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Lignin Peroxidase Activity Is Not Important in Biological Bleaching and Delignification of Unbleached Kraft Pulp by *Trametes versicolor*

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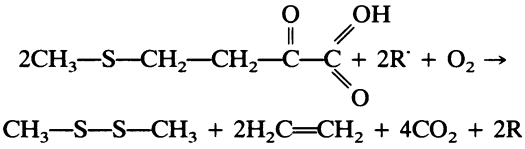
The discovery in 1983 of fungal lignin peroxidases able to catalyze the oxidation of nonphenolic aromatic lignin model compounds and release some CO_2 from lignin has been seen as a major advance in understanding how fungi degrade lignin. Recently, the fungus *Trametes versicolor* was shown to be capable of substantial decolorization and delignification of unbleached industrial kraft pulps over 2 to 5 days. The role, if any, of lignin peroxidase in this biobleaching was therefore examined. Several different assays indicated that *T. versicolor* can produce and secrete peroxidase proteins, but only under certain culture conditions. However, work employing a new lignin peroxidase inhibitor (metavanadate ions) and a new lignin peroxidase assay using the dye azure B indicated that secreted lignin peroxidases do not play a role in the *T. versicolor* pulp-bleaching system. Oxidative activity capable of degrading 2-keto-4-methiolbutyric acid (KMB) appeared unique to ligninolytic fungi and always accompanied pulp biobleaching.

Recently, it was shown that the white rot basidiomycete *Trametes* (*Coriolus*) *versicolor* has the ability to modify unbleached kraft pulps, raising their brightness from ~32 to ~60% and decreasing their lignin content (20, 37, 40). This biobleaching process might therefore be used to replace Cl_2 in a commercial pulp-bleaching sequence. Biobleaching is produced by mixing a small fungal inoculum with pulp (1 to 2% consistency) and incubating the mixture with shaking for 2 to 5 days. The treated pulp shows some reduction in viscosity but little or no decrease in overall paper physical quality (37). More recently, it was shown that hypha-pulp fiber contact is not required, i.e., the culture liquor contains everything necessary for biobleaching, but this liquor must be constantly or frequently "renewed" by contact with the fungal biomass (2). Determining the mechanism of this biobleaching is highly desirable, either to permit design of a cell-free delignification system or to optimize the present one.

A central question is the role of secreted lignin peroxidases (LPs). Although *T. versicolor* secretes both lignin peroxidase and manganese-dependent peroxidase (MnP) isozymes under certain conditions (13, 14, 21, 22), it is unknown whether these proteins or Mn(III) chelates (3) are important in our fungal unbleached kraft pulp bleaching and delignifying system. The role of LPs, if any, has proved remarkably difficult to determine for many reasons, including (i) the extremely complex and undefined nature of the pulp-mycelium-dilute nutrient slurry in which biobleaching occurs, (ii) the low sensitivity and great susceptibility to artifacts of the veratryl alcohol (VA)-based LP assay, (iii) the unknown structure of the residual kraft lignin substrate, (iv) the unknown nature of the delignifying and biobleaching reactions, (v) the small amount of lignin attacked and the long time required, and (vi) the lack of a simple rapid test for biobleaching activity.

Whether or not the biobleaching-pulp-delignifying mechanism involves LP, it almost certainly requires some moderately high-energy one-electron oxidations (19, 28, 43), and thus a good test for such oxidations might also be a good test for a central reaction in biobleaching. The one-electron oxidation of 2-keto-4-methiolbutyric acid (KMB) to ethylene, CO_2, and dimethyl sulfide (reaction 1) was felt to be a good choice for such a test because the ethylene evolved is generally stable, accumulating in the assay flask headspace, where it can easily be measured with sensitive gas chromatographic techniques.



$$CH_3—S—S—CH_3 + 2H_2C{=}CH_2 + 4CO_2 + 2R \quad (1)$$

Since the elucidation of the oxidation mechanism by Yang (47), KMB and its analogs methional and methionine have been shown to release ethylene specifically in response to strong electron-abstracting species, such as ·OH (5, 9, 12), lignin peroxidase (15–17, 24, 25), light-activated flavin mononucleotide (48), microsomal peroxidases (10, 31), Mn(III) chelates (2a), and species created with UV light and ionizing radiation (8, 30), as well as alkoxy (RO·) and alkyl peroxyl (ROO·) radicals (39).

In fact, much of the evidence supporting the oxy-radical hypothesis of fungal lignin biodegradation popular before the 1983 discovery of secreted lignin peroxidases (16, 43) was based on ethylene evolution from KMB, which was found to be coincident with lignolytic activity and believed to indicate generation of ·OH and related oxy radicals (15, 25) (despite earlier findings warning against making such assumptions about the evolution of ethylene from KMB or its analogs [10, 39, 47, 48]). The discovery that the LP proteins of the most intensively studied white rot fungus, *Phanerochaete chrysosporium*, readily oxidized KMB to ethylene in the presence of VA did much to weaken the oxy-radical hypothesis of lignin degradation and strengthen the hypothesis that LP

is central to the process (16, 17, 23–25). This newer perception is also shown by the fact that the KMB-to-ethylene oxidation has been recommended as an assay for LP, giving that reaction the dubious distinction of having been recommended as a quantitative assay for two entirely different substances, the hydroxyl radical (·OH) (9, 11, 12) and LP proteins (23), with both recommendations in *Methods in Enzymology*. After considerable initial enthusiasm for the hypothesis that LP was the key component in fungal delignification, its inability to extensively degrade lignin apart from the fungus and its tendency to polymerize more than fragment aromatic compounds (27, 29, 41) have emphasized that the mechanisms of fungal delignification remain unknown.

The present work was undertaken to determine whether LP is important in *T. versicolor*-mediated pulp bleaching and delignification and whether the KMB oxidation occurring concomitantly with biobleaching is LP dependent or a good indication of biobleaching. The work employs a new LP assay and a new LP inhibitor and strongly suggests that secreted LP does not play any role in the *T. versicolor*-mediated pulp bleaching system.

MATERIALS AND METHODS

Strains and growth conditions. *T. versicolor* 52 (ATCC 20869), a dikaryotic Paprican strain, was maintained on 2% malt agar. Biobleaching experiments were initiated with an inoculum prepared as follows. Three 1-cm-diameter punchouts from the periphery of a malt agar plate incubated for 5 days were placed in each of several 200-ml mycological broth (MB) aliquots in 500-ml polypropylene flasks. MB contained 10 g of Bacto Soytone liter^{-1}, 40 g of D-glucose (pH 5.0) liter^{-1}, and the following trace metals added in an acidic 1,000× concentrate: 1 μM $FeCl_3$, 40 μM Na_3 citrate, 1 μM $CuSO_4$, 5 μM $ZnCl_2$, 20 μM $MnSO_4$, 50 μM $MgCl_2$, 5 μM $CoCl_2$, 0.1 μM $NiCl_2$, and 0.5 μM $(NH_4)_6Mo_7O_{24}$. Each flask also contained a 2.5-cm glass bead and 0.25% hardwood kraft pulp (HWKP) from an eastern Canadian mill. The same pulp was used in the biobleaching. The inoculum flasks were incubated with shaking (at 200 rpm, a radius of shaking of 9 mm, and 25°C) for 5 days, and the resulting suspension was used to inoculate (15% [vol/vol]) sterile 500-ml flasks containing 200 ml (final volume) of water with 1 or 2% (dry weight) hardwood kraft pulp (HWKP). These biobleaching cultures were then shaken (200 rpm, 25°C) for 2 to 5 days. The eight fungal strains compared with *T. versicolor* 52 were also grown on MB and bleaching cultures prepared in the same manner. For all organisms, broth cultures were slurries of fungal fragments prepared as the inocula for biobleaching but lacking HWKP. Cultures expressing LPs secreted by *T. versicolor* were grown by using the medium of Jonsson et al. (22) and *Trametes* defined medium (TDM), which consisted of 33 mM D-glucose, 15 mM NH_4Cl or L-glutamine, 5 mM NaCl, 5 mM KH_2PO_4, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 10 mM 2,2-dimethylsuccinate, 0.8 mg of thiamine liter^{-1}, and 1 ml of acidic trace metal (1,000× concentrate) liter^{-1} (set to pH 5.0). The final trace metal concentrations were 20 μM $FeSO_4$, 2 μM $CuSO_4$, 5 μM $ZnCl_2$, 20 μM $MnSO_4$, 5 μM $CoCl_2$, 0.1 μM $NiCl_2$, and 0.5 μM $(NH_4)_6Mo_7O_{24}$.

The following fungi, which were used in the ethylene production comparison with *T. versicolor* 52, were from the Paprican (Pointe Claire, Quebec, Canada) culture collection (those derived from American Type Culture Collection [ATCC] strains are followed by their ATCC numbers): *Coniophora puteana*, *Saccharomyces cerevisiae*, *Aspergillus niger* (11414), *Neurospora crassa* (14692), *Candida utilis* (9950), *Mucor rouxii* (24905), *Trichoderma hamatum* (26798), and *Schizophyllum commune*.

Assays. Ethylene production from the one-electron oxidation of KMB was monitored by incubating 5 ml of the reaction mixture with 2 mM KMB in 50-ml flasks of known total volume with gastight rubber seals. After 60 min (usually) of incubation with shaking at 200 rpm and 25°C, 0.5 ml of the flask headspace gas was injected into a 12-ft (ca. 3.7-m) Porapak N column (oven temperature = 70°C) in a Hewlett-Packard 5890 gas chromatograph with a flame ionization detector (120°C) and ethylene quantitated with an integrator against standards. All trials used triplicate flasks, and results are expressed in nanomoles of ethylene in total flask headspace (mean ± standard deviation).

Laccase (phenoloxidase) activity was determined from the rate of oxidation of 5 mM 2,2′-azinobis-(3-ethyl benzthiazoline)-6-sulfonate (ABTS) in 0.1 M Na acetate, pH 5, measured at 420 nm. LP was measured in 50 or 100 mM Na tartrate, pH 4.5, with 2.0 mM VA and 0.1 mM H_2O_2 by the appearance of veratraldehyde as measured at 310 nm. Alternatively, the same assay system with 32 μM azure B replacing the 2 mM VA was used. Azure B oxidation was monitored by the decrease in A_{651}. Full details of this new LP assay are presented in the companion article (1). Oxidation of phenol red and Poly R_{478} in biobleaching systems was followed by measuring the ΔA_{559} and ΔA_{519}, respectively, on samples adjusted to pH 7 with 0.1 M Tris buffer. MnPs from *T. versicolor* and *P. chrysosporium* were measured in a mixture of 50 mM Na succinate, 50 mM Na lactate, 0.1 mM $MnSO_4$, 3 mg of gelatin ml^{-1}, 50 μM H_2O_2, and 0.1 mM phenol red, whose oxidation rate was measured at 431 nm. Horseradish, *Coprinus macrorhizus*, and *Arthromyces ramosus* peroxidases in 50 mM Na phosphate buffer (pH 7)–2 mM H_2O_2–1 mM 3,3-diaminobenzidine (DAB) were measured at 482 nm. Pulp brightness was measured on minihandsheets (2.5 by 4.5 cm) made from washed test pulps by using an apparatus with a 150-mesh screen. The sheets were dried for 48 h and measured at 457 nm with a Perkin Elmer λ 3 spectrophotometer with an integrating sphere reflectance attachment, referenced to a barium sulfate standard.

VA, veratric acid, and veratraldehyde were quantitated (5 to 7 μM resolution limit) with a μBondapak C-18 reverse-phase column (0.39 by 30 cm) on a Waters high-performance liquid chromatograph (HPLC) with a Hewlett-Packard 1040A diode array detector.

Enzyme preparation. *T. versicolor* 52-secreted LP was prepared from 15-day N-limited, O_2-sparged, gently shaking (70 rpm) 1-liter modified TDM liquid cultures in 2.8-liter Fernbach flasks (see Table 4). The culture liquors were filtered, set to pH 5.5, and mixed with bulk DEAE-Sephadex in 5 mM Na succinate buffer (pH 5.5). The DEAE-Sephadex was then poured into a column and washed with the buffer and LP peaks were eluted with a 0 to 500 mM NaCl gradient. LP peaks, of which there were several, were desalted, concentrated, and applied to a Mono-Q ion-exchange column. The resulting LP peaks (4) were pooled, and an attempt to remove residual laccase activity with Protein-Pak 125 (2 by 30 cm) sieve columns in series on a Waters HPLC was made. The resulting preparation showed a strong Soret A_{407} and high VA- and Azure B-oxidizing activity but still contained some laccase (ABTS-oxidizing) activity. Incomplete separation on the HPLC indicated that this ABTS activity

was due to a second protein, not a second activity of the LP proteins. *P. chrysosporium* LP was induced under conditions similar to those used for *T. versicolor* LP and then was ammonium sulfate precipitated, dialyzed, and microfiltered. The laccase (phenoloxidase) preparation from *T. versicolor* 52 was made by pooling activity peaks from a DEAE Biogel column prepared as described elsewhere (1). Commercial enzymes were employed with no further purification. LPs and MnPs from *P. chrysosporium* were also obtained (as partially purified mixtures of isozymes) from a commercial source (Tienzyme, Inc., State College, Pa.).

Reagents. Standard chemicals, including the LP inhibitor metavanadate, were from Fisher and Sigma. *Rhus* laccase was from Saito Co., Osaka, Japan, and *C. macrorhizus* peroxidase was from Chemical Dynamics Corp., South Plainfield, N.J. Sigma also supplied *A. niger* catalase, glutathione, Poly R_{478}, Azure B, and KMB.

RESULTS AND DISCUSSION

Fungal KMB oxidation. The validity of using ethylene evolution from KMB (or methional) as a measure of an important biobleaching reagent or reaction was investigated. *T. versicolor* and a diverse group of eight nonligninolytic fungi were each grown in the biobleaching format and examined for KMB oxidation. These fungi were strains of *C. puteana*, *S. cerevisiae*, *A. niger*, *N. crassa*, *C. utilis*, *M. rouxii*, *T. hamatum*, and *S. commune*. Although many of these strains as well as *T. versicolor* were tried with and without growth in the presence of 1% HWKP and 1 mM VA, only *T. versicolor* was capable of generating substantial amounts of ethylene from 2 mM KMB over 60 min (data not shown).

This result suggests that KMB is not oxidized by most fungi. Earlier work indicated that the oxidation of KMB by the white rot fungus *P. chrysosporium* was strongly dependent on the presence of LP and the induction of ligninolytic activity (16, 17, 24, 25). However, in our work with *T. versicolor*, substantial KMB-oxidizing activity was always present, regardless of the presence of pulp, culture age, or nitrogen limitation (inducers of ligninolytic activity and LP in *P. chrysosporium*). Addition of VA had no stimulatory effect on ethylene evolution in a biobleaching culture (Table 2). Good pulp bleaching was also not significantly altered by early addition of VA (on day 0 or 2 of a 5-day bleaching experiment) (data not shown). Production of ethylene from KMB by *T. versicolor* occurred at a constant rate in the biobleaching system, with the filtered liquor alone being much less active than the complete system (Fig. 1).

LP. When purified, mixed *T. versicolor* LP isozymes and H_2O_2 are applied to KMB directly, there is only a small reaction (which may be largely a heme-plus-H_2O_2 artifact), as is the case for laccase plus KMB per se; however, with the addition of VA, the reaction is enormously enhanced (Table 1). Thus, the LP-dependent evolution of ethylene from KMB used to explain ethylene evolution by ligninolytic *P. chrysosporium* cultures and discredit the oxy-radical hypothesis (16, 17, 24) is only an indirect oxidation of KMB. The data in Table 1 also demonstrate the inability of several "conventional" peroxidases and laccases to oxidize KMB, even in the presence of VA and H_2O_2.

Numerous VA-based assays for LP production by 2- to 6-day-old biobleaching cultures of *T. versicolor* detected no LP activity. However, there are several reports of both LP and MnP isozymes being produced by this organism in low-nitrogen, high-oxygen, still cultures with added detergent and VA, conditions found to be necessary for the expression of these enzymes (and ligninolytic activity) in *T. versicolor* and *P. chrysosporium* (1, 13, 21, 22). This behavior was confirmed with *T. versicolor* 52, with both MnP and LP appearing in a pulp-free culture in defined medium, with LP reaching the modest level of 24 U $liter^{-1}$. The following conditions and compounds not present in the pulp biobleaching system appeared to be necessary for detectable LP expression: (i) a low Mn concentration (2 μM), (ii) detergent (Tween 80), (iii) nitrogen limitation, (iv) longer incubation, (v) an elevated partial O_2 pressure, (vi) addition of VA, and (vii) and very gentle or no culture agitation (data not shown).

Could the VA assay be somehow missing LP in the 2- to 5-day-old biobleaching culture liquor but not in the longer-term N-limited cultures? Comparison of the KMB oxidizing behaviors of the two types of culture liquor is informative

TABLE 1. Abilities of various peroxidase and laccase (phenoloxidase) enzymes to oxidize KMB

Reaction mixture[a]	Ethylene (nmol $flask^{-1}$)
Na tartrate buffer[b]	<0.05
+ *T. versicolor* LP + VA + H_2O_2	246 ± 10.6
+ *T. versicolor* LP + H_2O_2	2.7 ± 0.2
+ *P. chrysosporium* LP + VA + H_2O_2	403 ± 4.2
+ *P. chrysosporium* LP + H_2O_2	5.4 ± 0.3
Na acetate buffer[c]	<0.05
+ *T. versicolor* laccase + VA	<0.05
+ *T. versicolor* laccase	<0.05
Na phosphate buffer[d]	<0.05
+ Horseradish peroxidase + H_2O_2 + VA	<0.05
+ Horseradish peroxidase + H_2O_2	<0.05
+ *C. macrorhizus* peroxidase + H_2O_2 + VA	<0.05
+ *C. macrorhizus* peroxidase + H_2O_2	<0.05
+ *A. ramosus* peroxidase + H_2O_2 + VA	<0.05
+ *A. ramosus* peroxidase + H_2O_2	<0.05
+ *Rhus* laccase + VA	<0.05
+ *Rhus* laccase	<0.05

[a] Trials were run as described in Table 2, footnote *a*, and Results. All enzymes displayed good activity in their standard assays.
[b] The Na tartrate assays were run in 50 mM Na tartrate, pH 4.5.
[c] The Na acetate assays were run in 0.1 M Na acetate, pH 5.0.
[d] The Na phosphate assays were run in 50 mM Na phosphate, pH 7.0.

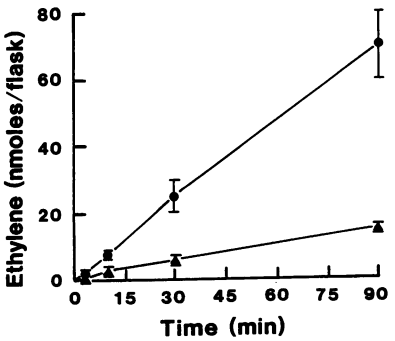


FIG. 1. KMB oxidation by a complete biobleaching culture of *T. versicolor* and its culture liquor. Symbols: ●, complete culture with 2% HWKP, fungal biomass, and 15% MB after 5 days in standard biobleaching format; ▲, filtered (Whatman no. 1 filter) culture liquor.

TABLE 2. Factors affecting KMB oxidation in TDM and MB biobleaching cultures of *T. versicolor*

Reaction mixture[a]	Ethylene (nmol flask^{-1}) in:	
	TDM culture	MB biobleaching culture
Sterile medium	5.0 ± 1.6	1.1 ± 0.2
Complete culture[b]	—[c]	75.0 ± 6.2
Culture liquor	7.0 ± 2.4	23.5 ± 5.8
Culture liquor + VA	—	13.8 ± 5.4
Culture liquor + H_2O_2	171 ± 19[d]	22.3 ± 2.4
Culture liquor + VA + H_2O_2	162 ± 5	15.1 ± 3.1
Culture liquor + 5 mM GSH	—	85.6 ± 20.0
Complete culture[e]	—	50.0 ± 5.5
Complete culture filtrate[e] (Whatman no. 1 filter)	—	25.4 ± 7.0
Complete culture filtrate[e] (Zytex filter)[f]	—	18.1 ± 3.4
Complete culture filtrate[e] (Zytex filter, boiled)	—	15.0 ± 1.9
Complete culture (no pulp)[e]	—	56.1 ± 4.9
Complete culture (no pulp) filtrate[e] (Whatman no. 1 filter)	—	30.2 ± 5.8
Washed pulp and culture, fresh 15% MB	—	18.5 ± 0.2
Pulp, no culture	—	0.4 ± 0.2

[a] Reaction mixtures (5 ml) all contained 2 mM KMB and were incubated for 60 min at 25°C with shaking at 200 rpm and analyzed for ethylene as described in Materials and Methods.

[b] Assays were run with triplicate standard biobleaching flasks on day 5 (brightness, 50.1% ± 3.8%) and with N-limited Fernbach flask cultures (1 liter of TDM) on day 14.

[c] —, it was not possible to assay small samples of culture because the biomass was in large masses in a clear liquor.

[d] Because the addition of 3 mM VA was a necessary part of the induction of VA in the TDM cultures, >1 mM VA was already present. No VA, veratric acid, or veratraldehyde could be detected by HPLC (detection limit, 5 to 7 μM) in any 5-day biobleaching culture.

[e] A second biobleaching experiment with somewhat lower levels of KMB oxidation.

[f] A fibrous Teflon filter (5- to 15-μm pore size).

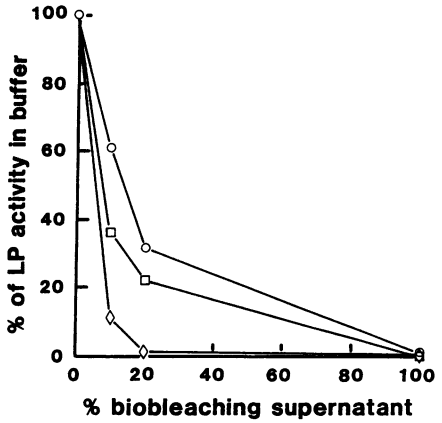


FIG. 2. LP inhibition by biobleaching culture liquor. The inhibition was measured by three separate LP assays: ○, KMB assay (ethylene produced in 60 min after adding 2 mM KMB, 2 mM VA, and 0.1 mM H_2O_2); □, standard VA assay; and ◇, azure B assay (ΔA_{651} after addition of 32 μM azure B). All assays contained 50 mM Na tartrate buffer made up in 10, 20, or 100% culture liquor (pH 4.5) and monitored in 1.5-ml cuvettes or standard 50-ml ethylene flasks with 5 ml of reaction mixture.

(Table 2). The biobleaching supernatant showed significantly more activity than the N-limited LP-expressing culture but was not stimulated by H_2O_2. In contrast, the initial very low level of KMB-oxidizing activity of the N-limited (TDM) culture liquor was dramatically increased by H_2O_2, as would be expected for an effect based on the activity of H_2O_2-requiring peroxidases. Added VA had no effect on the N-limited culture liquor, as the VA added during LP induction was still present. As reported elsewhere (2), the biobleaching culture liquor had no detectable endogenous H_2O_2 (≤1 μM), but as the K_m of H_2O_2 obtained for *T. versicolor* mixed LP isozymes is 0.5 μM (1), it is conceivable that traces of H_2O_2 are present and usable by tiny amounts of LP to produce the observed ethylene from KMB. However the ability of much of the filterable culture liquor KMB-oxidizing activity to withstand boiling (Table 2) indicates it is probably not due to LP or any other enzyme.

Could the LP be bound to pulp fibers in the biobleaching cultures and thus escape both detection in the VA assay for LP (usable only with clear liquids) and detection via VA or H_2O_2 stimulation of the filtered supernatant? Our studies showed that 28% of purified *T. versicolor* LP rapidly bound to HWKP and >95% rapidly bound to softwood kraft pulp fibers in buffer at the culture pH (4.5). Bleached HWKP also bound >95% of the LP. Since HPLC analysis had shown that there was no detectable VA, veratraldehyde, or veratric acid in the biobleaching cultures (2), VA and H_2O_2 were added. They had no stimulating effect, either alone or combined, on ethylene production; in fact, lower levels of VA usually somewhat depressed the KMB oxidation observed (Table 2), suggesting that VA and KMB might be competitors for the unknown oxidant(s) in the liquor. The results presented above and the inability to detect LP by using the VA-based assay mentioned above might also be explained by the masking or blocking of LP activity by some natural analog of VA competing as an LP substrate or accessory substance. If so, then addition of a high level of purified *T. versicolor* LP, or at least LP plus H_2O_2 and VA, ought to stimulate KMB oxidation.

Detectable LP activity completely disappeared when exogenous LP was added to a biobleaching culture. Biobleaching cultures were standard preparations of 2% HWKP in water with 15% MB containing mycelial flocs. They were incubated for 3 days for a final pH of 4.5, a brightness increase from 32.0% to 45.8% ± 0.5%, a laccase concentration of 0.017 U ml^{-1}, and no detectable LP. Ethylene was measured after 60 min in the standard assay. The amounts of ethylene (mean ± standard deviation, in nanomoles per flask) in the cultures were as follows: uninoculated HWKP, 0.35 ± 0.10; biobleaching culture, 109 ± 7.4; biobleaching culture plus 500 U of LP liter^{-1}, 114 ± 2.2; and biobleaching culture plus 500 U of LP liter^{-1}, 1 mM VA, and 0.4 mM H_2O_2, 92 ± 10.6.

Inhibition of LP in cultures. Addition of purified *T. versicolor* LP to filtered biobleaching culture supernatant liquor (pH 4.5) abolished LP activity as measured by the conventional VA-based LP assay (preceding paragraph and Fig. 2). To determine whether interference with the appearance of veratraldehyde rather than a true lack of LP activity was responsible, another colorimetric assay for LP, based on oxidation of the heterocyclic dye Azure B, was devised. Azure B is oxidized preferentially over VA by LP and is not susceptible to interference from H_2O_2-evolving VA oxidase-type enzymes (7, 18, 35, 36), and its oxidation is measured at a wavelength (651 nm) at which the strong UV absorbance of most culture liquors is not a problem. This assay is described more fully in another report (1). Figure 2 shows that in three

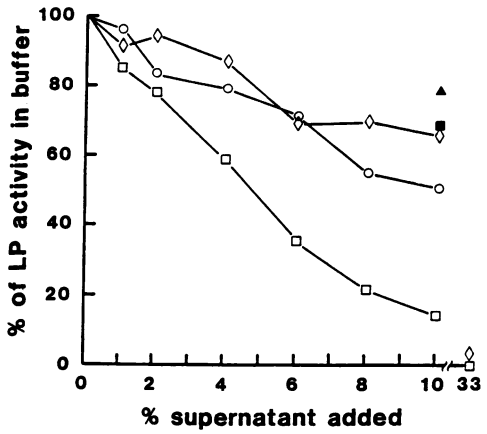


FIG. 3. Effects of filtered biobleaching culture liquor on detectable LP activity as determined by the VA assay. The same 5-day-old 15% MB biobleaching liquor used in the experiments whose results are illustrated in Fig. 2 was microfiltered (0.45-μm-pore-size filter), treated, and then added, up to a 33% (vol/vol) concentration, to 5 mM Na succinate buffer, pH 4.5. Symbols: □, undialyzed, filtered liquor; ○, undialyzed, filtered liquor plus 0.1% Tween 80; ◇, dialyzed (≥12 kDa), microfiltered liquor; ■, dialyzed, ultrafiltered liquor (≤100 kDa, ≥12 kDa); ▲, dialyzed, ultrafiltered liquor (≤100 kDa, ≥12 kDa) plus 0.1% Tween 80.

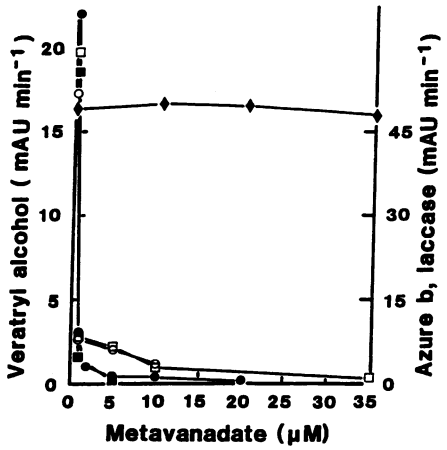


FIG. 4. Effect of Na metavanadate on the activity of fungal peroxidase and laccase (phenoloxidase) enzymes. Increasing amounts of peroxidase and Na metavanadate were added to various enzymes in buffer (50 mM Na tartrate, pH 4.5), and the resulting changes in enzymatic activity were recorded. Symbols: ●, *T. versicolor* LP, azure B assay; ■, *P. chrysosporium* crude LP, azure B assay; ○, *P. chrysosporium* crude LP, VA assay; □, *T. versicolor* LP, VA assay; ◆, *T. versicolor* mixed laccases, ABTS assay. Abbreviation: mAU, thousandths of arbitrary units.

separate assays, LP is powerfully inhibited by the biobleaching liquor. This, of course, could explain why none of the earlier VA-based assays could detect LP in cultures. Furthermore, if and only if this inhibition extends to LP activity on all potential LP substrates in the biobleaching system, then secreted LP, even when present, cannot play a role in biobleaching or delignification. Even 10% undialyzed culture liquor in an LP assay buffer reduced LP activity by >80% (Fig. 3). Recent work suggesting that clumping or aggregation of LP is important in its in vitro shaking-mediated loss of activity (44) and the requirement of LP-secreting fungal cultures for Tween-type surfactants for good LP expression prompted the application of 0.1% Tween 80 to the inhibition of LP by biobleaching liquor. The surfactant had a distinct protective effect on LP (Fig. 3), but this effect leveled off at 0.3 to 0.4% Tween 80 (data not shown). Furthermore, Tween 80 (0.1%) was unable to reverse the complete inhibition seen with liquor diluted 1:3, let alone undiluted liquor. Both high- and low-molecular-weight fractions of the filtered liquor contributed to its LP-inhibiting properties (Fig. 3). In contrast to this strong LP inhibition exhibited by biobleaching culture liquor, the filtered liquor of the 15-day nitrogen-limited pulp-free defined-medium *T. versicolor* cultures used to induce LP exhibited little or no LP inhibition, as indicated by the LP activity observed in direct assays of the liquor (data not shown). Recoveries during LP purification were also consistent with the results of these LP assays being approximately correct.

Pulp-culture medium LP inhibition occurred in both the high (≥1,000-Da)- and low (≤1,000-Da)-molecular-mass fractions (see Fig. 5) free of filterable particles, indicating that there is at least one mechanism of inhibition that does not involve simple adsorption of LP to pulp solids. This powerful inhibition should be kept in mind when one evaluates other reports giving the levels (or absence) of LP in pulp or culture media. For example, the authors of a recent survey of ligninolytic activity in fast-growing ligninolytic white rot fungi state that their results showed "that the fungi have similar ligninolytic systems although minor differences exist" (45). In fact, according to their reported results, *Trametes cingulata* showed very substantial labelled-lignin degradation (to $^{14}CO_2$) yet produced no detectible LP and only traces of VA, in sharp contrast to *P. chrysosporium*, *Chrysosporium pruinosum*, and *T. versicolor* (incubated under N-limiting, high-O_2 conditions). Only three of the seven ligninolytic species employed produced detectable LP (45).

Sodium metavanadate-mediated inhibition of LP. Recently, it was reported that micromolar concentrations of $NaVO_3$ can effectively inhibit rat intestinal, horseradish, and squash peroxidases but not lactoperoxidase or catalase (42). The mechanism apparently involves the binding of 0.6 to 1.7 V atoms, probably in the +5 valence state, per enzyme molecule (42). In that first report of the use of V ions as a peroxidase inhibitor, there was no mention of fungal proteins, so we tried a range of *T. versicolor* 52 and *P. chrysosporium* proteins. There was no inhibition of purified, mixed cellobiose quinone oxidoreductases from *T. versicolor*, mixed MnP isozymes from *P. chrysosporium* (Tienzyme preparation [data not shown]), or laccase isozymes from *T. versicolor* (Fig. 4). In contrast, the oxidation of VA by the LP enzymes of both *P. chrysosporium* and *T. versicolor* was extremely sensitive to the addition of even submicromolar Na metavanadate at the physiological pH of 4.5 (Fig. 4). A second partially purified mixture of *P. chrysosporium* LP isozymes (Tienzyme) gave essentially the same results as our crude *P. chrysosporium* LP used in the experiments whose results are shown in Fig. 4 (data not shown). When the pH was decreased to 2.5, the usual assay pH for LP, the K_i of metavanadate for *T. versicolor* LP fell to 24 nM, compared with 235 nM at pH 4.5 (Fig. 5). Therefore, Na metavanadate may prove to be a very useful tool in any study in which a role for LP proteins is proposed. However, trial of a variety of aromatic substrates showed that the inhibition of LP by metavanadate is not universal (Table 3). Oxidation by LP of those substrates (except

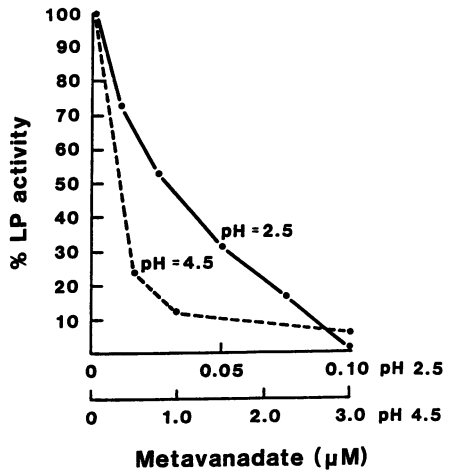


FIG. 5. Inhibition of *T. versicolor* LP proteins by Na metavanadate. The assay used a mixture of 0.1 mM H_2O_2, 2 mM VA, and 0.001 IU of LP in 50 mM Na tartrate at 25°C at pH 2.5 or 4.5.

ferulic acid) also oxidizable by horseradish peroxidase and *T. versicolor* laccase was affected little by 50 μM Na metavanadate. Conversely, oxidation of methoxybenzenes, two diaryl compounds, and other nonphenolics normally oxidized only by LP was strongly inhibited by VO_3^-, suggesting that the ion acts by decreasing the effective redox potential (or electron-abstracting vigor) of the LP active site. Two kraft black liquor lignin samples (≥12,000-Da fractions), one from a spruce and one from a hardwood kraft digestion, were also used. Both showed rapid oxidation (darkening at 465 nm) in the presence of laccase and LP. Na metavanadate (50 μM) had no effect on laccase activity but reduced LP oxidation of the two indulins by 40 and 53%, respectively.

The obvious question in the present study was this one: will metavanadate block biobleaching? Table 4 displays the results of using 50 μM filter-sterilized, unheated Na metavanadate as a treatment for or addition to the standard biobleaching system. This level was chosen because it is about 200 times the apparent K_i (VA) of VO_3^- for mixed *T. versicolor* LP isozymes (Fig. 5) but is still relatively low (a consideration because V salts reportedly inhibit a number of intracellular enzymes in other organisms [42]). The results (Table 4) show that metavanadate had no effect on biobleaching and little or no effect on ethylene production whether it was added immediately before the assay or present for the full 5 days of biobleaching. Before it could be concluded that secreted LP cannot function in the biobleaching system, it had to be demonstrated that after 5 days, LP-inhibiting V salts were still present in the liquor. Frozen, thawed aliquots of the liquor from the biobleaching cultures incubated for 5 days with 50 μM Na metavanadate were assayed by atomic absorption spectrophotometry and found to contain 46.7 ± 7.1 μM vanadium; i.e., essentially all the vanadium had remained in the liquor throughout the biobleaching experiment. Thus, there was no significant adsorption to fungus or fibers and little or no fungal V uptake. Microfiltration (0.45-μm-pore-size filters) of the freeze-thawed liquor decreased the V concentration to 29.6 ± 4.1 μM, which was not surprising since freeze-thawing caused some tiny flocs to form in the liquor. There was no detectable vanadium in the control biobleaching culture liquor. In mildly acidic aqueous solution, Na metavanadate can form a variety of other V species, especially polymers of VO_3^- such as $[H_2V_{10}O_{28}]^{-4}$, $[HV_{10}O_{28}]^{-5}$, and $[V_{10}H_{28}]^{-6}$ and even the pervanadyl ion VO_2^+. Thus, proof that after 5 days of biobleaching the liquor still contained a form of V strongly inhibitory to LP was required.

Examination of the fresh 0.45-μm-filtered liquor from triplicate 5-day pulp–*T. versicolor* biobleaching cultures with and without 50 μM vanadate showed that a 16% (vol/vol) addition of the vanadate-free liquor was required for 50% inhibition of *T. versicolor* LP activity in Na tartrate buffer at

TABLE 3. Inhibition of laccase and LP activity by 50 μM sodium metavanadate

Substrate	λ (nm)	Results[a] for:							
		P. chrysosporium LP[b]		Laccase		*T. versicolor* LP[b]			
						pH 4.5		pH 2.5	
		Oxidation	% Inhibition	Oxidation	% Inhibition	Oxidation	% Inhibition	Oxidation	% Inhibition
1,2-Dimethoxybenzene (veratrole)	273	+	>99	−		−		−	
1,2,3-Trimethoxybenzene	267	+	>99	−		−		−	
1,2,4-Trimethoxybenzene	253	+	87	−		+	80	+	95
3,4-Dimethoxybenzyl alcohol (VA)	310	+	98	−		+	98	+	>99
3,5-Dimethoxybenzyl alcohol	310	−		−		−		−	
Veratryl glycerol β-*O*-guaiacyl ether	310	+	>99	−		+	>99	+	>99
1-(3,4-Dimethoxyphenyl-2-phenylethane diol)	310	+	84	−		+	77	+	78
Azure B	651	+	>99	−		+	>99	+	>99
4-Coumaric acid (4-hydroxycinnamic acid)	288	+	34	+	21	+	0	+	0
DAB	482	+	0	+	0	+	0	+	0
O-Dianisidine (3,3′-dimethoxybenzidine)	460	+	19	+	10	+	0	+	0
Ferulic acid (4-hydroxy-3-methoxycinnamic acid)	312	+	51	+	0	+	0	+	0
Guaiacol (2-methoxyphenol)	420	+	25	+	15	+	0	+	0

[a] LP assays were run as described in the legend to Fig. 2 with 2 mM substrate. Laccase assays were run at pH 5.0 in 0.1 M Na acetate buffer. Oxidation indicates enzyme-dependent disappearance of substrate or appearance of product at the indicated wavelength within 5 min.

[b] Both LP preparations were mixtures of isozymes, as noted in Materials and Methods. In addition, the *T. versicolor* LP contained traces of laccase, so there might have been some VO_3^--mediated inhibition of laccase substrate oxidation by LP that was masked by this laccase activity.

TABLE 4. Importance of LP and effect of Na metavanadate in the *T. versicolor* pulp biobleaching system

Flask contents[a]	Final pH	Brightness (%)	Ethylene (nmol flask^{-1})
Sterile pulp + 15% MB	7.28	34.2	0.2 ± 0.1
Biobleaching culture	4.75	57.0 ± 1.4	61.6 ± 2.5
Biobleaching culture + metavanadate[b]	4.98	58.6 ± 0.9	50.1 ± 4.1
Biobleaching culture + metavanadate[c]			66.9 ± 1.3
Washed, biobleached pulp + biomass + 15% fresh MB[b]			18.5 ± 0.2
Washed, biobleached pulp + biomass (boiled) + 15% fresh MB[b]			0.8 ± 0.1

[a] Triplicate 500-ml flasks containing 200 ml of biobleaching culture (with 2% HWKP) were incubated for 5 days at 200 rpm as described in Materials and Methods. All flasks with culture and pulp showed substantial brightening on day 2.

[b] To complete biobleaching culture and pulp, 50 μM $NaVO_3$ (filter sterilized and unheated) was added on day 0. The contents of several biobleaching cultures were washed on Whatman filter paper with water and resuspended in fresh 15% MB with or without boiling for 5 min. The ethylene production over 60 min was measured as described in the text.

[c] Sodium metavanadate (50 μM) was added to triplicate aliquots of the control biobleaching culture only at the initiation of the 60-min KMB-ethylene incubation on day 5.

pH 4.5, while a 0.5% concentration of the vanadate-containing liquor gave the same level of inhibition. These trials were run at pH 2.5 and 4.5, and in all cases there was a linear relationship between LP inhibition and the amount of liquor added to the assay mixture. Thus, the ability of V ions to greatly enhance the LP-inhibitory properties of the biobleaching liquor had no detectable effect on the liquor's ability to biobleach.

Conclusions. On the basis of the foregoing results, it appears very unlikely that secreted lignin peroxidases play a significant role in *T. versicolor*-mediated pulp biobleaching for the following reasons. (i) Biobleaching culture supernatants are strongly inhibitory to LP, as measured in three different LP assays. One wonders how many of the numerous published VA assay-determined values for LP in other fungal cultures have been influenced by similar inhibitions. (ii) The fungus maintains the biobleaching cultures at pH 4.2 to 5.0, at which the LP proteins have only 10 to 25% of their optimal activity (as measured in buffers at pH 2.5 or 3.0). (iii) LP isozyme secretion can be induced in *T. versicolor* 52, but these proteins are detected only when all of the following conditions (not present in the biobleaching system) are met: (a) a low Mn concentration (2 μM), (b) a high flask headspace partial O_2 pressure, (c) no or very gentle shaking (60 to 75 versus 200 rpm), (d) a minimum of 7 days for the first appearance of LP, (e) growth-limiting nitrogen levels, and (f) the presence of a surfactant such as Tween 20. (iv) In many repeats of two different LP assays under many conditions, no LP was ever detected during biobleaching, even when the supernatant was dialyzed and concentrated. In contrast, MnP and laccase were always present. (v) KMB-oxidizing activity in biobleaching flasks, unlike LP-mediated KMB oxidation, was not greatly stimulated by added H_2O_2 and VA in any of a variety of combinations, nor was it repressed by the addition of high levels of catalase. (vi) No VA, veratraldehyde, veratric acid, or H_2O_2 could be detected in biobleaching cultures. However, levels just below the detection limit of the HPLC and the H_2O_2 assays used could still stimulate LP. (vii) Some of the KMB-oxidizing activity appearing in the biobleaching liquor supernatant appeared to survive autoclaving. (viii) Sodium metavanadate, a potent in vitro inhibitor of LP activity on many nonphenolic substrates, had no effect on a 5-day biobleaching treatment, although an assay at the end of the biobleaching experiment showed the liquor to be strongly inhibitory to LP and to contain more than 100 times the amount of V needed to inhibit more than 95% of the LP activity on VA. (ix) Fresh sodium metavanadate had little or no effect on the KMB-oxidizing activity seen in the liquor of a biobleaching culture. (x) Earlier work demonstrated that in our system, direct contact between pulp fibers and the hyphae of *T. versicolor* is not necessary for biobleaching, indicating that the agents of delignification, if not lignin degradation, are soluble (2), yet analysis of the liquor showed no LP.

No single one of the above facts is a sufficient basis on which to reject secreted LP as an important biobleaching component in the *T. versicolor* system, but taken together they seem to be conclusive. This of course does not preclude a role for intracellular peroxidases of the type extensively investigated by Lobarzewski et al. (32–34), although these proteins could not be central to cell-free biobleaching. These results also neither support nor deny a role for MnPs or Mn^{3+} chelates produced indirectly by laccase (3).

The results obtained in this study with the KMB oxidation reaction show that, at least in fungi similar to *T. versicolor* under nitrogen-sufficient conditions, ethylene production from KMB is unrelated to secretion of peroxidases and thus cannot be used as an LP assay, as has been suggested elsewhere (23). *T. versicolor* KMB oxidation also appears to be largely independent of the presence of pulp and partially cell associated (Table 2), suggesting that the mechanism is constitutive, yet none of eight diverse nonligninolytic fungi could rapidly oxidize KMB. Thus, the principal mechanism(s) of KMB oxidation in the *T. versicolor* biobleaching system remains unknown, as does its role, if any, in biobleaching and delignification. Likewise, the demonstration 42 years ago that horseradish and turnip peroxidases can rapidly generate Mn(III) complexes, but only in the presence of a phenolic such as *p*-cresol or phenol (26), suggested that phenoxy radicals are the proximal oxidants. This led to the finding that laccases are, like MnPs, able to indirectly generate the putative lignin-degrading Mn(III) complexes and ethylene from KMB, as LP and MnP can (3, 38). Clearly, if the goal is to understand in vivo biobleaching or delignification, examining fungal enzymes and single aromatic, phenolic, or lignin model substrates in isolation, as is commonly done, and not in various combinations, is a mistake.

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