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# The C $\alpha$ -C $\beta$ Bond Cleavage of the Secondary Metabolite Veratrylglycerol Catalized by A New Modified "Ligninase" Preparation from *Phanerochaete chrysosporium*\*<sup>1</sup>

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**Abstract**—The various culture conditions for production of lignin peroxidase were examined by use of the white-rot fungus *Phanerochaete chrysosporium* grown for 4 days in the cultures with high carbon and low nitrogen (HC/LN)-, low carbon and high nitrogen (LC/HN)-, or low carbon and medial nitrogen (LC/MN)-media. Among them, LC/MN-culture with 0.1% Tween 80 produced the greatest amount of ligninase activity (356 units/l). A secondary metabolite veratrylglycerol occurring in the extracellular culture fluid underwent C $\alpha$ -C $\beta$  bond cleavage in the presence of ligninase and hydrogen peroxide, yielding veratraldehyde and glycolaldehyde. The oxidation rates of a *threo*- and a mixture of *threo*- and *erythro*-forms of veratrylglycerol synthesized were about 60% of that of veratryl alcohol. A possible relevance of this cleavage reaction to the C $\alpha$ -C $\beta$  bond cleavage of arylglycerol- $\beta$ -aryl ether substructures of lignin polymer was discussed.

**Key words:** White-rot fungi, ligninase, veratrylglycerol, secondary metabolite, lignin biodegradation, peroxidase

## 1. Introduction

Current lignin biodegradation research has been receiving a keen attention toward fundamentals and applications of ligninolytic fungi and enzyme systems for processing lignocellulosic materials<sup>1)</sup>. We have been interested in the key enzyme reactions to explain the reason why lignin biodegradation is expressed as a part of secondary metabolism, which is initiated by the nitrogen starvation of the fungal culture<sup>2)</sup>. In view of the fact that the white-rot fungus *Phanerochaete chrysosporium* biosynthesizes veratryl alcohol and veratrylglycerol as its secondary metabolites in parallel with lignin degradation, a possible metabolic relationship between

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lignin biodegradation and veratryl alcohol biosynthesis has been first suggested by Shimada *et al.*<sup>3,4)</sup>. Thus, the secondary metabolic event of lignin biodegradation is now approached by studying secondary metabolism of L-phenylalanine, because both veratryl alcohol and veratrylglycerol are formed from this aromatic amino acid<sup>3)</sup>.

In this context, it is important to elucidate how the ligninolytic and the biosynthetic systems are connected in terms of mechanistic features of the enzymatic oxidation of veratrylglycerol, since lignin consists of mainly arylglycerol (C<sub>6</sub>~C<sub>3</sub>) units linked by C-C and ether bonds.

As to catalytic actions of the "ligninase" (lignin peroxidase) produced by *Phanerochaete chrysosporium*, the enzyme has been established to catalyze, at the incipient step, the one-electron transfer oxidation<sup>5~7)</sup>, yielding radical cation species of the substrate molecules, which undergo a variety of bond cleavage reactions. These degradative reactions are featured principally by the C<sub>α</sub>-C<sub>β</sub>-, the β-ether- and the aromatic ring-cleavages<sup>8~10)</sup>.

We report here in this investigation that, like synthetic lignin model substrates for arylglycerol-β-aryl ether substructure, the natural secondary product veratrylglycerol also undergoes C<sub>α</sub>-C<sub>β</sub> bond cleavage, yielding veratraldehyde and glycolaldehyde in the presence of the fungal peroxidase and hydrogen peroxide. The results are discussed in relation to a possible role of this metabolic pathway in lignin biodegradation. We also report here an alternative convenient method for preparation of "ligninase" from *P. chrysosporium*.

## 2. Materials and Methods

### 2.1 Chemicals

All chemicals used are in a reagent grade. Veratrylglycerol for a substrate was prepared from 3,4-dimethoxycinnamyl alcohol according to the method previously reported; *threo*-form and a mixture of *threo*- and *erythro*-isomers of this compound were synthesized by use of osmium tetroxide and *m*-chloroperbenzoate, respectively<sup>3)</sup>.

### 2.2 Culture conditions for "ligninase" preparations

*Phanerochaete chrysosporium* (BKM-F-1767, ATCC 24725) kindly supplied by Dr. Kirk was grown in the same media as reported by Kirk *et al.*<sup>11)</sup>, except that the following modifications were made: 2% and 0.2% glucose were used for high carbon (HC)- and low carbon (LC)-nutrient-containing-media, respectively; 12 mM, 6 mM, and 1.2 mM ammonium tartrate were used for high nitrogen (HN)-, medial nitrogen (MN)-, and low nitrogen (LN) nutrient containing-media, respectively; 7-fold amounts of minerals<sup>11)</sup>, Tween 80 (0.1% at the final concentration) and/or

oleic acid (0.035%) were added to some media to compare the enzyme activities with those produced in the cultures without these components.

HC/LN, LC/HN and LC/MN (60 ml/1-litre flask) media were inoculated with asexual spores of the white-rot fungus and incubated for 4 days at 38°C under normal atmosphere, which was flushed with 100% oxygen on days 2 and 3, and veratryl alcohol (1.5 mM final concentration) was added on day 2 after inoculation.

Culture filtrate was freed from the mycelial mat by filtration through paper towel and concentrated by use of Pelicon-Labo cassette apparatus fixed with millipore ultrafiltration membrane (10,000 molecular weight cutoff). The concentrated enzyme solution was stored at about 2°C in the refrigerator until its use. It retained the all enzyme activity for 3 weeks, unless it was frozen. Nearly 100% of the activity was also retained when it was stored in the presence of 20% glycerol and 10 mM veratryl alcohol at -20°C for the same period but the activity was lost in the absence of veratryl alcohol.

### **2.3 Enzymatic oxidation of veratrylglycerol**

For assay of "ligninase" activity, the enzyme stock solution was thoroughly dialyzed against 0.05 M tartrate buffer (pH 5.5) to remove veratryl alcohol. Enzyme proteins were determined by the Bio-Rad method<sup>12)</sup>. The enzyme activity was determined by measurement of the increase in the absorbance at 310 nm due to the formation of veratraldehyde from veratryl alcohol<sup>12)</sup>. The enzyme activity is expressed as the international unit ( $\mu$ moles of product formed/min) or one unit of activity produces  $\mu$ mole of veratraldehyde from the substrate per min.

The reaction mixture contained the substrate veratrylglycerol (10 mM), hydrogen peroxide (0.5 mM), 0.1 M tartrate buffer (pH 3.0), and the appropriate amount of the enzyme solution and was normally incubated at 30°C. The C<sub>α</sub>-C<sub>β</sub> cleavage reaction rate was followed by measurement of the absorbance at 310 nm due to the formation of veratraldehyde in the same way as "ligninase" was assayed with the veratryl alcohol substrate<sup>13)</sup>. When the enzyme activity was considerably weaker, the substrate was recommended to be added first to the reaction mixture and the reaction was started by addition of hydrogen peroxide. Otherwise, the reaction rate falls off rapidly due to inactivation of the enzyme.

### **2.4 Identification of Compounds**

For isolation of a 2,4-dinitrophenylhydrazone (DNPH) derivative of the glycolaldehyde product, the enzymatic reaction was carried out as follows. The reaction mixture (30 ml) containing 40  $\mu$ moles of the substrate, 25  $\mu$ moles of H<sub>2</sub>O<sub>2</sub>, the enzyme about 40 units, 0.1 M tartrate buffer (pH 3.0) incubated at 25°C for 30 min, thereafter 30 ml of 0.2% 2,4-dinitrophenylhydrazine in 50% ethanol or 0.1 M tartrate buffer (pH 3.0) was added to convert the aldehyde products to DNPH

derivatives. The hydrazones were extracted with ethyl acetate and purified by preparative TLC plates (silica gel 60 F-254) with the solvent (THF: Benzene, 3:100) according to Byrne<sup>14</sup>). Alternatively, the hydrazones were further confirmed by color changes formed with ethanolamine sprayed on the TLC-plate. A mono-DNPH derivative of glycolaldehyde was isolated as the major product and subjected to analyses by MS-and NMR-spectrometries. The mono-DNPH derivative was further acetylated for measurement of its NMR spectrum in comparison with the authentic specimen which had been prepared from commercial glycolaldehyde and 2,4-dinitrophenylhydrazine.

Thus, we avoided the drastic condition for DNPH-derivatization in order to isolate mono-DNPH derivative of glycolaldehyde, because it is further converted to bis-DNPH derivative, i.e. glyoxal DNPH, under the conventional reaction conditions reported<sup>14</sup>).

However, veratraldehyde product was routinely detected by TLC (silicagel F-254) with ethyl acetate/*n*-hexane (1:2) after extraction of the reaction mixture with ethyl acetate. This aromatic aldehyde was rigorously confirmed by measurement of its mass spectrum by GC-MS.

The compounds other than the aldehydes were detected by dipping the TLC plates into 0.5% phosphomolybdate-ethanol solution and heating them on the hot-plate.

## 2.5 Instrumental apparatus used

A Hitachi model 200-20 spectrophotometer was used for measurement of the absorbance at 310 nm. Mass-spectral data for DNPH-glycolaldehyde were obtained by the direct inlet method with a Shimadzu GC-MS QP-1000 apparatus (EI-MS, 70 eV) MS-data for veratraldehyde were obtained with the column; chemical bonded fused-silica capillary column Hicap CBP1 (non-polar methyl silicone polymer, Shimadzu, Japan) 12 m × 0.53 mm (i.d.). <sup>1</sup>H-NMR-spectra were taken with a Varian XL-200 FT-NMR spectrometer with tetramethylsilane as an internal standard.

## 3. Results

### 3.1 Production of fungal peroxidase

Since the attempts to produce "ligninase" in shaken-cultures were failed, we examined the various stationary cultures in reference to the earlier work reported<sup>15~21</sup>).

As the results are given in Table 1, we found that the white-rot fungus grown in LC/MN with aconitate buffer, 7-fold mineral components, 0.1% Tween 80 and 100% oxygen flushed produced the greatest amount of ligninase (356 units/l of

Table 1. Culture condition of *P. chrysosporium* and ligninase production

No.	Culture conditions			Activity (U/l)	Protein conc. (mg/l)	Specific activity (U/mg)
	Media	Buffer <sup>a)</sup>	Additives <sup>b)</sup>			
1	HC/LN	D	N	3.4	—	—
2	LC/MN	D	N	56.0	7.63	7.29
3	LC/HN	A	N	79.0	11.3	6.98
4	LC/MN	A	N	96.0	10.3	9.32
5	LC/MN	A	M	169.0	18.3	9.25
6	LC/MN	A	T	170.0	11.6	15.0
7	LC/MN	A	M, T	356.0	19.5	18.3
8	LC/MN	A	M, O	103.2	15.3	10.9
9	LC/MN	A	M, T, O	208.1	11.8	16.0

a) D: dimethylsuccinate buffer pH=4.5, A: aconitate buffer pH=4.3, b) N: no addition except for veratryl alcohol, M: 7 fold minerals, T: 0.1% (w/v) Tween 80, O: Oleic acid (350 mg/l)

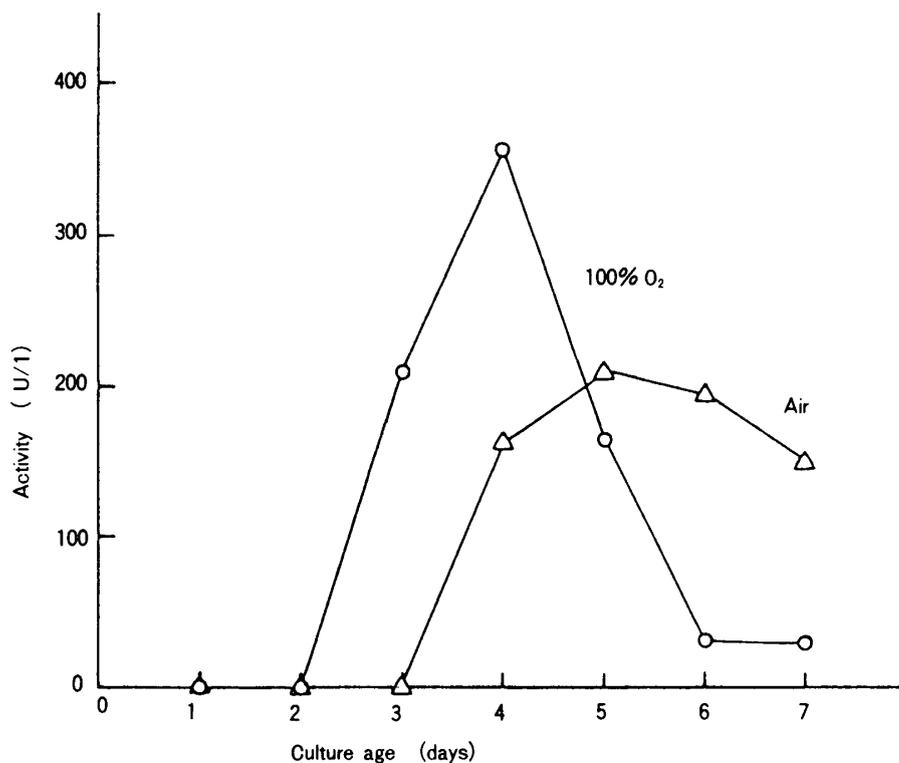


Fig. 1. Production of "ligninase" during cultivation of *Phanerochaete chrysosporium* in LC/MN culture.

culture fluid).

Fig. 1 shows two different production profiles of the ligninase enzyme when the white-rot fungus was grown in LC/MN cultures under air or 100% oxygen. The culture with 100% oxygen (No. 7 in Table 1) began to produce the enzyme

around day 2, reaching the maximum activity on day 4, and decreasing rapidly in the activity thereafter, whereas the other one began to increase after day 3, reaching the maximum activity on day 5, decreasing rather moderately. We found that addition of 0.1% Tween 80 is the most important for the production of the enzyme in larger quantity, since the fungus grown in LC/MN culture under air (21% oxygen) in the absence of 0.1% Tween 80 did not produce a significant amount of the enzyme throughout the culture period (data not shown). Tween 80 detergent might help secretion of fungal peroxidase by solubilization of mycelial membrane, since similar stimulation of the fungal peroxidases were solubilized with other detergent (CHAPS)<sup>4)</sup>.

Although the reported enzyme preparation procedures<sup>15-21)</sup> normally consume at least 6 days, this procedure takes only 4 days to obtain the highest ligninase preparation by the modifications of the original enzyme preparation methods of their culture conditions by taking account of the following factors: a) use of LC/MN, b) 100% oxygen flushings on days 2 and 3, c) additions of 0.1% Tween 80, 1.5 mM veratryl alcohol and 7-fold mineral solution.

### 3.2 Identification of products formed from veratrylglycerol

Veratraldehyde (II) was normally detected without such derivatization on the fluorescent TLC-plate. After the extraction of the reaction mixtures with ethyl acetate, glycolaldehyde (III) was isolated in a form of mono-DNPH derivative.

However, both cleavage products (II and III) were further analyzed by mass- or NMR-spectrometry. As shown in Fig. 2, the mono-DNPH derivative of glycol-

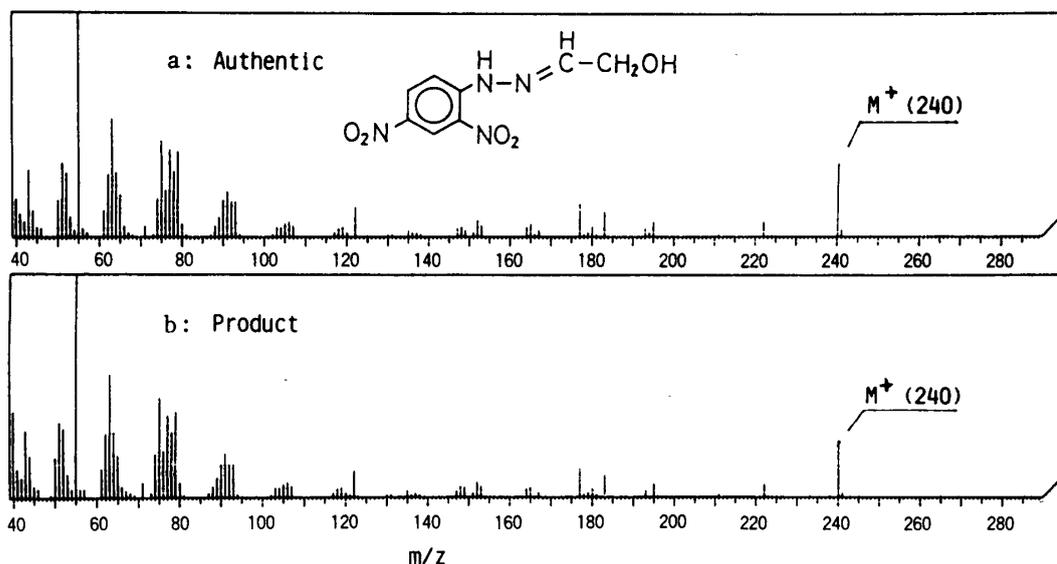


Fig. 2. Mass-spectra of the glycolaldehyde DNPH (a, authentic compound; b, obtained after enzymatic reaction).

aldehyde product (III) gives the MS-spectrum: m/z (%); 240 (M<sup>+</sup>, 34), 222 (7.7), 196 (7.8), 183 (11.6), 180 (5.2), 177 (16.2), 122 (14.0). This mass spectrum is consistent with that of the authentic specimen.

Likewise, the aromatic aldehyde (II) was identified by measurement of its mass-spectrum (M<sup>+</sup>, 166) in comparison with the authentic specimen.

NMR of DNPH glycolaldehyde: <sup>1</sup>H-NMR (CDCl<sub>3</sub>) δ (ppm): 4.55 (2H, d, J=4 Hz, -CH<sub>2</sub>-OH), 7.72 (1H, t, J=4 Hz, -N=CH-), 7.97 (1H, d, J=10 Hz, aromatic C<sub>6</sub>), 8.38 (1H, dd, J=10 Hz, 2 Hz, aromatic C<sub>5</sub>), 9.18 (1H, d, J=2 Hz, aromatic C<sub>3</sub>).

Alternatively, the NMR-analysis of the acetylated mono-DNPH derivative clearly indicates that one acetyl group was introduced into the mono-DNPH of glycolaldehyde. The control reaction system lacking the enzyme solution or the substrate did not give rise to glycolaldehyde DNPH.

Thus, the ligninase enzyme prepared as described above was shown for the first time to catalyze the oxidation of a natural metabolite veratrylglycerol (I) which, like β-O-4 dimeric lignin model, underwent C<sub>α</sub>-C<sub>β</sub> cleavage, yielding veratraldehyde (II) and glycolaldehyde (III) as shown in Fig. 3.

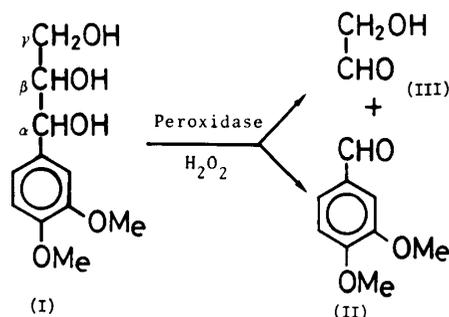


Fig. 3. The C<sub>α</sub>-C<sub>β</sub> bond cleavage of veratrylglycerol (I) catalyzed by fungal peroxidase.

### 3.3 Specificity of the fungal peroxidase to *erythro*- and *threo*-isomers of the veratrylglycerol substrates

Table 2 shows the enzymatic oxidation rates of veratryl alcohol and veratrylglycerol (I) in a *threo*-form and mixtures of *threo*- and *erythro*-forms. The ligninase

Table 2 "Ligninase" activity on veratryl and veratrylglycerol

Substrate	Activity	
	(μmole/min/ml)	(ratio)
Veratryl alcohol	0.90	(100)
Veratrylglycerol ( <i>threo</i> + <i>erythro</i> )	0.57	(63)
Veratrylglycerol ( <i>threo</i> )	0.57	(63)

At pH 3.0, 20°C.

does not show any preference to either of two samples of the substrate (I), both of which were oxidized in a rate of about 60% of that of veratryl alcohol substrate. However, 3,4-dimethoxycinnamyl alcohol did not serve as good substrate enzymatic reaction, although small amounts of veratrylglycerol and veratraldehyde were detected by the TLC analysis. The inactivation mechanism is not elucidated but the active center of the protoporphyrin prosthetic group might be destroyed by covalent bonding with the side chain of this substrate. A similar suicidal inactivation of hemoprotein cytochrome P-450 was also observed during epoxidation of the olefine substrate<sup>21)</sup>. Therefore, it is unlikely that "ligninase" peroxidase participates in conversion of 3,4-dimethoxycinnamyl alcohol to veratrylglycerol.

### 3.4 Localization of veratrylglycerol and veratryl alcohol

It is important to examine whether veratrylglycerol (I) is located in the extracellular fluid of culture. The mycelia and culture fluid fractions were separated and detection of veratrylglycerol together with the veratryl alcohol metabolite was attempted as previously reported<sup>3)</sup>. The analytical result clearly showed that both secondary metabolites occurs exclusively in the extracellular fluid fraction of the whole culture although the amount is much smaller than that of veratryl alcohol<sup>3)</sup>.

Alternatively, we attempted to detect the glycolaldehyde metabolite (III) in the whole HC-LN culture throughout cultivation period of 6 days, since it has not yet been reported to occur in the ligninolytic cultures of *P. chrysosporium*. However, we could not determine the occurrence of the aldehyde, although methylglyoxal and glyoxal were reported to occur in the culture fluid<sup>23)</sup>.

## 4. Discussion

This investigation is ultimately concerned with a basic question how white-rot fungi successfully "invented" the ligninolytic enzyme systems throughout their biochemical evolution processes, whereas brown-rot fungi do not have such a lignin-decomposing activity. Comparative studies of biosynthetic mechanisms of their secondary metabolites may help us to find a clue to answer this intriguing question. Recently we have given a possible explanation for the different secondary metabolic functions between the brown-rot fungus *Lentinus lepideus* and the white-rot fungus *Phanerochaete chrysosporium*<sup>24)</sup>.

In addition to *P. chrysosporium*, other white-rot fungi such as *Coriolus versicolor*<sup>25)</sup>, *Phelebia radiata*<sup>26)</sup>, and *Lentinus edodes*<sup>27)</sup>, have been reported to biosynthesize veratryl alcohol. However, enzymatic study on the oxidation of this fungal product veratrylglycerol has not yet been carried out, although the relevance of this enzymatic reaction to lignin degradation by "ligninase" has been suggested in the earlier paper<sup>3)</sup>.

This investigation has demonstrated for the first time that glycolaldehyde (III) and veratraldehyde (II) are produced as a result of the C<sub>α</sub>-C<sub>β</sub> bond cleavage of veratrylglycerol (I) in the presence of the fungal peroxidase and hydrogen peroxide. The result clearly indicates that this fungal product mimicks the C<sub>α</sub>-C<sub>β</sub> bond cleavage of synthetic lignin model compounds reported<sup>28,29</sup>). Furthermore, the present result (Table 2) shows that the fungal peroxidase does not exhibit the stereospecificity to *erythro*- and *threo*-isomers of veratrylglycerol, which is also consistent with the recent finding that "ligninases" of *Trametes (Coriolus) versicolor* do not distinguish but equally oxidize *erythro*- and *threo*-isomers of the β-O-4 substrate<sup>30</sup>).

The reaction mechanism for the C<sub>α</sub>-C<sub>β</sub> bond cleavage of this substrate is postulated as shown in Fig. 4, which is almost the same as that of the diol bond

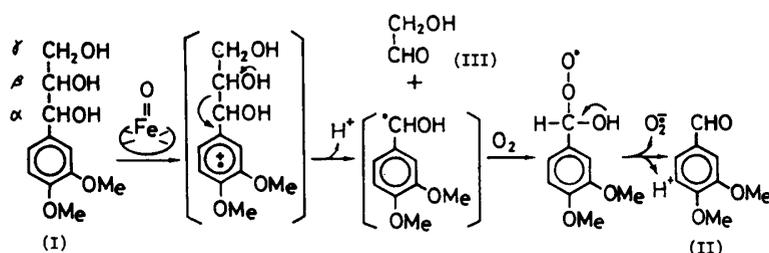


Fig.4. A possible reaction mechanism of the C<sub>α</sub>-C<sub>β</sub> bond cleavage of veratrylglycerol (I) catalyzed by the fungal peroxidase.

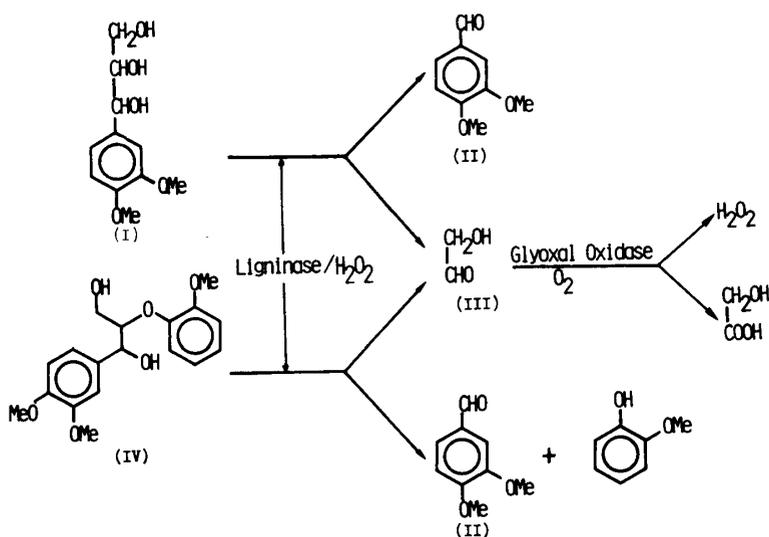
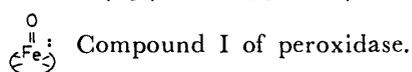


Fig. 5. A possible connection between the peroxidase-catalyzed oxidations of the secondary metabolite veratrylglycerol (I) and the synthetic β-O-4 lignin model compound (IV), both of which constitute hydrogen peroxide-generating system with glycolaldehyde (III) as the common cleavage product.

cleavage of diphenylglycol substrate reported by Hammel *et al*<sup>6)</sup>. Therefore, the results reported herein clearly indicate that the enzymatic oxidation of this fungal metabolite exhibits the  $C_{\alpha}$ - $C_{\beta}$  bond cleavage, one of the prominent features of the ligninase-catalyzed reactions, as shown in Fig. 5.

Since this metabolite was shown to occur together with veratryl alcohol exclusively in the extracellular fluid of culture of this fungus, these secondary metabolites are oxidized by the extracellular "ligninase" system. If lignin or other related compounds are exogenously added into the ligninolytic culture, "ligninase" is shared by these endogenous (natural) metabolites and exogenous (xenobiotic) compounds, including lignin in wood. In view of the above findings, regardless of the presence or absence of lignin, the "ligninase" enzyme systems might be primarily produced for oxidation of veratrylglycerol and veratryl alcohol which are biosynthesized from L-phenylalanine at the secondary metabolic stage of the white-rot fungi. It is inferred, therefore, that the fungal peroxidase catalyzing  $C_{\alpha}$ - $C_{\beta}$  cleavage of veratrylglycerol is identical with the enzyme called "ligninase" or "lignin peroxidase". The above interpretation is in good harmony with the present situation that "ligninase" activity is now routinely assayed by use of the veratryl alcohol substrate instead of using lignin polymer substrate. Again, it is interesting to notice that this secondary metabolite undergoes the ring cleavage reaction<sup>31,32)</sup>, which also exhibits the ring cleavage of the  $\beta$ -O-4 model substrates<sup>16)</sup>.

Furthermore, it is noteworthy, as shown in Fig. 5, that glycolaldehyde product formed is also the substrate for another enzyme or glyoxal oxidase, which oxidizes glycolaldehyde in the presence of dioxygen to yield hydrogen peroxide<sup>23)</sup>. Then, hydrogen peroxide formed is utilized for oxidations of the endogenous secondary metabolites and also of other exogenous lignin-related compounds. It is also noteworthy that this hydrogen peroxide generating system is manifested as the secondary metabolic event<sup>23)</sup>. Thus, as described above, the secondary metabolic pathway, to metabolize veratrylglycerol and veratryl alcohol, contains such key enzyme systems that produce hydrogen peroxide as the secondary metabolic event in the extracellular site.

Therefore, once veratryl alcohol biosynthesis is switched on, all the enzyme systems involved start to metabolize L-phenylalanine to these secondary metabolites and finally carbon dioxide. At this point, it is assumed that the "ligninase" systems are primarily involved in the secondary metabolism of L-phenylalanine. This hypothesis could rationally explain the reason why lignin biodegradation occurs as a secondary metabolic event in the absence of lignin.

In conclusion, the present investigation provides one of the examples for the key reactions to answer the intriguing question: what is the necessity of produc-

tion of "ligninase" as the secondary metabolic event in the absence of lignin? However, it is obscure that this interpretation holds for all white-rot fungi for the following reasons.

a) It is uncertain whether all the white-rot fungi biosynthesize the metabolite veratryl alcohol from veratrylglycerol. b) Some of the brown-rot fungi may have "ligninase" regardless of the absence of the veratryl alcohol biosynthetic system. c) There is a possibility that the C<sub>α</sub>-C<sub>β</sub> bond cleavage of veratrylglycerol is catalyzed by another enzyme probably within or outside the cell. d) Many different isozymes of peroxidases have their own roles for oxidizing specifically natural metabolites or lignin-related compounds. e) During the biochemical evolution of white-rot fungi, the very unique hemoprotein "ligninase" might have derived from a family of hemoproteins, originally independent of secondary metabolism of L-phenylalanine, and might have finally acquired the present enzyme activity to breakdown both the fungal (endogenous) metabolites and plant (exogenous) metabolite lignin in wood.

### References

- 1) E. ODIER: "Lignin Enzymic and Microbial Degradation" (Symposium international, Paris, France, 23, avril) pp. 302 (1987).
- 2) P. KEYSER, T.K. KIRK and J.G. ZEIKUS: *J. Bacteriol.*, **135**, 790 (1978).
- 3) M. SHIMADA, F. NAKATSUBO, T.K. KIRK and T. HIGUCHI: *Arch. Microbiol.*, **129**, 321 (1981).
- 4) M. SHIMADA and T. HIGUCHI: "Recent Advances in Lignin Biodegradation Research" (T. Higuchi, T.K. Kirk, and H.-m. Chang, eds) Uni Publisher, Tokyo, p.195 (1983).
- 5) P. KERSTEN, M. TIEN, B. KALYANARAMAN and T.K. KIRK: *J. Biol. Chem.*, **260**, 2609 (1985).
- 6) K.E. HAMMEL, M. TIEN, B. KALYANARAMAN and T.K. KIRK: *J. Biol. Chem.*, **260**, 8348 (1985).
- 7) T. HABA, M. SHIMADA, T. OKAMOTO, B. PANIPAN and T. HIGUCHI: *J. Chem. Soc. Chem. Commun.*, **1985**, 1323.
- 8) T.K. KIRK: *Ann. Rev. Rev. Microbiol.*, **41**, 465 (1987).
- 9) T. HIGUCHI: *Wood Research*, No. **73**, 58 (1986).
- 10) T. UMEZAWA: *ibid*, No. **75**, 21 (1988).
- 11) T.K. KIRK, E. SHULZ, W.J. CONNORS, L.E. LORENZ and J.G. ZEIKUS: *Arch. Microbiol.*, **117**, 277 (1978).
- 12) M. BRADFORD: *Anal. Biochem.*, **72**, 248 (1976).
- 13) M. TIEN, T.K. KIRK, C. BULL and J.A. FEE: *J. Biol. Chem.*, **261**, 1687 (1987).
- 14) G.A. BYRNE: *J. Chromatog.*, **20**, 528 (1965).
- 15) M. ASTHER, C. CAPDEVILA and G. CORRIEU: *Appl. Environ. Microbiol.*, **54**, 3194 (1988).
- 16) M. ASTHER, G. CORRIEU, R. DRAPRON and E. ODIER: *Enzyme Microb. Technol.*, **9**, 245 (1988).
- 17) J.A. BUSWELL, B. MOLLET and E. ODIER: *FEMS Microbiol. Lett.*, **25**, 295 (1984).
- 18) B.D. FAISON and T.K. KIRK: *Appl. Environ. Microbiol.*, **49**, 299 (1985).
- 19) A. JAGER, S. CROAN and T.K. KIRK: *Appl. Environ. Microbiol.*, **50**, 1274 (1985).
- 20) T.K. KIRK, S. CROAN, M. TIEN, K.E. MURLAGH and R.L. FARRELL: *Enzyme Microb. Technol.*, **8**, 27 (1986).
- 21) M.S.A. LEISOLA and A. FIECHTER: *FEMS Microbiol. Lett.*, **29**, 33 (1985).
- 22) P.R.O. de MONTELLANNO and K.L. KUNZE: *Biochemistry*, **20**, 7266 (1981).

- 23) P.J. KERSTEN and T.K. KIRK: *J. Bacteriol.*, **169**, 2195 (1987).
- 24) M. SHIMADA, A. OHTA, H. KUROSAKA, T. HATTORI, T. HIGUCHI and M. TAKAHASHI: "Plant Cell Wall Polymers" (N.G. Lewis and M.G. Paice, eds). *Am. Chem.Soc. Symp. Ser.*, **399**, 412 (1989).
- 25) S. KAWAI, T. UMEZAWA and T. HIGUCHI: *Wood Research*, No. **73**, 18 (1986).
- 26) A. HATTAKA, A. KANTELINEN, A. TERVILA-WILO and L. VIHKARI: in REF 1, p.185 (1987).
- 27) H. KOFUJITA, K. NABETA, H. OKUYAMA and M. MIYAKE: *Mokuzai Gakkaishi*, **35**, 268 (1989).
- 28) M. TIEN and T.K. KIRK: *Proc. Natl. Acad. Sci. USA*, **81**, 2280 (1984).
- 29) T. HABE, M. SHIMADA, T. UMEZAWA and T. HIGUCHI: *Agri. Biol. Chem.*, **49**, 3505 (1985).
- 30) L. JONSSON, O. KARLSSON, K. LUNDQUIST and P.O. NYMAM: *FEBS Lett.*, **247**, 143 (1989).
- 31) M.S.A. LEISOLA, B. SCHMIDT, U. THANEI-WYSS and A. FIECHTER: *FEBS Lett.*, **189**, 267 (1985).
- 32) M. SHIMADA, T. HATTORI, T. UMEZAWA, T. HIGUCHI and K. UZURA: *FEBS Lett.*, **221**, 327 (1987).