); C', *cis*- γ -lactone with 13% ^{18}O -incorporation from $^{18}\text{O}_2$ ($\text{III-}^{18}\text{O-b}$); D, *cis*- γ -lactone labeled with ^{18}O at the etheral oxygen of lactone ring moiety ($\text{III-}^{18}\text{O-E}$).

Table 8. Ratio of the relative intensities of the peaks at m/z 126 to that at m/z 124 in catalytic system with hemin

Lactone species	m/z 126 / m/z 124	
	Ratio	Relative value
III	0.032	1
III- $^{18}\text{O-a}$	0.04	1.3
III- $^{18}\text{O-b}$	0.065 (8%-incorporation)	2.0
	0.14 (13%-incorporation)	4.4
III- $^{18}\text{O-E}$	0.023	0.7

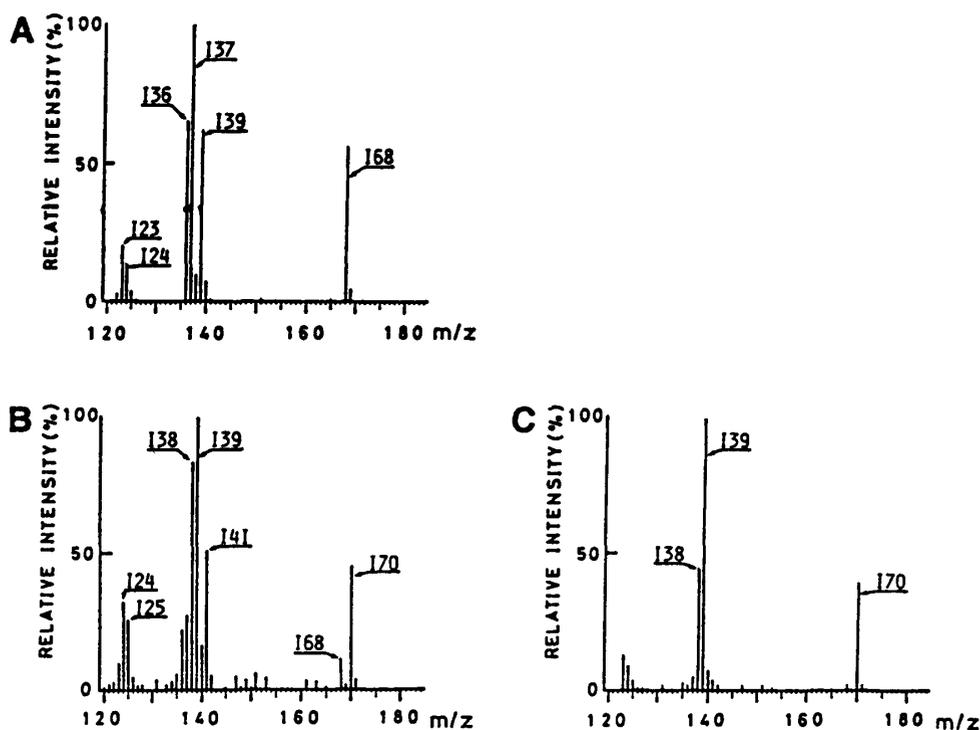


Fig. 24. Mass spectra of the *trans*- γ -lactone species formed in catalytic system with hemin.

A, normal *trans*- γ -lactone (IV); B, *trans*- γ -lactone oxygenated with ^{18}O from H_2^{18}O (IV- ^{18}O -a); C, *cis*- γ -lactone labeled with ^{18}O at the etheral oxygen of lactone ring moiety (IV- ^{18}O -E).

δ -Lactone (V)

Figure 26 shows the mass-spectra of the normal δ -lactone (V), δ -lactone oxygenated with H_2^{18}O (V- ^{18}O -a), δ -lactone labeled with ^{18}O atom at the etheral oxygen of lactone moiety (V- ^{18}O -E). Figure 27 shows the mass-chromatograms for (V), (V- ^{18}O -a), (V- ^{18}O -b) and (V- ^{18}O -E) at m/z 124 and 126, and the ratios m/z 126 / 124 are summarized in Table 10. The results show that the ratio m/z 126 / 124 (0.014) for (V- ^{18}O -E) is almost equal to that (0.015) for (V). On the contrary, the 126 / 124 ratios for (V- ^{18}O -a) and (V- ^{18}O -b) are 0.62 and 0.088, respectively, which are 41-fold and 5.9-fold greater than that for (V), respectively.

1.2.4 Discussion

The reaction mechanism for the aromatic ring opening of varatryl alcohol (I) with a hemin catalyst is discussed in relation to the similar enzymatic ring opening reaction.

The fact that the same type of lactones, (*cis*- γ -lactone (III), *trans*- δ -lactone (IV), δ -lactone (V)) are produced in the hemin catalyzed reaction revealed that hemin mimics the lignin peroxidase activity for the ring opening of the nonphenolic substrate (I)⁴⁵.

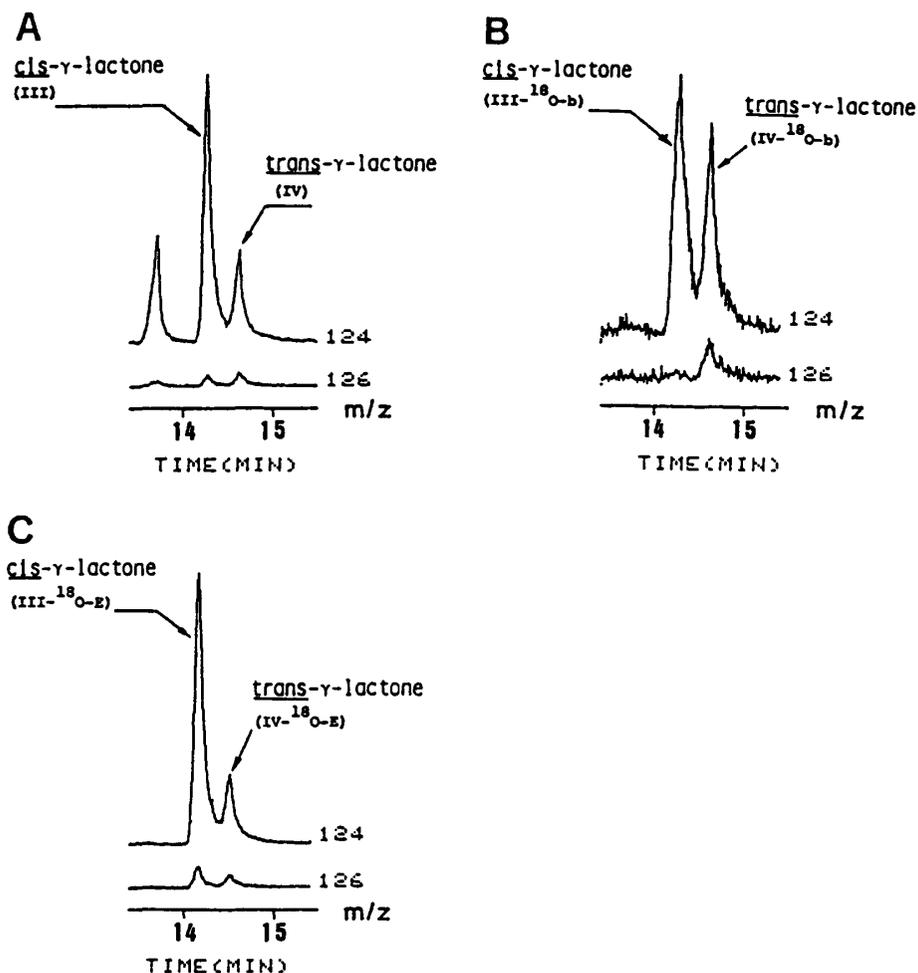


Fig. 25. Mass chromatograms of the fragment ions for m/z 124 and 126 of the *trans*- γ -lactone (IV).
 A, normal *trans*- γ -lactone (IV); B, *trans*- γ -lactone with 7% ^{18}O -incorporation from $^{18}\text{O}_2$ (IV- ^{18}O -b); C, *trans*- γ -lactone labeled with ^{18}O at the ether oxygen of lactone ring moiety (IV- ^{18}O -E).

Table 9. Ratio of the relative intensities of the peaks at m/z 126 to that at m/z 124 in catalytic system with hemin

Lactone species	m/z 126 / m/z 124	
	Ratio	Relative value
IV	0.17	1
IV- ^{18}O -a	N.D.	N.D.
IV- ^{18}O -b	1.73	10
IV- ^{18}O -E	0.16	0.9

N.D.; not detected.

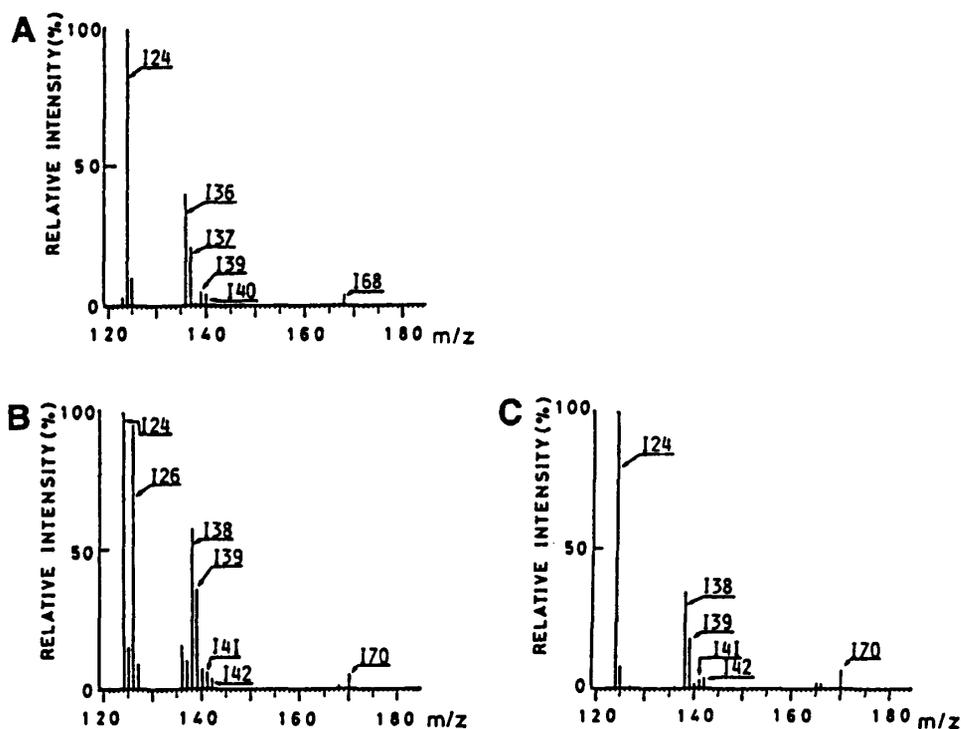


Fig. 26. Mass spectra of the δ -lactone species formed in catalytic system with hemin.

A, normal δ -lactone (V); B, δ -lactone oxygenated with ^{18}O from H_2^{18}O (V- ^{18}O -a); C, δ -lactone labeled with $^{18}\text{O}_2$ at the ethereal oxygen of lactone ring moiety (V- ^{18}O -E).

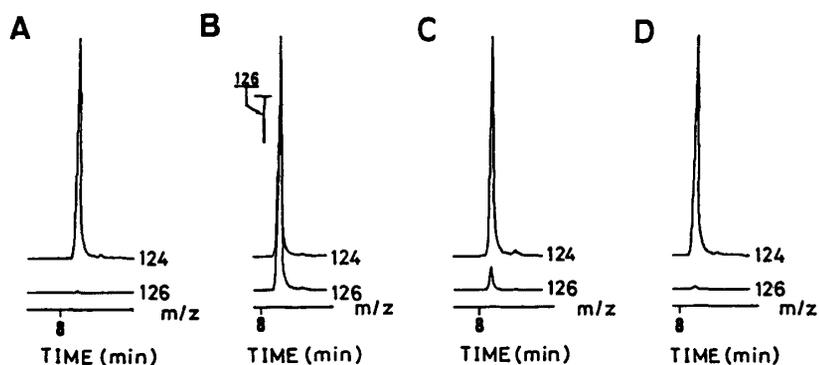


Fig. 27. Mass chromatograms of the fragment ions for m/z 124 and 126 of the δ -lactones.

A, normal δ -lactone (V); B, δ -lactone oxygenated with ^{18}O from H_2^{18}O (V- ^{18}O -a); C, δ -lactone with 13% ^{18}O -incorporation from $^{18}\text{O}_2$ (V- ^{18}O -b); D, δ -lactone labeled with ^{18}O at the ethereal oxygen of lactone ring moiety (V- ^{18}O -E).

The fact that the negligible amounts of the ring opening products were obtained from the non-aqueous system suggests that water is required for the aromatic ring opening reaction catalyzed by hemin. The tracer experiment with H_2^{18}O showed

Table 10. Ratio of the relative intensities of the peaks at m/z 126 to that at m/z 124 in catalytic system with hemin

Lactone species	m/z 126 / m/z 124	
	Ratio	Relative value
V	0.015	1
V- ^{18}O -a	0.62	41
V- ^{18}O -b	0.088	5.9
V- ^{18}O -E	0.014	1

in fact that water is directly involved as the reactant in the aromatic ring opening, because one oxygen atom from water was almost quantitatively incorporated into all the lactones produced.

Similarly, only one ^{18}O atom was found to be incorporated from $^{18}\text{O}_2$ into the lactone, but ^{18}O -incorporation in this system is much lower than those observed for the system with H_2^{18}O and for the enzyme system with $^{18}\text{O}_2$. Furthermore, (III), (IV) and (V) were shown to be formed under the oxygen-free atmosphere, which clearly indicates that dioxygen is not required for the ring opening in this non-enzymatic system. It is suggested that the other active oxygen radical species might react with veratryl alcohol (I) yielding the ring opening products in the hemin system. For example, *t*-butylhydroperoxy radical might be accounted for such an active radical species.

On the basis of the analyses of the mass fragmentations of (III), (IV) and (V) the regiospecific ^{18}O atom-incorporation from H_2^{18}O and $^{18}\text{O}_2$ into lactones in hemin-catalyzed reaction is also discussed. Tables 11, 12 and 13 show that the fragment ions for the ^{18}O -labeled products shifted by two mass units by ^{18}O atom incorporation as compared with the fragment ions of the unlabeled lactones. Since, the fragment ions of the labeled products were shifted in the same way as described in section 1.1 on the enzymatic reaction, it is postulated that same carbonyl oxygen is labeled with

Table 11. Major peaks in *cis*- γ -lactone species

unlabeled	^{18}O -labeled lactone	
	III- ^{18}O -a	III- ^{18}O -E
124	124	124
136	138	138
137	139	139
139	141	141
140	142	142
168	170	170

Table 12. Major peaks in *trans*- γ -lactone species

unlabeled	^{18}O -labeled lactone	
	IV	IV- ^{18}O -a
123	125	123
124	124	124
136	138	138
137	139	139
139	141	139
168	170	170

Table 13. Major peaks in δ -lactone species

unlabeled	^{18}O -labeled lactone	
	V	V- ^{18}O -a
124	126	124
125	127	125
136	138	138
137	139	139
139	141	141
140	142	142
168	170	170

^{18}O atom in the reaction system with H_2^{18}O as found in the enzyme system. Furthermore, since (III- ^{18}O -a) gave the fragment ions at m/z 126 and 124 with almost the same ratio (0.035) as that (0.032 and 0.023 for (III) and (III- ^{18}O -E), respectively) it is clear that ^{18}O atom was incorporated from H_2^{18}O into the carbonyl oxygen of lactone moiety of *cis*- γ -lactone regioselectively. Therefore, the same regioselective oxygenation as found in the enzyme system takes place for the case of non-enzymatic system.

On the other hand, since δ -lactone (V- ^{18}O -a) gave the fragment ions at m/z 126 and 124 with fairly different ratio as that for (V) and (V- ^{18}O -E), it is clear that ^{18}O atom was incorporated from H_2^{18}O into the same carbonyl oxygen of lactone moiety as that for (V- ^{18}O -a) formed in enzymatic system. However, there are slight difference in the ratios m/z 126 to m/z 124 for (III- ^{18}O -b) and (III) (III- ^{18}O -a) (Table 8), as well as (V- ^{18}O -b) and (V), (V- ^{18}O -a) (Table 10), because of low incorporation of ^{18}O atom from $^{18}\text{O}_2$.

The possible reaction mechanism for the aromatic ring opening by hemin is shown in Fig. 28. Figure 28 show essentially the same mechanism as proposed for the enzymatic ring cleavage reaction except for the involvement of *t*-butylhydroperoxy radical in this biomimetic reaction system.

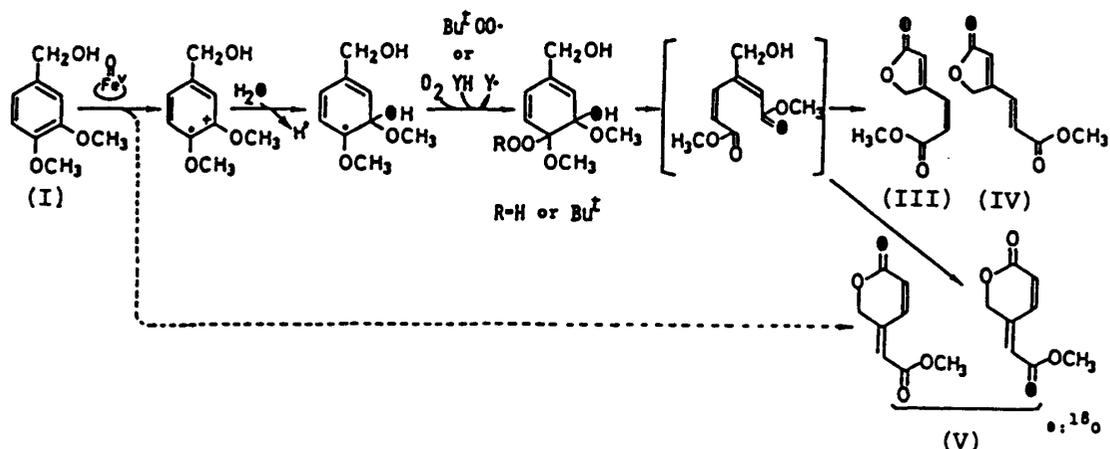


Fig. 28. Mechanisms for the aromatic ring opening of veratryl alcohol (I) to give lactones in the catalytic system with hemin.

Chapter 2

Degradation of Phenolic and Biphenyl Lignin Model Compounds by Lignin Peroxidase

Section 2.1

Degradation of Vanillyl Alcohol

2.1.1 Introduction

In chapter 1 is described the mechanism for aromatic ring opening reaction of veratryl alcohol (I) which is not only a natural substrate for lignin peroxidase but also the simplest nonphenolic lignin model compound. While it was reported that phenolic ring of the substrate is mainly converted to biphenyl structure by lignin peroxidase²⁵⁾. It has been further reported that lignin peroxidase systems catalyze repolymerization of lignin rather than depolymerization, although small amounts of lower molecular weight compounds were produced^{22,47-49)}. Therefore, it is of great importance to examine whether the phenolic moieties of the guaiacyl units undergo only condensation but not other reactions such as a ring opening similar to the cleavage of nonphenolic moieties of lignin model substrates.

Kawai *et al.*⁷⁰⁾ reported the aromatic ring opening of 4,6-di-*tert*-butylguaiacol substrate catalyzed by laccase of *C. versicolor* (L. ex Fr.) Quél. However, it is not known whether vanillyl alcohol (VII), which is a closer model of the guaiacyl lignin moiety than 4,6-*tert*-butylguaiacol undergoes a similar ring opening with laccase or a lignin peroxidase system.

In section 2.1 is described a new type of ring opening reaction of vanillyl alcohol (VII) to yield δ -lactone (V) by lignin peroxidase, although the phenolic ring opening reaction is not major⁵⁰⁾.

2.1.2 Experimental

Preparation of substrate and authentic compounds

Vanillyl alcohol (VII) was prepared from vanillin (VIII) by reduction with NaBH_4 in $\text{CH}_3\text{OH}/\text{THF}=1:2$, v/v, at 0°C and purified by TLC before use. The chemical structure of (VII) was confirmed by $^1\text{H-NMR}$ and GC-MS. δ -Lactone (V) was prepared in the previous chapter⁴⁵⁾. Dehydrodivanillyl alcohol (XI) was prepared as follows. Dehydrodivanillin (XX) prepared according to the method of Pew⁷¹⁾ was subjected to reduction with NaBH_4 in a 0.1–0.2 N NaOH solution at 0°C to give dehydrodivanillyl alcohol (XI). The crude crystal of (XI) was purified by TLC. The chemical structure of (XI) was confirmed by $^1\text{H-NMR}$ ⁵⁰⁾.

Preparation of lignin peroxidase

The stationary culture of *P. chrysosporium* strain BKM-F-1767 (ATCC 24725) was carried out according to the method of Kurosaka *et al.*⁷²⁾. The 60-ml cultures in 1-l erlenmeyer flasks were grown and the total volume of culture medium was 3.6l. The culture medium contained the following (g/l): glucose, 2; ammonium L-tartrate, 1; Tween 80, 1; KH_2PO_4 , 0.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.015; thiamine HCl, 0.0005; and 7 ml of trace elements solution¹⁵⁾. The culture was buffered at pH 4.3 with 10 mM sodium *trans*-aconitate. After inoculation of conidial suspensions, they were cultivated at 37°C under air. They were flushed with oxygen gas on days 2 and 3 for five min. One ml of 100 mM veratryl alcohol (I) solution in H_2O was added to each flasks on day 2. Culture fluid was collected on day 4 and filtered. The filtrate (2.8 l) was concentrated to 36 ml by use of a millipore ultrafiltration system (Pellicon Lab Cassette XX42 OLC KO; membrane, PTGC OLC M2 10,000-dalton pore size). The crude enzyme solution (34 ml) thus obtained was dialyzed against 20 mM potassium phosphate buffer (pH 6.5) for 24 h and applied to a DEAE-Bio-Gel A column (Bio-Rad; column, 1.6×20 cm, C16/20, Pharmacia) previously equilibrated with the same buffer. The column was washed with 96 ml of the same buffer, and the enzyme protein was eluted by use of a linear gradient method with 0–0.2 M NaCl in 20 mM phosphate buffer; the mixing and reservoir chambers contained 360 ml of 20 mM phosphate buffer and 360 ml of the same buffer with 0.2 M NaCl, respectively. All purification procedures were conducted at 4°C . 27 ml portion of the first major fraction (60 ml) out of six fractions with lignin peroxidase activity, was concentrated to 5.8 ml in a dialysis tube (Cellulose Tubing, 20/32, Visking Company) by use of polyethylene glycol (# 20,000, Nakarai Tesque). The concentrated enzyme solution was used after dialysis of the solution against 20 mM sodium succinate buffer (pH 5.7) for 24 h. Enzyme activity was determined as described in the previous chapter 1.

Degradation of vanillyl alcohol (VII) catalyzed by lignin peroxidase

HATTORI: Aromatic Ring Opening of Veratryl Alcohol

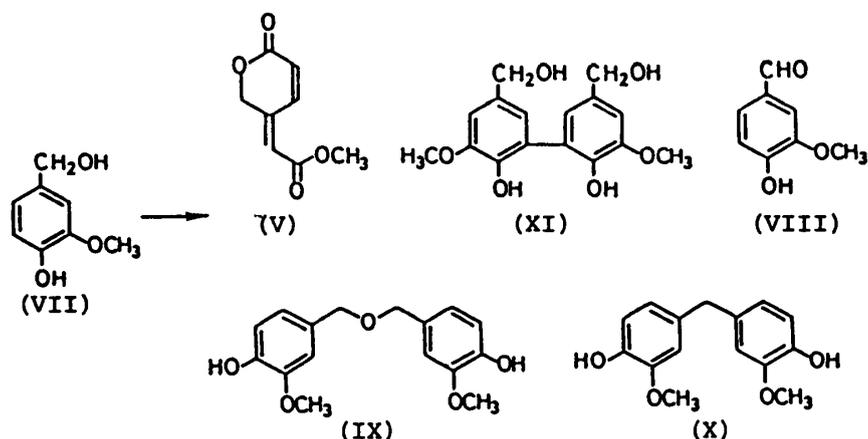


Fig. 29. Oxidation of vanillyl alcohol (VII) by the lignin peroxidase / H_2O_2 system.
(IX) and (X) were formed non-enzymatically.

Table 14. $^1\text{H-NMR}$ and Mass spectra of the products

Compounds	$^1\text{H-NMR}$ and Mass spectra
II	152 (M^+ , 89.5), 151 (100.0), 123 (18.6), 109 (16.1), 81 (22.6), and 65 (10.0).
V	168 (M^+ , 6.8), 137 (25.4), 136 (47.8), 124 (100), 111 (26.4), 109 (21.5), 108 (38.1), 81(48.0), 80 (37.8), and 79 (53.8).
XI-Ac	2.11 (3H \times 2, s, -OAc \times 2), 2.12 (3H \times 2, s, -OAc \times 2), 3.87 (3 H \times 2, s, -OCH $_3$ \times 2), 5.08 (2H \times 2, s, -CH $_2$ OAc \times 2), 6.84 (1H \times 2, d, $J=2\text{Hz}$, aromatic C $_2$ \times 2 or C $_6$ \times 2), 6.96 (1H \times 2, d, $J=2\text{Hz}$, aromatic C $_2$ \times 2 or C $_6$ \times 2). 432 (M^+ -C $_2\text{H}_2\text{O}$, 28.0), 331 (14.8), 330 (75.0), 271 (31.4), 270 (100), and 269 (19.3).
IX	3.90 (3H \times 2, s, -OHC $_3$ \times 2), 4.46 (2H \times 2, s, -CH $_2$ -O-CH $_2$ -), 5.61 (1H \times 2, dd, $J=8$, 1.3 Hz, aromatic C $_6$ \times 2), 6.89 (1H \times 2, d, $J=1.3$ Hz, aromatic C $_2$ \times 2), and 6.89 (1H \times 2, d, $J=8$ Hz, aromatic C $_5$ \times 2).
IX-Ac	374 (M^+ , 1.1), 332 (16.6), 180 (15.8), 138 (100.0), 137 (57.2), 123 (11.8), and 106 (13.5).
X	3.83 (3H \times 2, s, -OCH $_3$ \times 2), 3.83 (2H, s, -CH $_2$ -), 5.47 (1H \times 2, s, -OH \times 2), 6.65 (1H \times 2, dd, $J=8$, 2 Hz aromatic C $_6$ \times 2), and 6.83 (1H \times 2, d, $J=8\text{Hz}$, aromatic C $_5$ \times 2).
X-AC	344 (M^+ , 6.4), 302 (23.0), 261 (16.0), 260 (100), 229 (15.7), and 137 (11.8).

To a reaction mixture containing 23.4 mg of vanillyl alcohol (VII), 13 ml of 0.1 M sodium tartrate buffer (pH 3.0) and 2 ml of enzyme solution (3.6 μkat), 15 μl of 500 mM H_2O_2 solution in H_2O were added 20 times at 3 min intervals at 20°C in a water bath under air. After 1 h from the first addition of H_2O_2 , the reaction pro-

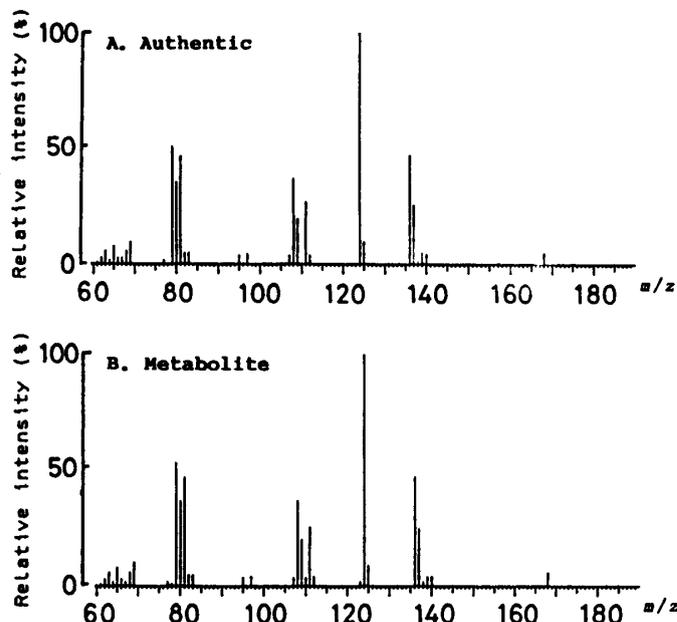


Fig. 30. Mass spectra of the authentic δ -lactone (V) (spectrum A) and metabolite δ -lactone (V) (spectrum B).

ducts extracted with ethyl acetate was subjected to TLC and the two bands on a TLC plate corresponding to vanillin (VIII) and dehydrodivanillyl alcohol (XI), respectively, were scraped off and extracted with 20% $\text{CH}_3\text{OH}/\text{CH}_2\text{Cl}_2$. The one extract containing (XI) was acetylated and analyzed by $^1\text{H-NMR}$ and MS. The other extract was subjected further to TLC. Two products corresponding to δ -lactone (V) and vanillin (VIII) were isolated and analyzed by GC-MS.

Other fractions obtained in the first TLC also were analyzed by $^1\text{H-NMR}$ and GC-MS. The control reactions, lacking lignin peroxidase, or both lignin peroxidase and H_2O_2 , also were carried out for comparison.

2.1.3 Results

Oxidation of vanillyl alcohol (VII) in the complete reaction system yielded dehydrodivanillyl alcohol (XI) as the major product, accompanied by vanillin (VIII) and the aromatic ring opening product δ -lactone (V) in smaller amounts (Fig. 29).

The control system lacking the enzyme yielded a negligible amount (less than 5%) of (V). However, the other control system lacking both the enzyme and H_2O_2 did not yield (V), (XI), or (VIII) but yielded other compounds which also were detected in the complete system. Two of these nonenzymatically formed compounds were identified as divanillyl ether (IX) and diphenylmethane (X) by $^1\text{H-NMR}$ and GC-MS, which is consistent with the result reported by Hemingson and Leary⁷³⁾. Analytical data for the identified products are summarized in Table 14. Mass spectrum of δ -lactone (V) obtained was identical with that of the authentic compound

as shown in Fig. 30.

2.1.4 Discussion

The fact that the enzymatic oxidation of vanillyl alcohol (VII) yielded the products (V), (XI), and (VIII) indicates that cleavage of the phenolic aromatic ring, coupling, and C α -oxidation, respectively occurred.

It is found that the lignin peroxidase of *P. chrysosporium* catalyzes the ring opening of the phenolic substrate, although the ring opening reaction was not major. This result also indicates that demethylation is not a prerequisite for ring opening of the phenolic substrate as well as that of non-phenolic ones reported previously⁷⁴⁾. Although Chen and Chang¹⁴⁾ suggested that the demethylation at C₃ might be important for the ring opening of lignin polymer. Furthermore, the observed ring opening of the guaiacyl substrate (I) strongly suggests a new possibility that the guaiacyl moieties of lignin polymer similarly undergo ring opening during the wood-decaying process (*in vivo*) in contrast to the repolymerization which frequently was observed in the *in vitro* systems. This new hypothesis is in good agreement with the fact observed by Kirk and Chang⁷⁵⁾ that the protolignin in wood did not undergo exclusively the C-C coupling to form 5-5' condensed products during natural wood decays caused by *C. versicolor* and *Polyporus anceps*.

However, further research is needed to evaluate the significance of the ring opening of phenolic moieties of lignin polymer during the natural wood degradation process.

Section 2.2

Degradation of Biphenyl Model Compounds

2.2.1 Introduction

The enzymatic oxidation of vanillyl alcohol (VII) *in vitro* resulted in mainly polymerization yielding dehydrodivanillyl alcohol (XI), although a small amount of aromatic ring opening product δ -lactone (V) was obtained⁵⁰⁾ (section 2.1). It is still uncertain whether during wood decay processes the phenolic ring of the protolignin undergoes exclusively repolymerization as it is observed *in vitro* system. In this context, it is important to elucidate whether both vanillyl alcohol (VII) and dehydrodivanillyl alcohol (XI) undergo the aromatic ring opening reaction. Furthermore, it has not been fully elucidated how lignin peroxidase degrades biphenyl structure.

In section 2.2 is described lignin peroxidase-catalyzed oxidation of several non-phenolic and phenolic lignin model substrates. Nonphenolic syringyl (XV) and biphenyl substrates (XXI) yielded the ring opening products similar to those obtained from veratryl alcohol (I) in the enzymatic degradation. No ring opening products were detected from the phenolic syringyl and biphenyl lignin substructure model compounds⁵¹⁾.

2.2.2 Experimental

Preparation of lignin peroxidase

Lignin peroxidase was a kind gift from Dr. Leisola. The enzyme was dialyzed against 20 mM sodium phosphate buffer (pH 6.5) for 24 h at 4°C before use. Enzymatic activity was determined as described in the section 1.1.

Preparation of substrates and authentic compound

Syringyl alcohol (XII) and 3,4,5-trimethoxybenzyl alcohol (XV) were prepared by reduction of syringaldehyde (XIII) and 3,4,5-trimethoxybenzaldehyde (XVIII) with NaBH₄ in THF/CH₃OH=2:1 at 0°C, respectively. Dehydrodivanillyl alcohol (XI) was prepared as described in previous section 2.1. Dehydrodiveratryl alcohol (XXI) and compound (XXV) were prepared by methylation of (XI) with CH₃I, K₂CO₃ in DMF at room temperature. The reaction products were purified to separate dehydrodiveratryl alcohol (XXI) and 5,5',6'-trimethoxy-3,3'-dihydroxymethylbiphenyl-1-ol (XXV) by TLC. (XXV) was identified by ¹H-NMR and GC-MS⁵¹). (XV-D) was prepared from (XII) by use of C²H₅I, K₂CO₃ in DMF at room temperature. The authentic 2-(2-hydroxy-5-hydroxymethyl-3-methoxyphenyl)-4-methoxycarbonylmethylene-2-penten-5-olide (XXIV) was prepared by the modified method of Dence *et al.*⁷⁶ from (XI). The authentic compound of (XXIV) was identified by comparison of its ¹H-NMR- and mass- spectra with one reported previously⁷⁷. (XXIV-Me) was prepared by methylation with CH₃I, K₂CO₃ in DMF at room temperature.

Enzymatic degradation of (XII) and (XV)

To a reaction mixture containing 0.15 m mol of (XII) or (XV), 15 ml of 0.1 M sodium tartrate buffer (pH 3.0) and lignin peroxidase (0.65 μkat), 15 μl of 500 mM H₂O₂ solution in H₂O was added 10 times at 3 min intervals at 25°C under air. After 30 min from the first addition of hydrogen peroxide, the reaction products extracted with ethyl acetate were separated by TLC. Each fraction was analyzed by ¹H-NMR and GC-MS. The fraction containing both (XVI) and (XVII) was further separated by TLC and the obtained two fractions were analyzed by ¹H-NMR and GC-MS. The substrate (XV-D) was also subjected to enzymatic degradation in the same way.

Enzymatic degradation of (XI), (XXI) and (XXV)

To a 0.05 m mol of (XI), (XXI) or (XXV) in 40 μl of DMF, 15 ml of 0.1 M sodium tartrate buffer (pH 3.0) was added while stirring and then lignin peroxidase (0.63 μkat) was added. To this solution, 15 μl of 500 mM H₂O₂ solution in H₂O was added 10 times at 3 min intervals at 25°C under air. 0.63 μkat of lignin peroxidase was further added after 15 min from the first addition of H₂O₂. After 30 min from the first addition of H₂O₂, the reaction products extracted with ethyl acetate were separated by TLC. After acetylation, each fraction was analyzed by ¹H-NMR

and GC-MS.

Identification of the product (XXVI)

To determine which hydroxymethyl moiety of (XXV) was oxidized to formyl group, (XXVI) was acetylated followed by reduction with NaBD₄ and the acetyl group of the product was removed by NaOCH₃ to obtain (XXV-D). (XXV-D) was further acetylated to give (XXV-D-Ac). The chemical shifts of ¹H-NMR assigned to methylene protons of both (XXV-D) and (XXV-D-Ac) were compared to those of authentic dehydrodivanillyl alcohol (XI), dehydrodiveratryl alcohol (XXI) and their acetate (XI-Ac) and (XXI-Ac).

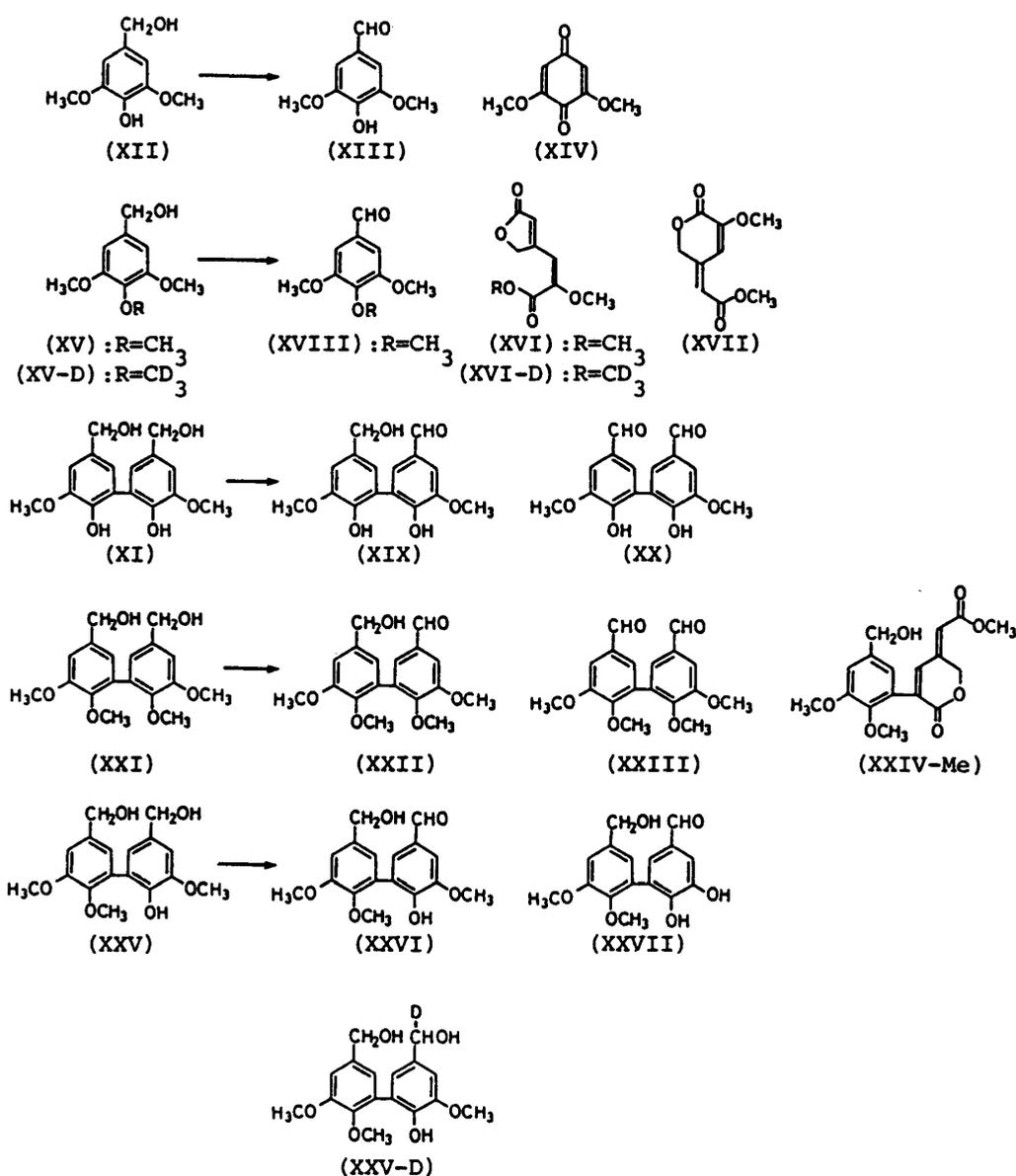


Fig. 31. Degradation of the substrates catalyzed by lignin peroxidase. (XXV-D) was derived from (XXVI) by reduction with NaBD₄.

Table 15a. ¹H-NMR spectra data for products

Compound	¹ H-NMR (CDCl ₃)
XVI	3.87 (3H, s, CHOOC-), 3.89 (3H, s, H ₃ CO-C(=C)-CO-), 5.14 (2H, dd, <i>J</i> =1.89, 0.71Hz, -COO-CH ₂ -), 6.14 (1H, td, <i>J</i> =1.89, 0.68Hz, -C=CH-COO-), 6.71 (1H, m, -CH=C(OCH ₃)-CO).
XVII	3.76 (3H, s, CH ₃ OOC), 3.86 (3H, s, CH ₃ O-C(=CH)-COO-), 4.98 (2H, d, <i>J</i> =1.7Hz, -COO-CH ₂ -), 5.68 (1H, m, CH ₃ OOC-CH=C-), 7.62 (1H, broad-s, -HC=C(OCH ₃)-COO-).
XXVI-Me	2.11 (3H, s, -OAc), 3.77 (3H, s, -COOCH ₃), 3.84 (3H, s, methoxyl group), 3.88 (3H, s, methoxyl group), 5.04 (2H, s, -CH ₂ -OAc), 5.08 (2H, d, <i>J</i> =1.23Hz, -COO-CH ₂), 5.93 (1H, m, =CH-COOCH ₃), 6.89 (1H, d, <i>J</i> =1.5Hz, aromatic C ₂ or C ₆), 6.96 (1H, d, <i>J</i> =1.5Hz, aromatic C ₆ or C ₂).
XXVI-Ac	2.12 (3H, s, -OAc), 2.14 (3H, s, -OAc), 3.58 (3H, s, C ₄ -OCH ₃), 3.92 (3H, s, -OCH ₃), 3.94 (3H, s, -OCH ₃), 5.06 (2H, s, -CH ₂ OAc), 6.82 (1H, d, <i>J</i> =1.8Hz, C ₂ -H or C ₆ -H), 6.95 (1H, d, <i>J</i> =1.8Hz, C ₂ -H or C ₆ -H), 7.50 (2H, s, C ₂ '-H and C ₆ '-H), 9.94 (1H, s, -CHO).
XXVII-Ac	2.12 (3H×2, s, alcoholic -OAc and phenolic C ₄ '-OAc), 2.32 (3H, s, phenolic C ₃ '-OAc), 3.56 (3H, s, C ₄ -OCH ₃), 3.92 (3H, s, C ₃ -OCH ₃), 5.06 (2H, s, -CH ₂ OAc), 6.84 (1H, d, <i>J</i> =1.9Hz, C ₂ -H or C ₆ -H), 6.96 (1H, d, <i>J</i> =1.9Hz, C ₂ -H or C ₆ -H), 7.77 (1H, d, <i>J</i> =1.9Hz, C ₂ '-H or C ₆ '-H), 7.82 (1H, d, <i>J</i> =1.9Hz, C ₂ -H or C ₆ '-H), 9.98 (1H, s, -CHO).

Table 15b. Mass spectra data for products

Compound	Mass spectra m/z (%)
XVI	198 (M ⁺ , 84.1), 170 (75.3), 154 (27.8), 153 (23.7), 142 (22.4), 141 (59.3), 139 (46.5), 138 (22.9), 127 (98.3), 126 (15.9), 125 (23.3), 124 (33.0), 123 (56.0), 111 (58.1), 98 (29.6), 95 (100.0), 83 (33.6), (33.6), 82 (19.3), 81 (34.8), 80 (15.0), 79 (24.4), 69 (35.3), 68 (19.3), 81 (34.8), 80 (15.0), 79 (24.4), 69 (35.3), 68 (33.4), 67 (49.0), 66 (89.1).
XVII	198 (M ⁺ , 93.6), 170 (59.7), 167 (29.0), 166 (57.2), 155 (34.9), 139 (50.6), 138 (100.0), 127 (39.4), 123 (84.5), 112 (25.6), 111 (66.7), 99.0 (16.0), 96 (16.0), 95 (55.4), 83 (34.8), 81 (16.9), 69 (22.0), 68 (55.7), 67 (43.7), 66 (35.3).
XXIV-Me-Ac	376 (M ⁺ , 100.0), 333 (49.5), 319 (48.7), 317 (45.5), 316 (21.8), 301 (40.8), 285 (20.5), 273 (21.8), 257 (45.1), 256 (25.8), 245 (22.1), 229 (34.2), 228 (23.3), 213 (23.6), 201 (32.2), 199 (22.3), 185 (24.9), 129 (21.1), 128 (36.8), 127 (24.2), 115 (62.5), 77 (24.8).
XXVI-Ac	402 (M ⁺ , 13.3), 300 (37.3), 285 (21.6), 270 (18.3), 269 (100.0).
XXVII-Ac	430 (M ⁺ , 32.4), 328 (47.7), 313 (11.4), 298 (15.7), 297 (79.5), 287 (24.6), 286 (37.4), 285 (12.0), 271 (21.4), 257 (10.7), 256 (18.1), 255 (100.0).

2.2.3 Results

The products obtained from the substrates (XII), (XV), (XI), (XXI) and (XXV) in the enzymatic degradation are given in Fig. 31. Analytical data of $^1\text{H-NMR}$ and Mass spectra for (XVI), (XVII), (XXVI-Me), (XXVI-Ac) and (XXVII-Ac) were summarized in Tables 15a and 15b.

Identification of the products (XVI), (XVII) and (XXIV-Me)

The aromatic ring opening products (XVII and XXIV) were not detected from phenolic model compounds (XII and XI) by GC-MS analysis, but oxidation products, 2,6-dimethoxy-*p*-benzoquinone (XIV) and the compound with *Ca*-carbonyl group (syringaldehyde (XIII), 5,5'-dimethoxy-6,6'-dihydroxy-3'-hydroxymethyl-3-biphenylcarbaldehyde (XXII), dehydrodivanillin (XX)) were obtained. On the contrary, (XV) and (XXI) yielded similar *Ca*-carbonyl products (3,4,5-trimethoxybenzaldehyde (XVIII), 5,6,5'6'-tetramethoxy-3'-hydroxymethyl-3-biphenylcarbaldehyde (XXII), dehydrodiveratraldehyde (XXIII)) accompanied by the aromatic ring opening products, such as, 5-Oxo-2H-furan-3(5H)-yl-2-methoxypropenoic acid methyl ester (XVI), 5-methoxy-6-Oxo-2H-pyran-3(6H)-yliden acetic acid methyl ester (XVII) and (XXIV-Me), respectively. The chemical structures of the lactone ring moiety of (XVI) and (XVII) were determined by use of (XV-D) of which methoxyl group at C₄ position was labeled with deuterium. The enzymatic degradation of (XV-D) yielded two aromatic ring opening products. $^1\text{H-NMR}$ and mass spectra of the two aromatic ring opening products showed that one of them was identical with (XVII) obtained from (XV). However, the other one was found to retain deuterium at the methyl ester moiety quantitatively. Thus (XVI) and (XVII) were confirmed to be five and six membered lactone, respectively. (XXIV-Me) and other degraded products were identified by comparison of their $^1\text{H-NMR}$ and mass-spectra with those

Table 16. Chemical shifts of $^1\text{H-NMR}$ spectra for methylene proton of XXV-D, XXVD-Ac and authentic compounds

Compound	methylene proton substituted on phenolic ring	methylene proton substituted on nonphenolic ring
XXV-D	4.636 (1H)	4.685 (2H)
XXV	4.635 (2H)	4.660 (2H)
XXI	—————	4.663 (4H)
XI	4.618 (4H)	—————
XXVD-Ac	5.088 (1H)	5.050 (2H)
XXV-Ac	5.097 (2H)	5.042 (2H)
XXI-Ac	—————	5.063 (4H)
XI-Ac	5.080 (4H)	—————

of authentic compounds.

Identification of the products (XXVI) and (XXVII)

Two products (XXVI and XXVII) were obtained in the enzymatic degradation of (XXV). (XXVI) was identified by $^1\text{H-NMR}$ and GC-MS. The chemical shifts of methylene protons of (XXV-D), (XI), (XXI), (XXV) and their acetate compounds were summarized in Table 16. The results show that the formyl group of (XXVI) is substituted not on nonphenolic ring but on phenolic ring. Each chemical shift assigned to -CDH- of (XXV-D) and (XXV-D-Ac) is in good accordance with methylene protons substituted on phenolic rings of (XI), (XXI), (XXV) and their acetate compounds, respectively. (XXVII) was identified by $^1\text{H-NMR}$ and GC-MS of its acetate. $^1\text{H-NMR}$ spectrum shows that (XXVII) includes two methoxyl groups. The chemical shifts (7.774, 7.821 and 9.978 assigned to the benzene ring and the formyl group of (XXVII-Ac), respectively) were lower than those (7.403 and 7.557 for aromatic ring, 9.946 for formyl group) of dehydrodivanillin (XX-Ac), which indicates that two of OAc and formyl group were substituted on the same benzene ring.

2.2.4 Discussion

It is showed that among the compounds tested only nonphenolic syringyl compound (XV) and biphenyl (XXI) compound which is a closer model to lignin than that reported previously⁷⁹⁾ yielded aromatic ring opening products (XVI and XVII, and XXIV-Me, respectively). Recently, Yokota *et al.*⁷⁹⁾ also reported the aromatic ring opening reaction of nonphenolic biphenyl lignin model compound. The present results also demonstrated that the aromatic ring opening reaction catalyzed by lignin peroxidase preferentially occurs on nonphenolic rings.

It is important to investigate how the biphenyl substructure in protolignin is degraded by lignin peroxidase and whether such a biphenyl structure is really formed during the white-rot decay process. The present study showed that the nonphenolic biphenyl structure is degraded *via* aromatic ring openings. As for the degradation of the phenolic ring of the biphenyl substructure, Umezawa *et al.*⁸⁰⁾ reported that the phenolic ring of dehydrodivanillic acid is degraded in the culture of *P. chrysosporium* to give aromatic ring opening products and a catechol structure. The degradation of dehydrodivanillin by alkalophilic bacteria also was reported⁸¹⁾. Interestingly, the catechol product (XXVII) was isolated by the enzymatic oxidation of the biphenyl substrate (XXV). Neither vanillyl alcohol nor syringyl alcohol gave such a catechol structure by lignin peroxidase (Data not shown). As proposed by Chen and Chang¹⁴⁾ it is suggested that the biphenyl structure is degraded *via* the formation of not only Ca-oxidation but also catechol structures by lignin peroxidase or unknown oxygenase.

Further research is being conducted on the enzymatic degradation of the phenolic ring of lignin in relation to biodegradation by white-rot fungi.

Chapter 3

Role of Veratryl Alcohol in Lignin Biodegradation

3.1 Introduction

In chapter 1 is described the mechanism for aromatic ring opening reaction of veratryl alcohol (I) catalyzed by lignin peroxidase and hemin catalyst in relation to both metabolism of veratryl alcohol (I) by *P. chrysosporium*, and mechanism for aromatic ring opening reaction of lignin biodegradation. Since veratryl alcohol (I) is a secondary metabolite of *P. chrysosporium*^{17,39)} and *C. versicolor*⁸²⁾, it is also interesting note that veratryl alcohol (I) may play multiple roles in white-rot decay process, such as a radical mediator⁵⁵⁾ or protecting agent against lignin peroxidase inactivation^{47,53 54)}. Recently, Kondo and Imamura⁵⁶⁾ reported that veratryl alcohol (I) added to the ligninolytic cultures of *P. chrysosporium* is converted to veratryl β -D-xyloside (XXXIII) in the presence of holocellulose or xylan.

The present author suspected that veratryl β -D-xyloside (XXXIII) might be also a radical mediator as veratryl alcohol (I) in the white-rot decay process.

The objective of chapter 3 is to investigate possible roles of veratryl alcohol (I) and veratryl β -D-xyloside (XXXIII) in lignin biodegradation. The enzymatic oxidation of three substrates; 1,2-bis(4-methoxyphenyl)propane-1,3-diol (XXVIII), 1-(3,4-dimethoxyphenyl)-2-(4-methoxyphenyl)propane-1,3-diol (XXX), 4-ethoxy-3-methoxyphenylglycerol- β -guaiacyl ether (XXXI) were carried out in the presence of the veratryl alcohol (I) or veratryl β -D-xyloside (XXXIII)^{57,58)}.

3.2 Experimental

Preparation of substrates and authentic compounds

1,2-Bis(4-methoxyphenyl)propane-1,3-diol (XXVIII), 1-(3,4-dimethoxyphenyl)-2-(4-methoxyphenyl)propane-1,3-diol (XXX) and 4-ethoxy-3-methoxyphenylglycerol- β -guaiacyl ether (XXXI) were kind gifts from Dr. Umezawa and Mr. Habe. 4-^[2H₃]Methoxybenzaldehyde (XXIX-D), 4-ethoxy-3-^[2H₃]methoxybenzaldehyde (XXXII-D) and 3-methoxy-4-^[2H₃]methoxybenzaldehyde (II-D) were prepared from *p*-hydroxybenzaldehyde, and 4-ethoxy-3-hydroxybenzaldehyde, respectively by methylation with C²H₃I (Merck, ²H: min. 99%)/K₂CO₃ in DMF. 3,4-Di^[2H₃]methoxybenzaldehyde (II-D2) prepared from protocatechualdehyde by methylation with C²H₃I was reduced by NaBH₄ to give 3,4-di ^[2H₃]methoxybenzyl alcohol (I-D). Since 4-^[2H₃] Methoxybenzaldehyde (XXIX-D) was spontaneously oxidized to the corresponding acid, the internal standard solution of (XXIX-D) was prepared before each experiment.

Preparation of the lignin peroxidase

P. chrysosporium (BKM-F-1767, ATCC 24725) was grown as described in section

2.1⁷²⁾. The concentrated extracellular fluid was dialyzed against 20 mM sodium phosphate buffer (pH 6.5) to remove veratryl alcohol (I) and used for the oxidation of lignin model compounds. Purified lignin peroxidase obtained from the same culture condition of *P. chrysosporium*⁸³⁾ was a kind gift from Mrs. Akamatsu and used to obtain Lineweaver-Burk plots. The lignin peroxidase activities were assayed as described in section 1.1.

Preparation of veratryl β -D-xyloside (XXXIII)

The reaction mixture containing acetobromoxylose (102 mg, 0.3 mmol), veratryl alcohol (I) (202 mg, 1.2 mmol), Hg(CN)₂ (190 mg, 0.75 mmol), molecular sieves 4A (450 mg) and 9 ml of anhydrous 1,2-dichloroethane was stirred at 50°C under N₂ for 30 min. The products were extracted with ethyl acetate, washed with saturated NaHCO₃ solution and saturated NaCl solution successively. The organic layer was dried over anhydrous Na₂SO₄ and evaporated. The condensate was subjected to TLC and each fraction was analyzed by ¹H-NMR. The fraction containing veratryl β -D-xyloside acetate (XXXIII-Ac) was further purified by TLC. Veratryl β -D-xyloside acetate (XXXIII-Ac) was identified with ¹H-NMR and MS spectra⁵⁸⁾. NaOCH₃ was used for deacetylation to give veratryl β -D-xyloside (XXXIII) which was purified by TLC.

Enzymatic degradation of (XXVIII) or (XXX)

The reaction mixture (2.5 ml) containing substrate (XXVIII or XXX, 0.4 mM) taken from 0.1 M DMF solution of (XXVIII or XXX), lignin peroxidase (6.6 nkat), H₂O₂ (0.5 mM) and 0.1 M sodium tartrate buffer (pH 3.0) was incubated at 20°C for 10 min. After the addition of 0.25 μ mol of 4-[²H₃] methoxybenzaldehyde (XXIX-D) for (XXVIII) or 0.5 μ mol of 3-methoxy-4-[²H₃] methoxybenzaldehyde (II-D) for (XXX) as an internal standard, the products were extracted with ethyl acetate. The condensate was acetylated for 12 h and the acetylated products were analyzed by GC-MS. The quantitative analysis for 4-methoxybenzaldehyde (XXIX) and 3,4-dimethoxybenzaldehyde (II) derived from (XXVIII) and (XXX), respectively was conducted by mass fragmentgraphy. The area of molecular ion peaks of both products and authentic compound were compared. The enzymatic degradation of products and authentic compound were compared. The enzymatic degradation of substrate (XXVIII) or (XXX) with 0.8, 1.2 and 2.4 mM were also conducted in the same procedure.

Enzymatic degradation of (XXVIII) or (XXX) in the presence of veratryl alcohol (I) or veratryl β -D-xyloside (XXXIII)

The reaction mixture with veratryl alcohol (I) or veratryl β -D-xyloside (XXXIII) is denoted as system 1 or 2, respectively. The reaction mixture (2.5 ml) containing substrate (XXVIII, 1.2 mM) taken from 0.1 M DMF solution of (XXVIII), lignin

peroxidase (6.6 nkat), veratryl alcohol (I) or veratryl β -D-xyloside (XXXIII) (0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mM, respectively), H₂O₂ (0.5 mM) and 0.1 M sodium tartrate buffer (pH 3.0) was incubated at 20°C for 10 min. After the addition of 0.25 μ mol and 0.5 μ mol of 4-[²H₃] methoxybenzaldehyde (XXIX-D) and 3-methoxy-4-[²H₃] methoxybenzaldehyde (II-D) as an internal standard, respectively, the products were extracted and acetylated. The acetylated products were analyzed by GC-MS as described above. (XXX) was also oxidized in the presence of 3,4-di[²H₃] methoxybenzyl alcohol (I-D) in the same way.

Degradation of (XXXI) with veratryl alcohol (I) or veratryl β -D-xyloside (XXXIII)

The reaction mixture (2.5 ml) containing substrate (XXXI, 0.2 mM) taken from 16.7 mM DMF solution of (XXXI), lignin peroxidase (6.6 nkat), veratryl alcohol (I) or veratryl β -D-xyloside (XXXIII) (0.033, 0.067, 0.1, 0.133, 0.167 and 0.2 mM, respectively), H₂O₂ (0.25 mM) and 0.1 M sodium tartrate buffer (pH 3.0) was incubated at 20°C for 10 min. After the addition of 0.042 μ mol and 0.017 μ mol of 4-ethoxy-3-[²H₃] methoxybenzaldehyde (XXXII-D) and 3-methoxy-4-[²H₃] methoxybenzaldehyde (II-D) as an internal standard, respectively, the products were extracted, acetylated and analyzed in the same procedure described above.

Degradation of veratryl β -D-xyloside (XXXIII) or veratryl alcohol (I)

The reaction mixture (2.5 ml) containing veratryl alcohol (I) or veratryl β -D-xyloside (XXXIII) (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mM, respectively) taken from 0.05 M DMF solution of the substrate, lignin peroxidase (6.6 nkat), H₂O₂ (0.5 mM) and 0.1 M sodium tartrate buffer (pH 3.0) was incubated at 20°C for 10 min. After the addition of 0.5 μ mol of 3-methoxy-4-[²H₃] methoxybenzaldehyde (II-D) as an internal standard, the products were extracted and analyzed in the same procedure described above.

Inhibition of lignin peroxidase by (XXVIII)

The initial rates for the lignin peroxidase activity were determined at the various concentrations of the veratryl alcohol (I) in the presence of 0.25 mM and 0.5 mM (XXVIII) and the absence of (XXVIII). The Lineweaver-Burk plots were obtained by plotting reciprocals of the initial rates (A absorbance at 310 nm/min) against those of the various substrate concentrations (mM). The same amount of the enzyme activity (3.3 kkat) was used for each assay. Purified enzyme (1.7 nkat) was also used to obtain Lineweaver-Burk plots.

3.3 Results

Table 17 shows differences in products formed from substrates (XXVIII) and (XXX) when they were oxidized in the absence of veratryl alcohol (I). Substrate (XXVIII) did not yield a C α -C β bond cleavage product, whereas substrate (XXX)

Table 17. The amounts of anisaldehyde (XXIX) and veratraldehyde (II) formed from substrates (XXVIII) and (XXX), respectively.

Concentration of (XXVIII) and (XXX) [mM]	Anisaldehyde (XXIX) formed from (XXVIII) (μmol)	Veratraldehyde (II) formed from (XXX) (μmol)
0.4	0	0.19
0.8	0	0.33
1.2	0	0.48
2.4	0	0.71

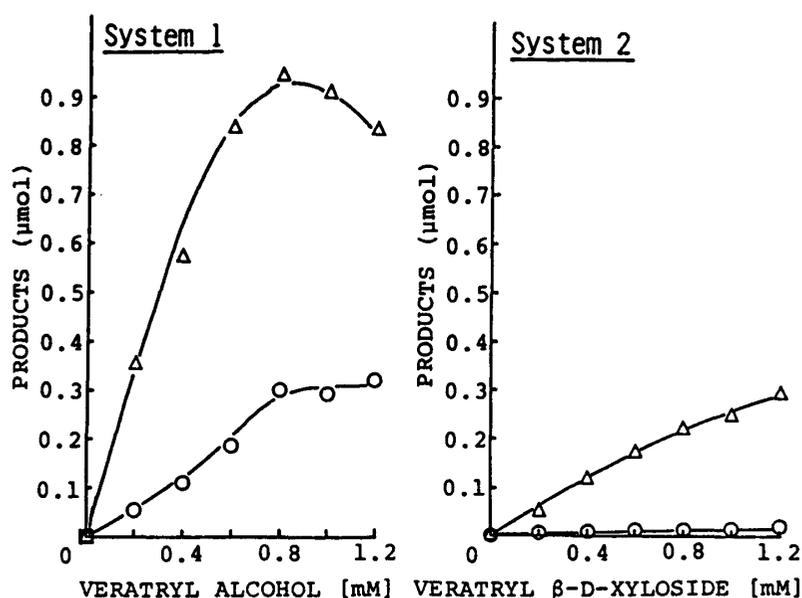


Fig. 32. Enzymatic degradation of 1,2-bis(4-methoxyphenyl)-propane-1,3-diol (XXVIII) with veratryl alcohol (I) (system 1) or veratryl β -D-xyloside (system 2).

(Δ): anisaldehyde (XXIX) formed from (XXVIII), (\circ): veratraldehyde (II) formed from systems 1 and 2.

gave veratraldehyde (II) as cleavage product.

However, the addition of veratryl alcohol (I) or its xyloside (XXXIII) to the enzymatic reaction mixture (System 1 and System 2, respectively; see Experimental) enhanced $C\alpha$ - $C\beta$ bond cleavage of (XXVIII). Figure 32 shows the amount of both anisaldehyde (XXIX) and veratraldehyde (II) formed in systems 1 and 2. System 1 containing veratryl alcohol (I) as a mediator gave 3 to 4 times anisaldehyde (XXIX) as a $C\alpha$ - $C\beta$ cleavage product than did system 2 containing veratryl β -D-xyloside (XXXIII). The ratio of the amounts of veratraldehyde (II) formed in system 2 to that of veratraldehyde (III) formed in system 1 was found to be 0.026, which indicates that the veratryl β -D-xyloside (XXXIII) is much more stable than is veratryl alcohol (I). The ratios of amounts of veratraldehyde (III) formed in systems 1 and 2 to those

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Table 18a. The ratios of the amounts of veratraldehyde (II) formed in system 1 to those of veratraldehyde (II) formed in the absence of (XXVIII)

Concentration of (I) [mM]	Veratraldehyde formed		[B] × 100/[A]
	in system 1 lacking (XXVIII) (μmol) [A]	in system 1 (μmol) [B]	
0.2	0.47	0.05	10.8
0.4	0.92	0.11	11.8
0.6	1.02	0.18	18.1
0.8	1.01	0.30	29.6
1.0	0.99	0.29	29.7
1.2	0.99	0.32	32.5

Table 18b. The ratios of the amounts of veratraldehyde (II) formed in system 2 to those of veratraldehyde (II) formed in the absence of (XXVIII)

Concentration of (XXXIII) [mM]	Veratraldehyde formed		[B] × 100/[A]
	in system 2 lacking (XXVIII) (μmol) [A]	in system 2 (μmol) [B]	
0.2	0.10	2.43×10^{-3}	2.4
0.4	0.18	3.39×10^{-3}	1.8
0.6	0.28	4.43×10^{-3}	1.6
0.8	0.35	5.40×10^{-3}	1.6
1.0	0.39	6.47×10^{-3}	1.6
1.2	0.45	8.03×10^{-3}	1.8

of veratraldehyde (III) formed in systems 1 and 2 without substrate (XXVIII) are given in Tables 18a and 18b. Tables 18a and 18b clearly indicate that addition of (XXVIII) inhibits the oxidation of veratryl alcohol (I) and its xyloside (XXXIII) to veratraldehyde (II). The Lineweaver-Burk plots were prepared to analyze the type of inhibition by (XXVIII) (Figs. 33a and 33b). Figs. 33a and 33b show that the addition of (XXVIII) alters both the K_m value and the maximal velocity with respect to the oxidation of veratryl alcohol (I). As the concentration of (XXVIII) increased the K_m value of veratryl alcohol (I) increased, whereas the maximal velocity decreased. Therefore, the inhibition pattern is a "mixed" type which comprises both competitive and noncompetitive characters.

The amounts of anisaldehyde formed from (XXVIII) also was increased by the addition of veratryl alcohol (I) in the presence of a smaller concentration of H_2O_2 (25 μM) as summarized in Table 19.

Figure 34 shows the oxidation of (XXX) with lignin peroxidase in the presence

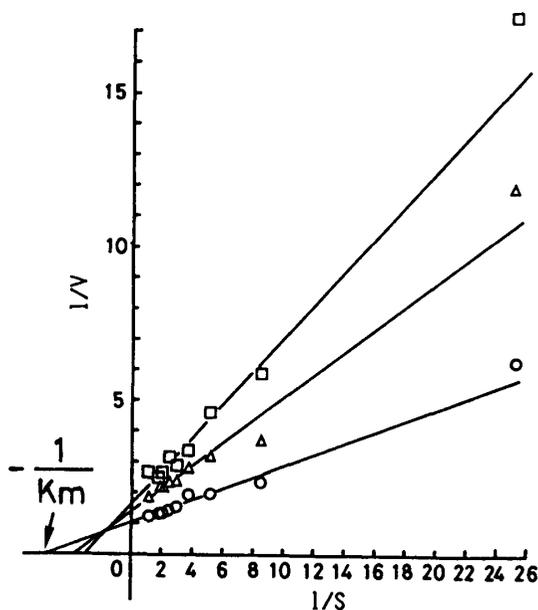


Fig. 33a. Lineweaver-Burk plots for lignin peroxidase activities in the presence and absence of different concentrations of 1,2-bis(4-methoxyphenyl)propane-1,3-diol (XXVIII).
 (○): 0 mM (XXVIII), (△): 0.25 mM (XXVIII), (□): 0.5 mM (XXVIII); 1/V, reciprocals of the reaction rates, 1/s, reciprocals of the veratryl alcohol (mM).

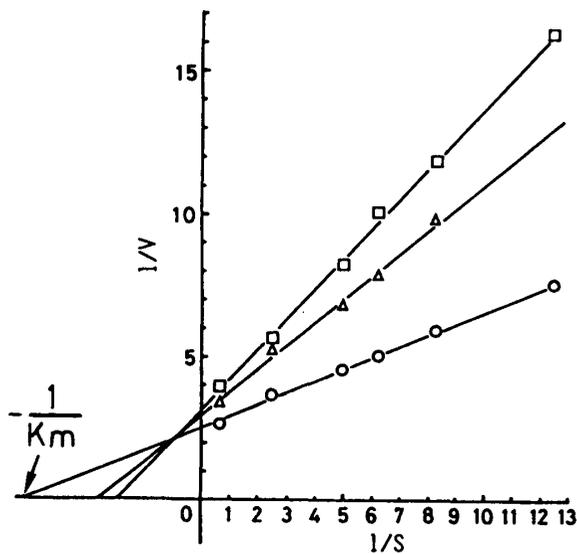


Fig. 33b. Lineweaver-Burk plots for purified lignin peroxidase activities in the presence and absence of different concentrations of 1,2-bis(4-methoxyphenyl)propane-1,3-diol (XXVIII).
 (○): 0 mM (XXVIII), (△): 0.2 mM (XXVIII), (□): 0.5 mM (XXVIII). The reaction mixture contained purified lignin peroxidase (1.7 nkat) and hydrogen peroxide (0.5 mM).

Table 19. The amounts of anisaldehyde (XXIX) and veratraldehyde (II) formed from (XXVIII) and veratryl alcohol (I), respectively, in the presence of H_2O_2 (25 $\mu\text{mol/l}$).

Concentration of veratryl alcohol (I)	Anisaldehyde formed from (XXVIII)	Veratraldehyde (II) formed from veratryl alcohol (I)
[mM]	(nmol)	(nmol)
0	4.4	—
0.2	49.6	21.5
0.4	45.8	24.7

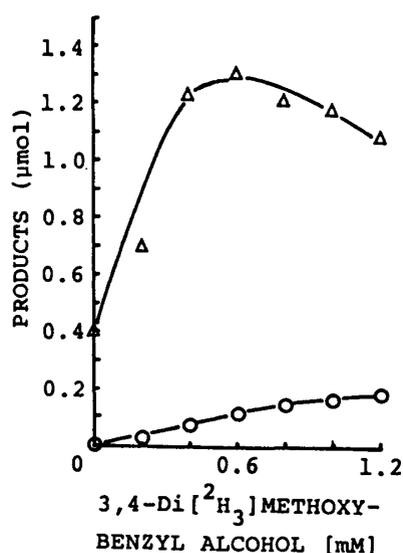


Fig. 34. Enzymatic degradation of 1-(3,4-dimethoxyphenyl)-2-(4-methoxyphenyl)propane-1,3-diol (XXX) with veratryl alcohol (I). (Δ): veratraldehyde formed from (XXX), (\circ): 3,4-Di[$^2\text{H}_3$]methoxybenzaldehyde (II-D2) formed from 3,4-Di[$^2\text{H}_3$]methoxybenzyl alcohol (I-D).

of 3,4-Di[$^2\text{H}_3$]methoxybenzyl alcohol. Although, (XXX) was oxidized in the absence of 3,4-di[$^2\text{H}_3$]methoxybenzyl alcohol by lignin peroxidase, but $\text{C}\alpha\text{-C}\beta$ cleavage reaction of (XXX) to yield veratraldehyde (II) was enhanced by the addition of 3,4-di[$^2\text{H}_3$]methoxybenzyl alcohol.

Figure 35 shows the oxidation of (XXXI) with lignin peroxidase in the presence of veratryl alcohol (I) and its xyloside (XXXIII). The addition of veratryl alcohol or its xyloside did not affect the oxidative degradation of substrate (XXXI).

3.4 Discussion

Harvey *et al.*⁵⁵⁾ suggested that veratryl alcohol acts as a radical mediator during the oxidation of anisyl alcohol in the lignin peroxidase system, whereas Valli *et al.*⁸⁴⁾ emphasizes the enzyme-protecting role of veratryl alcohol (I) in the enzymatic re-

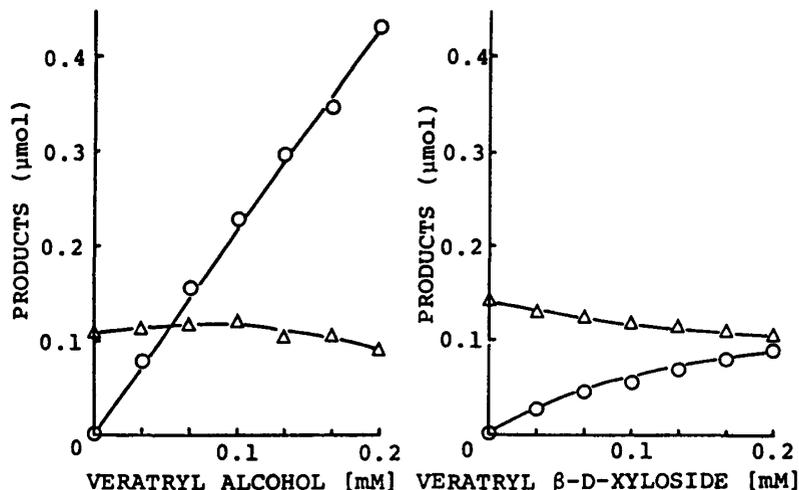


Fig 35. Enzymatic degradation of 4-ethoxy-3-methoxyphenylglycerol- β -guaiacyl ether (XXXI) with veratryl alcohol (I) or veratryl β -D-xyloside. (Δ): 4-ethoxy-3-methoxybenzaldehyde (XXXII) formed from (XXX-I), (\circ): veratraldehyde (II) formed from veratryl alcohol (I) and veratryl β -D-xyloside, respectively.

action. As reported by Valli *et al.*⁸⁴⁾ during the oxidation of anisyl alcohol in the presence of veratryl alcohol (I), both compounds might compete for the same binding site of the enzyme. The apparent inhibition by substrate (XXVIII) of the oxidation of veratryl alcohol (I) to veratraldehyde (II) can be examined by use of enzyme kinetics. However, no attempts to analyze the inhibition mechanism for the oxidation of dimeric lignin model compounds have been reported yet, although noncompetitive inhibition of veratryl alcohol by oxalic acid⁸³⁾ and competitive inhibition of veratryl alcohol (I) by anisyl alcohol⁸⁴⁾ were reported. Thus a similar inhibition analysis was attempted by use of Lineweaver-Burk plots to clarify the role of veratryl alcohol (I).

The results (Figs. 33a and 33b) indicate that the inhibition pattern observed for the oxidation of veratryl alcohol (I) is neither a competitive nor a noncompetitive type, but a mixed type of the two. This inhibition type suggests that veratryl alcohol (I) acts not only as a protector against the inactivation of lignin peroxidase but also as a radical mediator. (XXVIII) and veratryl alcohol (I) seem to compete for the same binding site of lignin peroxidase. Furthermore, the finding that anisaldehyde was produced from (XXVIII) in larger amount than veratraldehyde (II) formed from veratryl alcohol (I) (Fig. 32) is in agreement with the assumption that cation radical of veratryl alcohol (I) mediates possibly a one electron oxidation of (XXVIII). Veratryl β -D-xyloside (XXXIII) also was found to enhance a similar one electron oxidation of (XXVIII). Veratryl β -D-xyloside (XXXIII) is more stable than veratryl alcohol during reaction.

Valli *et al.*⁸⁴⁾ suggested that the ratio of H_2O_2 to enzyme determines whether the

oxidation of anisyl alcohol is stimulated by the presence of veratryl alcohol (I). However, the present results showed that the enzymatic degradation of β -1 model compound (XXVIII) is accelerated by the addition of veratryl alcohol (I) at either large (0.5 mM) or small (25 μ M) H₂O₂ concentrations. Furthermore, the present results also show that the enzymatic degradation of β -1 model compounds (XXVIII and XXX) is accelerated by the addition of veratryl alcohol (I), whereas β -O-4 model (XXXI) was not affected by the (I). However, the increase in the yield of the C α -C β cleavage product formed from another model of β -O-4 substrate (4-ethoxy-3-methoxyphenylglycerol- β -syringyl ether) by lignin peroxidase was reported⁸⁵⁾. Therefore, it seems that veratryl alcohol (I) present in the enzymatic reaction system affects differently the yield of the products, depending on the chemical structures of the substrates.

It is thus concluded that veratryl alcohol (I) and its xyloside (XXXIII) act as a radical mediator in the degradation of lignin substructure model compounds under specified condition. A study of the different effects of veratryl alcohol (I) on the oxidation of β -O-4 compounds is being conducted in relation to the oxidation of similar substrates with different aromatic substitutions. Furthermore, the detection of cation radical of veratryl alcohol (I), its xyloside (XXXIII) or β -1 substrate (XXVIII) remain to be investigated to confirm that veratryl alcohol and its xyloside actually act as a radical mediator.

Conclusion

The objectives of this study are as follows: 1. Elucidation of the reaction mechanisms for aromatic ring opening reaction of veratryl alcohol (I) by lignin peroxidase (Chapter 1). 2. Elucidation of aromatic ring opening reaction of phenolic structure by lignin peroxidase (Chapter 2). 3. Elucidation of the role of veratryl alcohol (I) in the white-rot decay process (Chapter 3). Since veratryl alcohol (I) is synthesized^{17, 39)} and metabolized¹⁷⁾ by white-rot fungi in lignin biodegradation process, the significance of veratryl alcohol (I) metabolism was discussed in relation to lignin biodegradation. Veratryl alcohol (I) is the simplest nonphenolic lignin model compound as well as a secondary metabolite by lignin degrading fungi. Objectives 1 and 2 are important to elucidate the reaction mechanism for aromatic ring opening reaction of nonphenolic and phenolic ring of lignin, respectively, although the mechanisms for aromatic ring opening of β -O-4 lignin substructure dimer have been demonstrated^{31, 59)}.

In chapter 1 the degradation mechanisms for aromatic ring opening reaction of veratryl alcohol (I) by lignin peroxidase and hemin catalyst were described. The chemical structures of aromatic ring opening products, *cis*- γ -lactone (III), *trans*- γ -lactone (IV) which were tentatively identified by Leisola *et al.*³⁸⁾ were confirmed. A new δ -lactone (V) was first isolated as the aromatic ring opening product. In the enzymatic

aromatic ring opening of veratryl alcohol (I) (Section 1.1) one ^{18}O atom each from ^{18}O -labeled H_2^{18}O and $^{18}\text{O}_2$ was incorporated regiospecifically into C-3 and C-4 positions of the aromatic ring of veratryl alcohol (I), respectively to give *cis*- γ -lactone (III). Therefore the reaction mechanism involving O_2^- via dioxetane intermediate proposed by Schoemaker *et al.*⁶¹⁾ was eliminated. The similar tracer experiment with hemin (Section 1.2) showed the same regiospecific oxygenation of veratryl alcohol (I) to give *cis*- γ -lactone (III). The result indicates that the regiospecific oxygenation is not governed by protein moiety but chemical property of the cation radical of veratryl alcohol (I)⁴⁵⁾. On the contrary, random incorporation of two ^{18}O atoms into δ -lactone (V) from H_2^{18}O and $^{18}\text{O}_2$ were observed. Thus, the reaction mechanisms for the ring opening reaction of veratryl alcohol (I) to yield δ -lactone (V) still remains to be investigated.

In chapter 2 the degradation of vanillyl alcohol (VII) (Section 2.1) and biphenyl compounds (Section 2.2) was described. It is found that lignin peroxidase catalyzes phenolic ring opening reaction of guaiacyl model compound, vanillyl alcohol (VII), although the ring opening reaction was not major⁵⁰⁾. Vanillyl alcohol (VII) mainly polymerized by lignin peroxidase *in vitro* system yielding dehydrodivanillyl alcohol (XI). The expected phenolic ring opening products (XXIV) and (XVII) were not detected in the enzymatic degradation of dehydrodivanillyl alcohol (XI) and syringyl alcohol (XII). It seems aromatic ring opening reaction catalyzed by lignin peroxidase preferentially occurs on nonphenolic ring but not phenolic ring⁵¹⁾.

Thus, there must be some mechanisms to prevent lignin from its polymerization during white-rot decay process. It is suggested that the guaiacyl phenolic ring opening reaction occurs in the degradation of lignin polymer in white-rot decay process, since protolignin seems to be degraded without undergoing so many coupling of phenolic units. Indeed, quite recently, Hammel and Moen⁸⁶⁾ showed the lignin degradation by lignin peroxidase *in vitro* system without so many couplings.

In chapter 3 the role of veratryl alcohol (I) and veratryl β -D-xyloside (XXXIII) was described. It was found that addition of both (I) or veratryl β -D-xyloside (XXXIII) enhanced Ca-C β bond cleavage of β -1 substructure model compound (XXVIII). The rate of degradation of (XXVIII) was greater with veratryl alcohol (I) than with veratryl β -D-xyloside (XXIII). However, veratryl β -D-xyloside (XXXIII) was much stable than veratryl alcohol (I) during reaction. The Lineweaver-Burk plots obtained suggested that veratryl alcohol (I) not only prevents lignin peroxidase from inactivation as proposed by Haemmerli *et al.*⁴⁷⁾, Tonon *et al.*⁵³⁾, and Wariishi and Gold⁵⁴⁾, but also acts as a radical mediator as proposed by Harvey *et al.*⁵⁵⁾.

On the contrary the addition of veratryl alcohol (I) or veratryl β -D-xyloside

(XXXIII) did not enhance the C α -C β cleavage of β -O-4 substructure model (XXXI), although Umezawa and Higuchi⁸⁵⁾ observed the enhancement of C α -C β bond cleavage of 4-ethoxy-3-methoxyphenylglycerol- β -syringyl ether by the addition of veratryl alcohol (I). Therefore it is concluded that veratryl alcohol (I) and veratryl β -D-xyloside (XXXIII) act as mediator in lignin biodegradation under the specific condition⁸⁶⁾. Further direct evidence to support the formation of cation radical species of veratryl alcohol (I), its xyloside (XXXIII) or β -1 substrate (XXVIII) is required.

Veratryl alcohol (I) is synthesized during white-rot decay process and plays multiple roles such as radical mediator and protective reagent and metabolized via aromatic ring opening reaction. Leisola *et al.*⁸⁷⁾ also pointed out the metabolism of veratryl alcohol (I) via aromatic ring opening reaction and conversion to quinones. Such a ring opening reaction catalyzed by lignin peroxidase also occurs in synthetic lignin polymer (DHP)⁸⁸⁾. However, phenolic ring involved in lignin polymer is more difficult to be opened than non phenolic ring by lignin peroxidase.

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