

Formation and Action of Lignin-Modifying Enzymes in Cultures of *Phlebia radiata* Supplemented with Veratric Acid

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Transformation of veratric (3,4-dimethoxybenzoic) acid by the white rot fungus *Phlebia radiata* was studied to elucidate the role of ligninolytic, reductive, and demeth(ox)ylating enzymes. Under both air and a 100% O₂ atmosphere, with nitrogen limitation and glucose as a carbon source, reducing activity resulted in the accumulation of veratryl alcohol in the medium. When the fungus was cultivated under air, veratric acid caused a rapid increase in laccase (benzenediol:oxygen oxidoreductase; EC 1.10.3.2) production, which indicated that veratric acid was first demethylated, thus providing phenolic compounds for laccase. After a rapid decline in laccase activity, elevated lignin peroxidase (ligninase) activity and manganese-dependent peroxidase production were detected simultaneously with extracellular release of methanol. This indicated apparent demethoxylation. When the fungus was cultivated under a continuous 100% O₂ flow and in the presence of veratric acid, laccase production was markedly repressed, whereas production of lignin peroxidase and degradation of veratryl compounds were clearly enhanced. In all cultures, the increases in lignin peroxidase titers were directly related to veratryl alcohol accumulation. Evolution of ¹⁴CO₂ from 3-O¹⁴CH₃- and 4-O¹⁴CH₃-labeled veratric acids showed that the position of the methoxyl substituent in the aromatic ring only slightly affected demeth(ox)ylation activity. In both cases, more than 60% of the total ¹⁴C was converted to ¹⁴CO₂ under air in 4 weeks, and oxygen flux increased the degradation rate of the ¹⁴C-labeled veratric acids just as it did with unlabeled cultures.

The occurrence of veratryl (3,4-dimethoxybenzyl) compounds is often associated with the expression of ligninolytic activities in white rot fungi. In particular, the role of veratryl alcohol in the regulation of lignin biodegradation has been the subject of intensive study (6, 24, 25, 35). Veratryl alcohol is synthesized and excreted by at least the following four lignin-degrading white rot fungi: *Phanerochaete chrysosporium* (28), *Pycnoporus cinnabarinus* (16), *Coriolus (Trametes) versicolor* (22), and *Phlebia radiata* (21). Addition of veratryl alcohol stimulates the development of lignin peroxidase (ligninase) production both in *Phanerochaete chrysosporium* (8, 25) and in *Phlebia radiata* (15). Veratryl alcohol is oxidized by lignin peroxidase in an H₂O₂-dependent catalysis reaction (37) to veratraldehyde and other products (13, 34). During lignin biodegradation, this compound may also act as an electron transfer mediator through radical cation formation (14).

For more than 20 years, the metabolism of veratric acid (3,4-dimethoxybenzoic acid) has been investigated in various white rot fungi, including *Fomes fomentarius* (19), *Polyporus (Polystictus, Coriolus, Trametes) versicolor* (19, 38), *Polystictus (Trametes) sanguineus* (10, 29), *Phanerochaete chrysosporium* (12), and *Pycnoporus cinnabarinus* (16). Aromatic carboxylic acids, such as vanillic, syringic, isovanillic, and veratric acids, are released in considerable amounts during white rot decay in wood (7). However, the excretion of veratric acid or its transformation from other aromatic compounds by wood-rotting fungi has not been reported, although Shimada et al. (36) proposed recently that this compound may be an intermediate in the biosynthesis of veratryl alcohol.

Veratric acid, as well as other aromatic carboxylic acids, is probably not a suitable substrate or electron donor for lignin peroxidase (33). However, white rot fungi transform veratric acid by a variety of reactions, including reduction (10, 16, 29, 33, 38), demeth(ox)ylation (12, 16, 19, 29), decarboxylation (16), aromatic ring cleavage (16, 33), and quinone formation (16, 33), all of which are also important in lignin biodegradation by these fungi.

In studies of lignin demeth(ox)ylation mechanisms, which were suggested by Frick and Crawford (9), veratric acid has been used as a simple model compound. With *Pycnoporus cinnabarinus*, which produces laccase (phenol oxidase) but not lignin peroxidase, both demethylated and decarboxylated products derived from veratric acid were detected (16). The nature of the carbon source in the medium directed the degradation so that glucose enhanced the reduction of veratric acid, whereas cellulose yielded demethylated and decarboxylated products (16).

Experiments with vanillic (4-hydroxy-3-methoxybenzoic) acid and *Sporotrichum pulverulentum (Phanerochaete chrysosporium)* (2, 3) led to the identification of several enzymes which catalyze reduction, demethylation, decarboxylation, and aromatic ring cleavage. An intracellular NAD(P)H-dependent quinone reductase (5) and a decarboxylating enzyme (4) were also characterized.

The white rot fungus *Phlebia radiata* efficiently degrades [U-¹⁴C]lignin of poplar wood to ¹⁴CO₂ under a 100% oxygen atmosphere. Addition of an extra carbon source, cellulose or glucose, enhances the degradation of [¹⁴C-ring]lignin of poplar by up to 65% (18). Of the array of extracellular lignin-modifying enzymes produced by *Phlebia radiata*, three lignin peroxidases (31), one manganese-dependent peroxidase (Mn-peroxidase) (17), and one laccase type of

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phenol oxidase (31) have recently been purified and characterized.

However, very little is known about the routes adopted by this fungus for the metabolism of aromatic compounds. In this paper, we show that veratric acid has a stimulating effect on the production of these extracellular enzymes by *Phlebia radiata*. Depending on the transformation route for veratric acid, the reductive and demeth(ox)ylating enzyme activities which we observed are also described.

MATERIALS AND METHODS

Fungus and inoculum. *Phlebia radiata* Fr. strain 79 (= ATCC 64658) was isolated at the Department of Microbiology, University of Helsinki, Helsinki, Finland (18). The inoculum was prepared by growing the fungus on 2% (wt/vol) malt agar plates at 28°C for 14 days, after which small agar pieces (ca. 0.5 cm²) were cut out and transferred to low-nitrogen (2.0 mM nitrogen) ADMS medium (18). Following growth of the 50-ml cultures in 250-ml flasks at 28°C for 7 days, the resulting mycelial mats were broken with a Waring blender to give a homogenized inoculum suspension.

Aromatic compounds. Veratric acid (3,4-dimethoxybenzoic acid), veratraldehyde (3,4-dimethoxybenzaldehyde), and veratryl alcohol (3,4-dimethoxybenzyl alcohol) were obtained from Fluka. Vanillyl (4-hydroxy-3-methoxybenzyl) alcohol and vanillic (4-hydroxy-3-methoxybenzoic acid) were obtained from Fluka, and vanillin (4-hydroxy-3-methoxybenzaldehyde) was obtained from E. Merck AG. Isovanillyl (3-hydroxy-4-methoxybenzyl) alcohol and isovanillin (3-hydroxy-4-methoxybenzaldehyde) were purchased from Aldrich Chemical Co., Inc., and isovanillic (3-hydroxy-4-methoxybenzoic acid) was obtained from Sigma Chemical Co. Veratryl alcohol was distilled before use (24). The purity of these compounds was confirmed by high-performance liquid chromatography (HPLC) before they were used.

Cultivation with unlabeled veratric acid. In all experiments low-nitrogen (2.0 mM N) ADMS medium (pH 4.5) (18) supplemented with 56 mM (1%, wt/vol) glucose as the carbon source was used. This medium was prepared at five times the normal strength and was filter sterilized by using Sterivex-GS filter units (pore size, 0.22 µm; Millipore Corp.). Conical flasks (100 ml) containing 10 ml of medium were inoculated with 0.4% (vol/vol) homogenized mycelium. Stationary cultures were grown at 28°C for 7 to 22 days either under air or under a 100% O₂ atmosphere. Oxygenated cultures were grown by using the same gas distribution system used for ¹⁴C-labeled cultures (18). Veratric acid (182 mg) was dissolved in 50 ml of 0.1 M NaOH; this preparation was adjusted to pH 5.5 with 0.1 M HCl, diluted to 100 ml with water to yield a 10 mM veratric acid solution, and filter sterilized. Veratric acid was added after 48 h of cultivation to a final concentration of 1.0 mM. A corresponding NaOH-HCl solution (pH 5.5) was added to control flasks without veratric acid.

Determination of enzyme activities. Enzyme activities were measured by using a Shimadzu model 160A UV-visible spectrum programmable spectrophotometer. Culture fluids from parallel sample flasks (two to four flasks) were separately filtered through Whatman no. 4 filter paper. Laccase (phenol oxidase, benzenediol:oxygen oxidoreductase; EC 1.10.3.2) activity was determined with syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine; EGA-Chemie) by using a modification of the method (27) of Ander and Eriksson (1). The reaction mixture (1 ml) contained 100 µl of crude sample in 0.1 M citrate-phosphate buffer (pH 5.0). The

reaction was initiated by adding 25 µM syringaldazine (dissolved in ethanol), and the change in A₅₂₅ at 25°C was recorded for 3 min. Oxidase (phenol oxidase) activity was measured by monitoring the conversion of 2,2-azino-di-3-ethylbenzothiazoline-6-sulfonic acid (Boehringer Mannheim Biochemicals) (31) in 0.1 M sodium tartrate buffer (pH 3.0) at 25°C and 436 nm for 3 min. Lignin peroxidase activity was determined by the veratryl alcohol oxidation method in the presence of H₂O₂ (23). Reaction mixtures, which were maintained at 37°C, contained 0.1 M sodium tartrate buffer (pH 3.0), 0.4 mM veratryl alcohol, and 1.65 ml of culture filtrate in a total volume of 3 ml. The reaction was initiated by adding 0.2 mM H₂O₂, and the increase in A₃₁₀ was measured for 3 min. Laccase, oxidase, and lignin peroxidase activities are expressed below in katal (moles per second).

Mn-peroxidase activity was assayed by using the phenol red method (11), which was modified by omitting the NaOH supplement and measuring the change in A₅₂₀ for 5 min at 30°C. Reaction mixtures (1 ml) contained 25 mM lactate, 0.1 mM MnSO₄, bovine serum albumin (1 mg/ml; Sigma), phenol red (0.1 mg/ml; Merck), and 0.5 ml of culture filtrate in 20 mM sodium succinate buffer (pH 4.5). The reaction was initiated by adding 0.1 mM H₂O₂. Mn-peroxidase activity is expressed below in ΔA₅₂₀ per minute per liter.

Methanol formation. The methanol concentrations in the culture fluids were analyzed by using a model 3800 gas chromatograph (DANI S.P.A.) equipped with a flame ionization detector and a model D2000 integrator (Hitachi). The glass column (2 mm [inside diameter] by 2 m) used was packed with Carbowax C-0.3% Carbowax-0.1% H₃PO₄ (60/80 mesh; Supelco). N₂ was used as the carrier gas. At the beginning the column temperature was 70°C; the temperature was increased to 150°C after 1 min. The retention time of methanol was 0.6 min. The area response of the eluted methanol peak was determined by using an external standard method and analysis grade methanol (Merck). Culture filtrates were clarified by using Acro LC-13 membrane filter units (pore size, 0.2 µm; Gelman Sciences, Inc.) prior to injection (2 µl).

Sample treatment for HPLC analysis. Pooled culture fluids were extracted three times with an identical sample volume (4 to 20 ml) of HPLC-grade dichloromethane (Rathburn) and dried over Na₂SO₄ for 1 h. After filtration through Whatman no. 2 filter paper, extracts were evaporated to dryness under a vacuum at 35°C, and the resulting residue was dissolved in 1 ml of HPLC-grade acetonitrile (Rathburn). Samples were filtered (pore size, 0.2 µm) prior to analysis.

HPLC analysis. HPLC was carried out by using a model 1090M liquid chromatograph (Hewlett-Packard Co.) equipped with an automated variable-volume injector, a diode array UV-visible spectrum detector, and a model 1046A fluorescence detector, all of which were controlled by HP ChemStation (Hewlett-Packard). The reversed-phase column was a Novapak-C₁₈ column (3.9 mm [inside diameter] by 15 cm; Waters Associates, Inc.). All analyses were run with gradient elution by using HPLC-grade acetonitrile (Rathburn) and 1.0 mM H₃PO₄ (pH 3.2). The gradient was partially linear, acetonitrile (vol/vol) increasing as follows: 11% (0 min), 25% (5 min), 25% (6 min), 40% (8 min), 60% (11 min), and 60% (15 min). The analysis time was 15 min, the flow rate was 0.75 ml/min, the temperature was 40°C, and the injection volume was 10 µl. Chromatograms at two UV wavelengths (280 and 254 nm) and at one fluorescence channel (excitation at 238 nm and emission at 340 nm) were monitored for each run. The integrated peaks were collected, and their spectra were determined. Authentic aromatic compounds were used as ref-

erences, and their retention times, UV spectra, and fluorescence responses were compared with those of sample peaks to aid identification. Eluted, identified compounds were quantified by using the external standard method.

Cultivation with ^{14}C -labeled veratric acids. Both $3\text{-O}^{14}\text{CH}_3$ - and $4\text{-O}^{14}\text{CH}_3$ -labeled veratric acids (specific activities, 5.1×10^7 and 9.0×10^7 Bq/mg, respectively) were obtained from Konrad Haider, Institut für Pflanzenernährung und Bodenkunde, Bundesforschungsanstalt für Landwirtschaft, Braunschweig, Federal Republic of Germany. Culture flasks (10 ml of medium per 100 ml) containing ca. 850 Bq per flask were incubated either under synthetic air (20% O_2) or under 100% O_2 by using the gas distribution system described by Hatakka and Uusi-Rauva (18). Evolved $^{14}\text{CO}_2$ was trapped and analyzed as described previously (16, 18).

RESULTS

Reduction of veratric acid. Reduction reactions were detected in *Phlebia radiata* cultures immediately following the addition of veratric acid on day 3 of cultivation. Figure 1b shows that reduction of veratric acid under an air atmosphere yielded veratraldehyde and veratryl alcohol. The supply of veratric acid was totally depleted after 5 days. Traces of products other than veratryl compounds were found only occasionally. Conversion of veratric acid to veratraldehyde and to veratryl alcohol was almost stoichiometric under air, and the total amount of veratryl compounds decreased only 20 mol% during 3 weeks of cultivation.

Under an oxygen atmosphere, both reduction and degradation of veratric acid proceeded very rapidly, and 90 mol% of all veratryl compounds were consumed within 7 days (Fig. 2b). During the following 2 weeks, only small amounts of veratryl alcohol and veratraldehyde were detected. This rapid exhaustion of veratric acid may have been due in part to the strong degradation of veratryl alcohol by lignin peroxidase (Fig. 2a) (see below) and in part to the uptake of veratryl compounds or intermediates by the fungal hyphae.

In control flasks without veratric acid, *Phlebia radiata* produced small amounts of veratryl alcohol (Table 1). Oxygen flux clearly stimulated veratryl alcohol production up to 18 μM . Also, small amounts of veratraldehyde were detected in the controls, presumably arising from the action of lignin peroxidase. No veratric acid was detected in controls maintained under air or oxygen.

Production of laccase. Phenol oxidase activities were determined by using two substrates, syringaldazine for the laccase assay and 2,2-azinodi-3-ethylbenzothiazoline-6-sulfonic acid for the oxidase assay (see above). Although the oxidase assay was more sensitive and gave almost five times higher values than the laccase assay (Fig. 1a), identical activity curves were obtained with both methods, indicating that syringaldazine and 2,2-azinodi-3-ethylbenzothiazoline-6-sulfonic acid were probably oxidized by the same enzymes.

Figure 1a shows that under air the addition of veratric acid was followed by a rapid increase in phenol oxidase activity. The level of oxidase activity increased to 9,000 nkat/liter in 3 days, whereas the level of laccase activity simultaneously reached 1,900 nkat/liter. After this, both activities rapidly decreased, and no phenol oxidase activity was detected after 10 days (i.e., 7 days following the addition of veratric acid). Production of a laccase type of phenol oxidase was clearly associated with veratric acid metabolism, since in control cultures laccase and oxidase values remained comparatively low (Table 1).

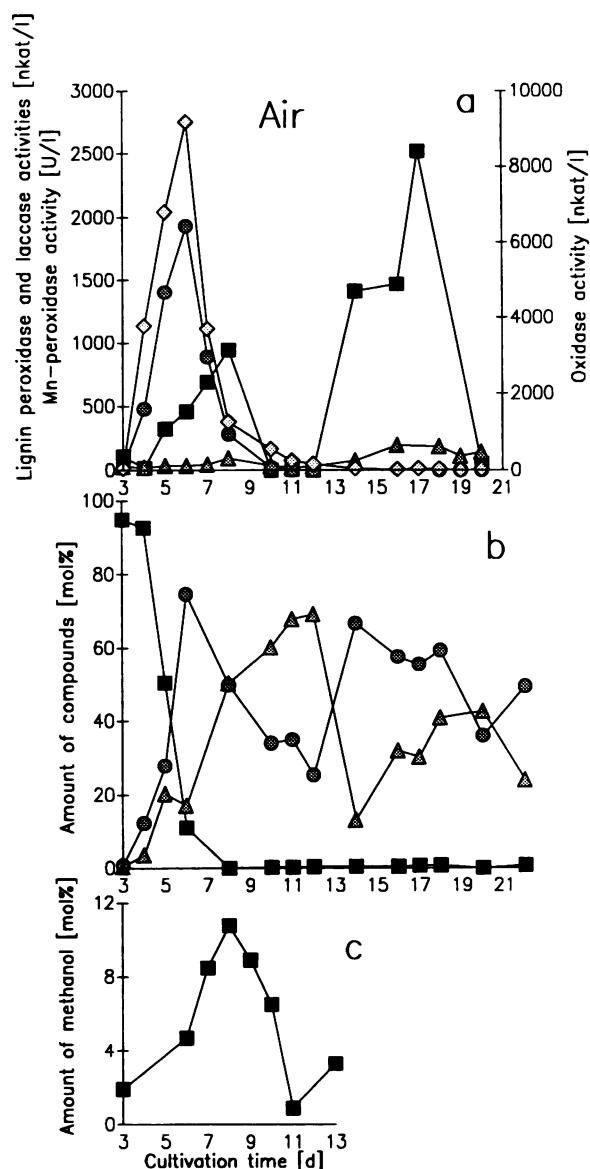


FIG. 1. *Phlebia radiata* cultivated under air: production of extracellular ligninolytic enzymes (a), conversion of 1.0 mM veratric acid (b), and production of methanol (c). (a) Symbols: ■, lignin peroxidase; ▲, Mn-peroxidase; ●, laccase; ◇, oxidase. (b) Symbols: ■, veratric acid; ▲, veratraldehyde; ●, veratryl alcohol. For conversion of 1.0 mM veratric acid and production of methanol, values are expressed in moles percent ([moles of compound detected/moles of veratric acid added that were recovered] \times 100). d, Days.

In *Phlebia radiata* cultures incubated under oxygen, phenol oxidase activity was not similarly stimulated (Fig. 2a). Indeed, in 24 h laccase production increased to only one-tenth of the levels obtained under an air atmosphere (Fig. 1a and 2a), and activity completely disappeared within 2 days. In control flasks (Table 1) suppression of phenol oxidase activity by oxygen was not so apparent.

Production of lignin peroxidase. Following the effective reduction of veratric acid via veratraldehyde to veratryl alcohol, the level of lignin peroxidase activity increased to almost 1,000 nkat/liter in cultures grown under air or oxygen (Fig. 1 and 2). In both cases, the same pattern was observed;

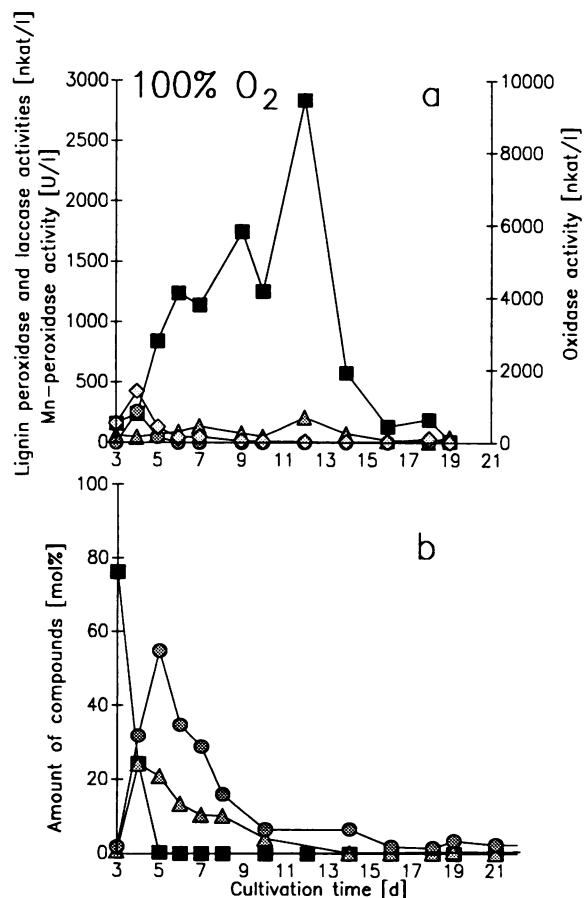


FIG. 2. *Phlebia radiata* cultivated under 100% oxygen: activities of extracellular ligninolytic enzymes (a) and conversion of 1.0 mM veratric acid (b). For an explanation of the symbols see the legend to Fig. 1. d, Days.

when ca. 80 mol% of added veratric acid was converted to veratryl alcohol, lignin peroxidase activity began to increase. Under an oxygen atmosphere, this increase continued, reaching maximum levels (2,800 nkat/liter) after 7 days (Fig. 2a). Simultaneously, a strong depletion of veratryl alcohol occurred (Fig. 2b). However, the amount of veratraldehyde formed by the action of lignin peroxidase was

TABLE 1. Accumulation of veratryl compounds and the production of ligninolytic enzymes by cultures of *Phlebia radiata* grown on basic medium without veratric acid

Cultivation atmosphere	Growth time (days)	Accumulation of:		Activity of:			
		Veratryl alcohol (μM) ^a	Veratraldehyde (μM) ^a	Lignin peroxidase (nkat/liter)	Mn-peroxidase (ΔA_{520} /min per liter)	Laccase (nkat/liter)	Oxidase (nkat/liter)
Air	3	0	0.1	23	30	26	51
	4	2.8	0.3	221	28	5	115
	12	8.6	1.7	185	31	4	27
Oxygen	3	7.6	2.5	80	8	61	955
	8	13.6	1.4	137	35	0	58
	18	18.0	1.7	97	34	0	46

^a Concentration as determined by HPLC.

much less than expected, given the veratryl alcohol concentration, indicating that other products were also formed. Traces of lactone and quinone types of compounds (13, 34) were detected by HPLC, although in relatively low amounts.

Under an air atmosphere, lignin peroxidase activity peaked twice during 3 weeks of cultivation (Fig. 1a). After reduction of veratric acid to veratryl alcohol to a level corresponding to 80 mol%, lignin peroxidase activity was detected. Enzyme levels began to decrease when the concentration of veratraldehyde exceeded that of veratryl alcohol (more than 50 mol% on day 8) (Fig. 1b). Veratraldehyde accumulated in the medium, although lignin peroxidase activity was no longer detectable on days 10 to 12. Subsequently, reduction of veratraldehyde occurred, and, simultaneously with the increase in veratryl alcohol levels, the level of lignin peroxidase activity increased to more than 2,500 nkat/liter; after this, enzyme levels again rapidly declined (Fig. 1a and b). In the course of this second lignin peroxidase stimulation cycle the concentration of veratraldehyde did not increase above the veratryl alcohol concentration. Under air, the total concentration of veratryl compounds remained high (>80 mol%) even after 22 days (Fig. 1b), and lignin peroxidase yielded mostly veratraldehyde (i.e., only traces of other products were detected).

Production of Mn-peroxidase. Mn-peroxidase activity appeared synchronously with lignin peroxidase activity and showed a similar cycle pattern under both culture conditions used (Fig. 1a and 2a). The levels of activity remained relatively low because of the assay method used (see above). The phenol red oxidation assay was apparently not sensitive enough to detect minor differences in enzyme levels.

Demeth(ox)ylation of veratric acid. Only trace amounts (<1.0 mol%) of phenolic intermediates were found extracellularly following the addition of veratric acid. Two of these compounds were identified as vanillic acid and vanillin. However, the strong stimulation of laccase activity under air suggested that these compounds were present in higher concentrations (Fig. 1a). Strong production of laccase probably resulted in the conversion of the phenolic compounds immediately following formation by postulated demeth(ox)ylating enzymes. Since no isovanillyl compounds were detected, demethylation appeared to take place preferentially at position 4. Demethylation may also have occurred intracellularly as a result of fungal uptake of veratryl or intermediate compounds.

Figure 1c shows the extracellular accumulation of methanol from veratric acid by *Phlebia radiata* cultivated under air. Methanol release followed laccase production and continued and even increased when laccase activity disappeared (Fig. 1a and c). On the other hand, production of lignin peroxidase and Mn-peroxidase appeared simultaneously with methanol release. This pattern indicated that demethoxylation and methanol accumulation may have occurred in part because of the action of these peroxidases. The total amount of methanol released after 13 days (ca. 15 mg/liter; 450 μM) accounted for 25 mol% of the initial amount of veratric acid, assuming that demethoxylation occurred at both position 3 and position 4. Since ca. 80 mol% of veratryl compounds was present at that time (Fig. 1b), this suggests that both methoxyl groups were released. Thus, only a minor part of the veratryl compounds was demethoxylated.

Degradation of ¹⁴C-methoxyl-labeled veratric acids to ¹⁴CO₂ was almost independent of the position of the label (Fig. 3 and 4). However, for the first 10 days of ¹⁴CO₂ evolution, the four parallel flasks showed more difference under air than under oxygen, and the oxygen level also

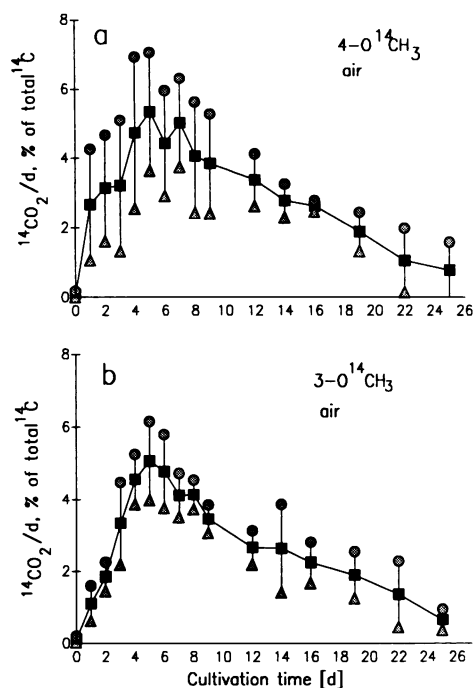


FIG. 3. Evolution of $^{14}\text{CO}_2$ from 4- O^{14}CH_3 -labeled veratric acid (a) and 3- O^{14}CH_3 -labeled veratric acid (b) by *Phlebia radiata* cultivated under air. Symbols: \blacksquare , mean of four flasks; \blacktriangle , minimum value; \bullet , maximum value. d, Day(s).

strongly influenced the rate of methoxyl removal. Under air, more than 65 to 70% of the applied activity from both 4- O^{14}CH_3 - and 3- O^{14}CH_3 -labeled veratric acids accumulated as $^{14}\text{CO}_2$, whereas under oxygen the extent of degradation increased to almost 80% in 4 weeks (Table 2). In all cases an insignificant part of the activity applied was associated with the mycelium. Only slightly more $^{14}\text{CO}_2$ was released from veratric acid labeled at position 4 under both culture conditions (Table 2).

Evolution of $^{14}\text{CO}_2$ from both labeled veratric acids reached the maximum level (5% of the total ^{14}C per day) on day 5 in cultures incubated under air (Fig. 3). In cultures incubated under oxygen, demeth(ox)ylation began immediately, and the level of evolved $^{14}\text{CO}_2$ (7 to 9% of the total ^{14}C per day) reached a maximum on day 2 (Fig. 4). The more efficient degradation of veratric acid in cultures incubated under oxygen was probably due to the strong production of lignin peroxidase under these conditions (Fig. 2a). Nevertheless, the early and intense demeth(ox)ylation of veratric acids in cultures incubated under oxygen (Fig. 4) must also have proceeded by a different enzyme system, since substantial lignin peroxidase secretion started later (i.e., after 5 days of incubation) (Fig. 2a). Conversely, in cultures incubated under air, production of laccase occurred simultaneously with $^{14}\text{CO}_2$ evolution, and both parameters reached maximum levels after 6 days of cultivation (Fig. 1a and 3).

DISCUSSION

Rapid reduction of veratric acid to veratraldehyde and veratryl alcohol began immediately following addition of the acid to cultures of *Phlebia radiata* and occurred in cultures incubated under both air and oxygen atmospheres. In fact, veratric acid was more rapidly reduced as well as consumed

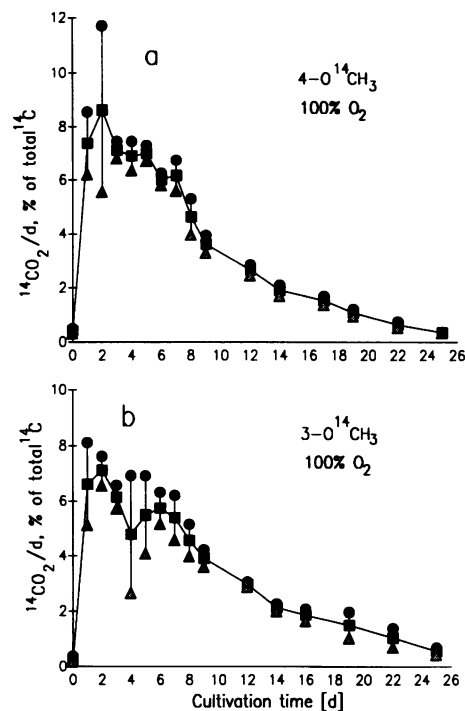


FIG. 4. Evolution of $^{14}\text{CO}_2$ from 4- O^{14}CH_3 -labeled veratric acid (a) and 3- O^{14}CH_3 -labeled veratric acid (b) by cultures of *Phlebia radiata* maintained under a continuous 100% O_2 flow. For an explanation of the symbols see the legend to Fig. 3. d, Day(s).

in oxygenated cultures, implying that oxygen, perhaps indirectly, stimulates production and/or activity of the reductive enzymes and the enzymes which catalyze the transformation of veratryl compounds. To date, an NADPH-dependent veratraldehyde-reducing oxidoreductase has been described in *Polystictus (Coriolus) versicolor* (38) and has been purified from *Phanerochaete chrysosporium* (33). However, the veratric acid oxidoreductase, which is also NADPH dependent, is either very labile (38) or difficult to purify (33), suggesting that the enzyme may be associated with the cell membrane and thus easily lost during purification.

Reduction of veratric acid has been demonstrated in several lignin-degrading white rot fungi (10, 16, 29, 33, 38). Vanillic acid is similarly reduced to vanillin and to vanillyl alcohol by fungal cultures (3, 16, 19), and fungal reduction of *para*-hydroxybenzoic acid has also been reported (16). These results indicate that the NADPH-dependent aromatic carboxylic acid and aromatic aldehyde oxidoreductases,

TABLE 2. Distribution of the ^{14}C label from [^{14}C]methoxyl-labeled veratric acid in cultures of *Phlebia radiata* after 27 days of cultivation^a

Atmosphere	^{14}C label	$^{14}\text{CO}_2$ evolved (%)	^{14}C in growth liquor (%)	^{14}C associated with mycelium (%)	^{14}C recovered (%)
Air	4- O^{14}CH_3	72.3 \pm 5.6	16.4 \pm 4.8	2.3 \pm 0.8	91.1 \pm 1.7
	3- O^{14}CH_3	64.7 \pm 9.7	17.8 \pm 1.9	1.9 \pm 0.3	84.4 \pm 7.9
Oxygen	4- O^{14}CH_3	78.3 \pm 0.9	13.7 \pm 1.5	1.8 \pm 0.1	93.8 \pm 0.7
	3- O^{14}CH_3	76.5 \pm 0.8	15.6 \pm 0.3	2.0 \pm 0.3	94.2 \pm 0.7

^a Values are percentages of the activity applied and are means \pm standard deviations for four flasks.

such as veratric acid and veratraldehyde reductases, may have a broad specificity toward simple aromatic, C_α-carbonylated compounds.

Secretion of veratryl alcohol by *Phlebia radiata* (in this work up to 18 μM in cultures incubated under an oxygen atmosphere) has also been reported previously (21). Our more recent results show that veratryl alcohol is produced de novo from glucose by this fungus. Veratryl alcohol synthesis in *Phlebia radiata* presumably occurs through phenylalanine as in *Phanerochaete chrysosporium* (35, 36), comprising various reactions such as reductions, methylations, and C_α-C_β cleavage. It has been proposed that the veratric acid and veratraldehyde oxidoreductases also participate in the biosynthesis of veratryl alcohol (33, 36). Thus, specific methyl transferases acting on positions 3 and 4 of the aromatic ring during veratryl alcohol biosynthesis may also reverse their activity and function as methyl-removing agents (i.e., demethylases).

In this study, a relatively high concentration of glucose (1%, wt/vol) was used as a carbon source in the medium. Since glucose promotes the reduction of aromatic acids by other white rot fungi (2, 3, 16) but represses decarboxylation (16) and demethylation (2, 16), the strong reduction compared with weak demethylation observed with *Phlebia radiata* is partly explained by glucose repression.

Demeth(ox)ylation was monitored by following ¹⁴CO₂ evolution from specifically ¹⁴C-methoxyl-labeled veratric acids and by following formation of methanol from unlabeled veratric acid. Both methods indicated that demeth(ox)ylation occurred; this conclusion was supported by the appearance of trace amounts of vanillyl compounds. Extracellular methanol probably arose through the action of several enzymes, including lignin peroxidase, Mn-peroxidase, and laccase. However, the direct demethoxylation of veratryl compounds by these enzymes has not been reported. Lignin peroxidase yields methanol from methoxybenzenes and β-O-4 dimeric compounds (6, 24), and Mn-peroxidase oxidizes various phenolic compounds (6, 11). Laccase is capable of removing methoxyl groups from phenolic compounds (1, 2, 6, 24, 26) that are preferentially hydroxylated at position 4 and possess 3- or 5-methoxyls (26). Thus, veratric acid may first be demethylated at position 4, possibly by the action of a specific veratrate-O-demethylase (32). Evidence for the existence of a similar enzyme that demethylates specifically at position 4 has been obtained previously with *Pycnoporus cinnabarinus* (16).

The strong, sharp stimulation of laccase (phenol oxidase) production in the early phases of *Phlebia radiata* cultures, which clearly resulted from veratric acid supplementation, is not characteristic of white rot fungi. For example, in *T. versicolor*, which is considered a typical laccase-producing white rot fungus, laccase excretion starts relatively late and continues for several days (26). However, the effect of nitrogen limitation on the onset of fungal laccase production has not been studied before. We have previously observed this early production of laccase in low-nitrogen cultures of *Phlebia radiata* supplemented with veratryl alcohol (20). In the case of veratric acid especially, this enhancement of laccase production may be a response to a relatively high concentration (1.0 mM) of a moderately toxic component. Laccase may act as a detoxifying enzyme, since oxidation reactions catalyzed by laccase result in polymerized products (6, 24, 26), thereby leading to removal of toxic monomers from the growth medium. Traces of unidentified quinone polymers were found in our experiments, especially in

cultures incubated under an air atmosphere, where increased laccase activity was mostly evident.

In the presence of veratric acid, lignin peroxidase activity was clearly enhanced in cultures incubated under both air and oxygen atmospheres following the reduction of veratric acid to veratryl alcohol. A similar effect of atmosphere on enzyme activities was not evident without veratric acid. In *Phanerochaete chrysosporium* the stimulation of lignin peroxidase by veratryl alcohol is thought to appear at the synthesis stage (8, 23, 24), probably by induction of certain lignin peroxidase isoenzymes (23). In this study, the stimulation of lignin peroxidase production by *Phlebia radiata* appeared primarily to be a consequence of a high concentration of veratryl alcohol. In oxygenated cultures especially, veratryl alcohol was rapidly oxidized and consumed by lignin peroxidase. In cultures of *Phlebia radiata* maintained under an air atmosphere, the major product was veratraldehyde, whereas mostly other products were formed in oxygenated flasks. Since traces of lactone and quinone types of compounds (13, 34) were detected in oxygenated cultures, most of the veratryl compounds were presumably consumed through the concerted action of lignin peroxidase and intracellular enzymes following fungal uptake of these intermediates (33).

Veratric acid did not enhance the production of Mn-peroxidase in the same way as veratryl alcohol did when it was added to *Phlebia radiata* cultures (17, 30). Furthermore, the strongly enhanced production of laccase and lignin peroxidase caused by the addition of veratric acid may have repressed the synthesis of Mn-peroxidase. The role of Mn-peroxidase under these conditions is unclear; if the enzyme is unable to act on veratric acid or intermediate products, it may serve primarily as a producer of H₂O₂ for lignin peroxidase (6, 11, 24).

Under these culture conditions, transformation of veratric acid in *Phlebia radiata* proceeded via reduction and demethylation to vigorous consumption by extracellular oxidizing enzymes, such as lignin peroxidase and laccase. Some of the enzymes that catalyze demeth(ox)ylation or reduction are probably intracellular. These enzymes have been described only in a few white rot fungi, and their participation in the activities observed in this work are under further investigation.

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