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A New Assay for Lignin-Type Peroxidases Employing the Dye Azure B

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The discovery in 1983 of fungal "ligninases" capable of catalyzing the peroxidation of nonphenolic aromatic lignin components has been seen as a major advance in understanding how certain basidiomycete fungi can completely degrade lignin. The ability of these lignin-type peroxidases to convert millimolar concentrations of veratryl alcohol to veratraldehyde, indicated by a change in the A_{310} of veratraldehyde, has become the standard assay for routine quantitation of LP activity. A new assay based on the oxidation of micromolar concentrations of the dye Azure B is presented. Although it is as simple and rapid as the veratryl alcohol assay, it appears to overcome some of the shortcomings of that assay. In particular, interference from UV- and short-wavelength visible-light-absorbing materials is greatly reduced and assay specificity is improved.

The discovery in 1983 of fungal lignin-type secreted peroxidases (lignin peroxidases [LP]) (13, 34) has been heralded as a major advance in understanding how the white-rot basidiomycetes degrade lignin. LP has now been found in many (but not all) white-rot fungi and in certain procaryotes (31). The initial detection and quantitation of LP activity were made by oxidation of a β-*O*-4 model compound (34). Later, a gas chromatographic assay of the ethylene released by the one-electron oxidation of 2-keto-4-methiolbutyric acid (KMB) by *Phanerochaete chrysosporium* LP was used. However, β-*O*-4 model compounds must be synthesized, and the KMB assay is considerably more laborious than most cuvette-spectrophotometer assays. As a result, when a simple assay based on the oxidation of veratryl (3,5-dimethoxybenzyl) alcohol (VA) to veratraldehyde was introduced in 1984 (35), it was widely adopted. To date, nearly all of the hundreds of articles appearing on the subjects of LP, ligninolytic activity, and white-rot basidiomycetes have employed some variation of the VA assay for routine quantitation and detection of LP activity.

LP activity can also be detected by cleavage or one-electron oxidation of a wide variety of nonphenolic diarylpropanes (13, 15, 35), β-*O*-4-linked lignin model compounds (15, 35, 37), other methoxybenzenes (19, 20), aromatic ring cleavages (17, 25, 37), and Cα-Cβ cleavages (15, 17, 34). However, since these methods generally offer increased complexity and no apparent advantages, VA oxidation has remained the universal assay. Certain polymeric dyes have been employed as LP or lignin degradation indicators in multiday cultures (12, 14), and radiolabelled CO_2 or soluble materials released from natural and synthetic lignins and lignin models likewise have been used for specific purposes (21, 29), but both methods are too slow, cumbersome, or expensive to be used as routine assays. The best existing alternative is probably the original KMB-ethylene assay since it is very sensitive and can be used in turbid or UV-absorbing samples where the VA assay is useless. However, it is slower, short-term rates cannot be monitored, and a number of oxidants other than LP are known to oxidize KMB, releasing ethylene. These oxidants include liver microsomes and normal liver metabolic systems (10, 24), an Mn^{2+}-phenol-pyrophosphate-SO_3^{2-} system (39), singlet oxygen (23), H_2O_2 and ascorbic acid (8, 9), Mn^{3+} chelates (3a), UV light (23), and, most notably, hydroxyl free radicals (·OH) for which ethylene production from KMB is frequently employed as an assay (5, 8, 23). Previous work has also shown KMB oxidation to be characteristic of active cultures of *Trametes versicolor* (but not of eight nonligninolytic fungi), even when no LP could be detected by other assays (2, 4).

Assays employing VA are, in contrast, simple and rapid and enable one to easily monitor reaction rates and the effect of additions on rates. However, VA assays have the following shortcomings: (i) veratric radical intermediates and the product veratraldehyde may be reduced by many fungal or medium components concomitant with the peroxidative oxidation of VA; (ii) measurement is at 310 nm, a wavelength at which phenolics and other aromatics typical of fungi and lignin-containing materials absorb very strongly, making assays in complex systems difficult; (iii) optical dispersion is high at 310 nm, making the assay much more sensitive to turbidity than one measuring in the visible range; (iv) the assay has a relatively low sensitivity; (v) an interfering substrate (methyl-3-methoxy-4-hydroxybenzoate) is found in some commercial VA preparations (36); and (vi) at least some white-rot basidiomycetes secrete nonperoxidizing flavin-cofactored VA oxidases (VAO), which also oxidize VA to veratraldehyde (6, 16, 26, 27).

Work on the mechanisms of biological bleaching of kraft brownstock by *T. versicolor* made the deficiencies of the VA assay very obvious; therefore, on the basis of earlier reports of the use of N-limited *P. chrysosporium* and LP to degrade azo and heterocyclic textile dyes in industrial wastes (11, 30), a survey of potential assay substrates was performed. This report describes the result, i.e., a new rapid cuvette assay for LP proteins which lacks many of the problems of VA-based assays.

MATERIALS AND METHODS

Enzyme assays. The VA assay for LP used 50 mM Na tartrate buffer (pH 2.5 or 4.5)–0.1 mM H_2O_2–2 mM VA in a 1.0-ml reaction volume, and activity was monitored at 310 nm (35). The dye-based LP assays were run similarly, with 1 ml of 50 mM Na tartrate (pH 4.5 or 2.5)–0.1 mM H_2O_2–32

μM Azure B (or other dye), with activity monitored at an appropriate wavelength (a few nanometers below the λ_{max}). The Azure B LP assay, as developed here, contained (final concentrations) 32 μM Azure B and 100 μM H_2O_2 in 50 mM Na tartrate buffer (pH 4.5, 25°C). The dye and H_2O_2 were made up as 100× stock solutions, and 10 μl of each was added to the 1.0-ml final reaction volume. The optical density (OD) decrease was read at exactly 651 nm. Since the absolute A_{651} of 32 μM Azure B was about 1.56, the chart recorder span was set for 1.4 to 1.6 OD units. For monitoring extended or very rapid reactions, the offset was reduced as the reaction proceeded or the full scale (span) was increased to 0.4 or 1.0 OD unit.

The 3,3′-diaminobenzidine assay for peroxidatic activity used 50 mM Na phosphate (pH 7.0)–1.0 mM 3,3′-diaminobenzidine–2.0 mM H_2O_2 in a 1-ml volume, and the activity was monitored at 482 nm. *T. versicolor* laccase was quantitated in 1 ml of 100 mM Na acetate buffer (pH 5.0) containing 1 mM 2,2′-azino-bis-(3-ethyl benzthiazoline)-6-sulfonate and measured at 420 nm (38). *Pleurotus* VAO was measured in 1.0 ml of 50 mM Na tartrate buffer (pH 5.0) with 1 mM VA (6). All assays, and isobestic point and absorbance peak determinations were done at 25°C by using a Perkin-Elmer λ3 UV-visible scanning spectrophotometer, calibrated for wavelength with a holmium oxide filter and for absorbance by using Gilford 202 OD standards at 550 nm. Most enzyme assays were run on a chart recorder at a full scale of 0.20 OD unit (200 milliabsorbance units).

The manganese peroxidase (MnP) assay (total volume, 1.0 ml) contained 0.2 mM $MnSO_4$, 0.1 mM H_2O_2, and 0.0025% phenol red, all in 50 mM buffer (pH 4.5). Buffers employed were sodium malonate, sodium tartrate, sodium oxalate, and a mixture of 25 mM sodium lactate and sodium succinate. The reaction was monitored at 431 nm (22).

Enzymes. *T. versicolor* LP was a mixture of at least three isozymes prepared from *T. versicolor* 52 (dikaryon) (ATCC 20869). The fungus was grown on a simple defined medium (TDM) (2) containing 0.1% Tween 20, limiting N (as NH_4Cl), and high O_2 with slow shaking (70 rpm) for 15 days in 1-liter aliquots in 2.8-liter Fernbach flasks. The LP in the supernatant was bulk adsorbed to DEAE-Bio-Gel equilibrated with 5 mM Na succinate buffer (pH 5.5), the gel was poured into a column and washed with the same buffer, and then peaks were eluted with a 0 to 0.5 M NaCl gradient. LP activity was concentrated, desalted, and run onto a Mono-Q (Pharmacia) column with the same buffer, and peaks were eluted on a 0 to 0.5 M NaCl gradient. LP peaks were pooled, desalted, and concentrated by ultrafiltration and then stored in small aliquots at −20°C. This final preparation showed a prominent 407-nm Soret absorbance and contained three major LP peaks, no MnP, and traces of laccase activity. *P. chrysosporium* 439 (from ATCC 20696) LP was induced on a simple defined medium (2) by using conditions similar to those used for *T. versicolor*, and the supernatant was filtered (pore size, 0.45 μm), dialyzed against water, and concentrated. This crude preparation retained good activity for at least several months at 4 and 20°C. A second *P. chrysosporium* LP preparation was obtained commercially. VAO I and II were purified as previously described (6) from *Pleurotus sajor-caju* 405 (Paprican collection). MnP was purified to homogeneity on a sodium dodecyl sulfate-polyacrylamide gel from *T. versicolor* 52J grown under nitrogen-sufficient conditions (when no LP could be detected) by a method nearly identical to that used for *T. versicolor* LP. For LP, a crude commercial preparation of *P. chrysosporium* MnP was also obtained and used. *Coprinus macrorhizus* peroxidase was purchased from the Chemical Dynamics Corp., South Plainfield, N.J., and not further purified. *Arthromyces ramosus* and horseradish peroxidases were from Sigma Chemical Co., and *P. chrysosporium* LP and MnP were from Tienzyme, Inc., State Park, Pa.

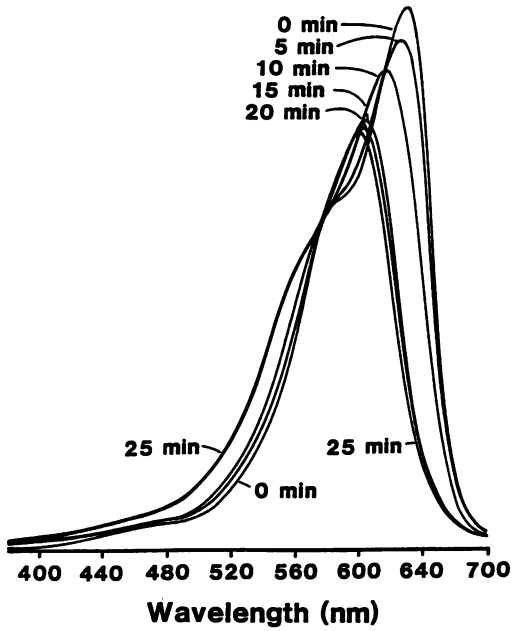

FIG. 1. Successive visible absorption spectra recorded just before and during the first 25 min of oxidation of 32 μM Azure B by 0.001 IU of *T. versicolor* LP and 0.1 mM H_2O_2 in 50 mM Na tartrate (pH 4.5).

Manganic pyrophosphate was prepared by the classical procedure of Kenten and Mann (18) by using $MnCl_2$, MnO_2 and 8-hydroxyquinoline-deferrated Na pyrophosphate; reacted overnight; and then microfiltered and quantitated by the A_{478} peak, assuming a molar extinction coefficient (ΣM) of 107. Such preparations always contain some Mn^{2+}. All dyes and other chemicals were from Sigma, Aldrich, and Fisher. The Poly dyes are stable nontoxic high-molecular-weight dyes designed by the Dynapol Corp. as food colorants and sold by Sigma.

RESULTS

Substitution of micromolar concentrations of Azure A, B, or C for the millimolar concentrations of VA in the LP assay gave reaction rates that were constant (an unchanging slope) over many minutes and that were equal to or greater than the observed VA oxidation rates under the same conditions. The Azure dye-based assays measure the disappearance of the substrate, i.e., the OD decrease caused by a hyperchromic shift of the major visible absorbance peak of the substrate (Fig. 1), not the increase at 310 nm due to the appearance of the VA assay product (veratraldehyde). Previously, the addition of Poly R-478 and phenol red (0.005%) to kraft pulp delignifying and biobleaching cultures of *T. versicolor* 52 showed that the system was capable of completely decolorizing these dyes over 18 h (3), but no short-term decolorization of Poly R-478 by purified LP from *T. versicolor* occurred here (Table 1). Phenol red was not tried because of its degradation by MnP proteins (22). Reportedly, Orange II will react with a crude *P. chrysosporium* LP preparation

TABLE 1. Oxidation of various dyes by *T. versicolor* LP[a]

Substrate	Concn	λ_{max} (nm)	λ Measured (nm)	ΔOD (short term)[b]
Poly R-478	0.005%	520	520	0
Poly S	0.005%	444	444	0
Poly Y	0.005%	470	470	0
Orange II	57 μM	489	489	0
Tropaeolin O	32 μM	398	398	1+
Azure A	32 μM	626	640	2+
Azure B[c]	32 μM	647 (600)	651	4+
Azure C	32 μM	619	623	3+

[a] Assays using *T. versicolor* mixed LP (≈0.002 U assay^{-1}), 50 mM Na tartrate buffer (pH 4.5, 25°C), and 0.1 mM H_2O_2 at 0.20 OD unit full scale were run in a 1.0-ml volume.

[b] Reactions ranged from no detectable reaction over 10 min (0) to a rapid change in OD (4+).

[c] The millimolar extinction coefficient (ΣmM) (651 nm) of Azure B under assay conditions is approximately 48.8, with a shoulder at 600 nm.

(11), but no reaction (over a 10-min period) was seen with the purified mixed *T. versicolor* LP isozymes. Tropaeolin O, while weakly reactive with the LP preparation, has two phenolic hydroxyl groups and therefore reacted slowly with laccase as well. Since the goal was to find a substrate that reacted with all LP but only LP proteins, both of these dyes were rejected. The remainder of the work employed Azure B only, which was selected because it had the most rapid reaction with LP and the longest measurement wavelength of the three Azure dyes.

To be of any value, a substrate for an LP assay must show reasonably high specificity for this class of enzymes or at least not respond to anything else commonly occurring in LP-containing biological systems. The fact that the Azure dyes (including Azure B) are designed for stability in textiles and are consequently a problem waste for the textile industry, in part because of their relative resistance to microbial degradation, is an encouraging characteristic (1, 7, 28). Table 2 shows that Azure B, like VA and KMB, will not react significantly with laccase per se or with the intracellular peroxidases from horseradish or the basidiomycete *C. macrorhizus*. The *A. ramosus* peroxidase is secreted by a soil fungus not known to be ligninolytic (33), and it also did not oxidize either VA or Azure B. The lack of reaction of *T. versicolor* laccase with the Azure dyes is interesting, given the reaction of structurally similar nonphenolic chlorpromazine with *Polyporus anceps* laccase (32). Azure B also did not react with Mn^{3+} pyrophosphate or MnPs in various buffers, which indicates that, at least under these conditions, it cannot be oxidized by the Mn^{3+} chelates of lactate, succinate, malonate, pyrophosphate, oxalate, or tartrate. However, the nature of the Mn^{3+} chelate and the Mn^{3+}/chelator ratio will have major effects on Mn^{3+} reactivity, so there may be conditions under which certain Mn^{3+} complexes will react with Azure B.

The specificity of VA for LP-type enzymes fails with a group of VAO found in *Pleurotus* (6, 16), *Bjerkandera* (26), and *Pichia* (27) species. These flavin enzymes are not peroxidatic but oxidize VA to veratraldehyde, thereby producing, instead of consuming, H_2O_2. Under conditions giving high VA oxidation rates, Azure B showed no reaction with either of two *P. sajor-caju* VAO isozymes. Thus, Azure B appears to be more selective for LP than VA.

Azure B performed in a manner comparable to that of VA when either crude *P. chrysosporium* or purified mixed *T. versicolor* LP was used, both at the traditional assay pH (2.5) and at a physiological pH (4.5). Repeated spectral scans obtained during the *T. versicolor* LP-Azure B reaction (Fig. 1) showed that the substrate with a λ_{max} of 647 nm underwent a hyperchromic shift to a relatively stable product with a λ_{max} of 617 nm. Thus, the assay measurement was at a λ of 651 nm to take advantage of the hyperchromic shift and the nearly linear lower slope of the Azure B absorption peak. Both Azure A and C dyes underwent similar hyperchromic shifts in response to LP oxidation, which was not surprising given the similarity of their structures.

Figure 1 also suggests that a single substrate (Azure B) was oxidized to a single product, judging from the apparent isobestic point at 595 nm. In contrast, Cripps et al. reported

TABLE 2. Specificity of Azure B oxidation[a]

Catalyst or oxidant	pH	H_2O_2 (mM)	Reaction in oxidized substrate[b]		
			Veratryl alcohol (2 mM)	Azure B (32 μM)	Other (2 mM)
Horseradish peroxidase	7.0	2	0	0	4+ (DAB)
C. macrohizus peroxidase	7.0	2	0	0	4+ (DAB)
A. ramosus peroxidase	7.0	2	0	0	4+ (DAB)
P. chrysosporium LP	2.5	0.1	4+	4+	
P. chrysosporium LP	4.5	0.1	2+	2+	
T. versicolor LP	2.5	0.1	4+	4+	
T. versicolor LP	4.5	0.1	2+	2+	
T. versicolor MnP[c]	4.5	0.1	0	0	4+ (PR)
P. chrysosporium MnP[d]	4.5	0.1	0	0	4+, 3+, 2+ (PR)
T. versicolor laccase	5.0	0	0	0	4+
P. sajor-caju VAO I	5.0	0	4+	0	4+ (ABTS)
P. sajor-caju VAO II	5.0	0	4+	0	
Mn^{3+} pyrophosphate (1 mM)	4.5	0	ND	0	
Na tartrate buffer (50 mM)	4.5	0.1	0	0	2+ (KMB)

[a] Assays were run as described in Materials and Methods using 1-ml assay volumes at 25°C.

[b] Reactions ranged from no detectable reaction (0) to very rapid reaction (4+). Abbreviations: DAB; 3,3′-diaminobenzidine; ABTS, 2,2′-azino-bis(-3-ethyl benzthiazoline)-6-sulfonate; PR, phenol red oxidation; ND, not determined. Results obtained with the commercial and laboratory-produced *P. chrysosporium* LP were identical.

[c] Assay (1.0 ml) was run in 50 mM Na malonate with 0.0024% phenol red.

[d] Assays were run using 50 mM malonate, 50 mM tartrate, 50 mM oxalate, and 25 mM succinate–25 mM lactate.

that an LP-containing supernatant from a *P. chrysosporium* culture yielded four colored products from Azure B, as measured by thin-layer chromatography (11). Whether the product species seen in Fig. 1 was produced and then further degraded by the LP isozymes or other factors in the *P. chrysosporium* supernatant is unknown; in any case, the assay is accurate as long as the rate measured is the disappearance of the Azure B (substrate) and not the appearance of a specific product.

When using a hydrophilic chromophore like Azure B as an assay substrate, pH effects on the color λ_{max} and extinction coefficient (ΣM) must be considered. The A_{647} of Azure B reportedly increases by 15% when the pH is decreased from pH 5.0 to 3.5 (11). However, when 50 mM Na tartrate buffer at pH 4.5 (pK, 4.16) and ≤20% (vol/vol) enzyme or culture fluid (typically pH 4.1 to 4.6) are used, the assay pH is ±0.1 of the buffer pH. Figure 2A displays the ability of Azure B to inhibit VA oxidation by LP, while Fig. 2B presents the opposite experiment, the inhibition of Azure B oxidation by VA at the realistic pH of 4.5. Clearly, *T. versicolor* LP preferentially oxidizes micromolar concentrations of Azure B over millimolar concentrations of VA. Both inhibition effects showed rather poor linearity with concentration, so K_i values were not calculated.

Purified LP enzymes cannot directly oxidize KMB but the oxidation proceeds rapidly in the presence of VA, as indicated by the evolution of ethylene (2). Increasing amounts of KMB competitively inhibit the appearance of veratraldehyde apparently because a peroxidase-generated veratryl intermediate is the proximal oxidant of KMB and is, in the process, rereduced to VA, thus preventing the appearance of veratraldehyde (2). Substitution of Azure B for VA reduces this KMB interference (Fig. 3). Since a relatively high-energy oxidation is required to oxidize KMB, it is not unreasonable to presume that the proximal veratryl oxidizing species reduced by KMB will also be reduced by many biological molecules and that therefore the Azure B LP assay will be less affected by many reductants interfering with the VA-based assay.

The optimal concentrations of the two LP substrates, H_2O_2 and Azure B, employed in the assay were determined (Fig. 4). The concentration of H_2O_2 used previously in the VA assay (100 μM) proved to be entirely satisfactory, as did the initial concentration of Azure B (32 μM).

Reducing the Azure B concentration from 32 to 4 μM had no effect on the initial reaction rate, indicating that the apparent K_m for Azure B of the mixed *T. versicolor* LP was ≤2 μM. Reducing the Azure B concentration to 4 or 8 μM therefore would affect only the usable extent of the 651-nm color change, not the initial rate. In the case of spectrophotometers unable to offset 1.4 OD units and to continue to give a smooth baseline at 0.2 OD unit full scale, it may be preferable to use 8 μM Azure B and an offset of 0.2 OD unit, or 0.4 OD unit full scale and no offset at all, although the total extent of measurable LP-driven oxidation will be reduced accordingly.

Although the Azure B assay wavelength (651 nm) should permit assay of moderately turbid samples, the fact that Azure B is a dye means that its adsorption by sample particulates must be considered. Hardwood kraft brownstock (0.25% [wt/vol]) added to 32 μM Azure B in standard assay buffer adsorbed 20.0 μM (62.5%) Azure B in 5 min. Highly purified cellulose (Solka-Floc) bound 7.5 μM (23.4%) in 5 min, while a large mass of fresh agar plate-grown *T. versicolor* mycelium bound 4.0 μM (12.5%) Azure B under the same conditions. Thus, at least some solids will interfere with the assay via adsorption. Samples should therefore be centrifuged or filtered before assay unless the solids present are known to not adsorb Azure B.

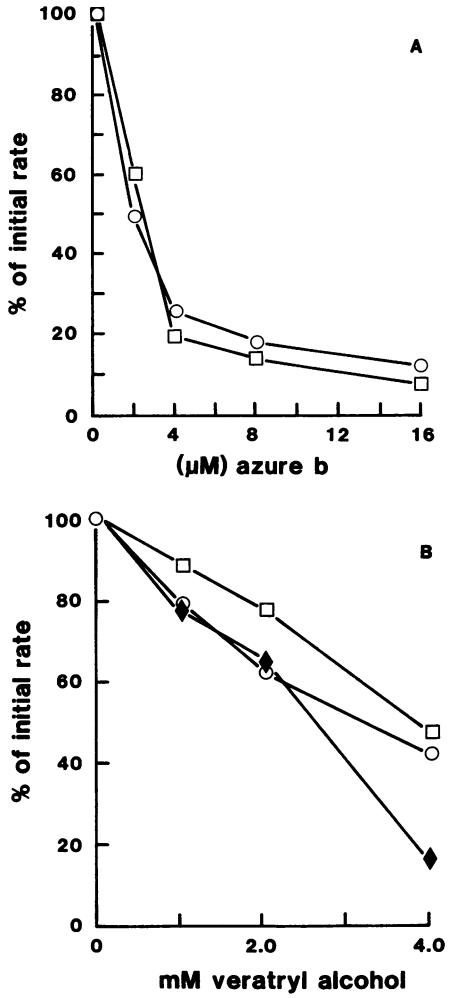

FIG. 2. (A) Inhibition of the LP-catalyzed oxidation of VA by Azure B. LP assay conditions were as described in Materials and Methods, and the reaction rate was measured at 310 nm. Symbols: ○, 4 mM VA; □, 2 mM VA. (B) Inhibition of the LP-catalyzed oxidation of Azure B by the addition of VA. Assay conditions were as described in Materials and Methods, and the rate was measured at 651 nm. Symbols: □, 32 μM Azure B; ○, 16 μM Azure B; ◆, 4 μM Azure B.

Repeated pulsing of the assay buffer containing 32 μM Azure B plus LP with 2 μM H_2O_2 showed that the apparent K_m (H_2O_2) for a mixture of three *T. versicolor* LP isozymes was 0.5 μM, and that for each nanomole of H_2O_2 consumed, 0.98 nmol of Azure B disappeared. Thus, one H_2O_2 was consumed, two water molecules were produced, and two electrons were abstracted per unit of Azure B oxidized. Since peroxidases are typically one-electron-abstracting oxidants, the oxidation reaction has at least two steps. The LPs are not necessarily involved in both oxidative steps or in all of the H_2O_2 consumption. Since such a multistep reaction, especially one involving aryloxy radical intermediates, might be expected to have an enzyme concentration-reaction rate relationship other than 1:1, the response of the assay to increasing amounts of *P. chrysosporium* and *T. versicolor*

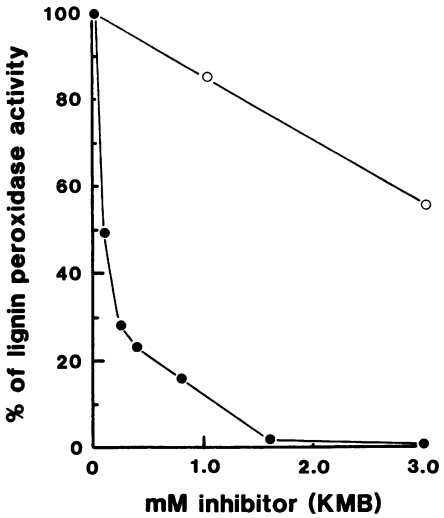

FIG. 3. Inhibition of LP-mediated VA and Azure B oxidation by KMB. Symbols: ○, Azure B (32 μM) assay; ●, VA (2 mM) assay.

LP was examined (Fig. 5). Curvature of the initial reaction rate lines for Azure A, B, and C dyes showed that the greater the LP activity present, the higher the apparent turnover number (specific activity) of the enzymes. That this is primarily an effect of the Azure A, B, and C substrates and not H_2O_2 or the LP proteins can be seen by the straight-line relationship obtained under identical conditions but with VA as the substrate. This effect gives the appearance of the Azure B assay being significantly more sensitive at higher LP concentrations (in terms of ΔOD at 651 nm min^{-1} unit of LP^{-1}) but similar to the VA assay at lower concentrations. Thus, when the Azure B assay is used, it is advisable to run an initial calibration curve.

Because the VA and Azure B assays are identical except for the LP substrate employed, reproducibility should be dependent upon the same errors in measurement and duplication of weights, volumes, temperature, and pH as well as similar mixing, cuvette, and instrument variations. The reproducibility of the Azure B assay run six times on partially purified *P. chrysosporium* LP (≈0.001 IU ml^{-1}) was ±4.47% (standard deviation) in our study.

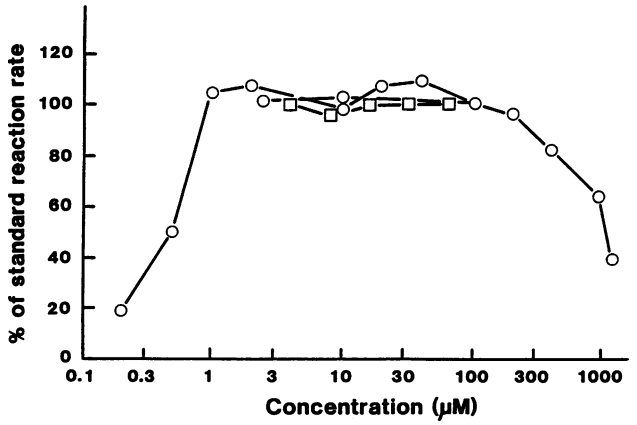

FIG. 4. Dye and H_2O_2 concentration dependence of the Azure B LP assay. Assays were run as described in Materials and Methods (50 mM Na tartrate [pH 4.5] in a 1-ml reaction mixture, at 25°C). Symbols: ○, H_2O_2 concentration (the two lines represent two separate sets of experiments); □, Azure B concentration. The rate obtained by using 0.1 mM H_2O_2 and 32 μM Azure B was arbitrarily designated 100%.

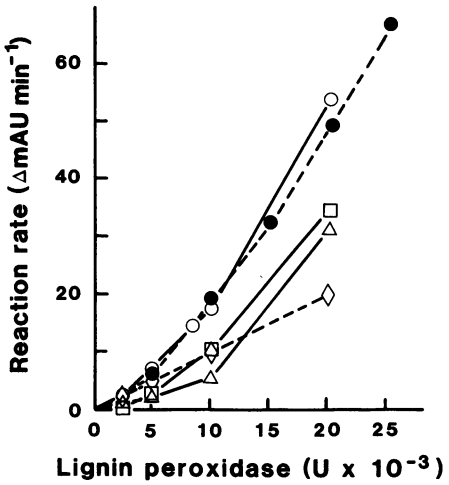

FIG. 5. Enzyme concentration-reaction rate relationships in LP assays using different substrates. Assays were performed as described in Materials and Methods but used both *T. versicolor* LP and a *P. chrysosporium* crude LP preparation (in one case) and were run using the three Azure dyes (32 μM) and VA (2 mM) as substrates. The oxidation rates spanned the range normally used in the assay. Symbols: ○, Azure B (651 nm); □, Azure C (619 nm); △, Azure A (640 nm); ◇, VA (310 nm); and ●, Azure B (651 nm) (*P. chrysosporium* LP). A unit of LP produced a ΔOD at 310 nm of 1.0 min^{-1} with VA as the substrate.

DISCUSSION

A novel spectrophotometric assay for LP is described which appears to have the following advantages over the present VA-based assay. (i) Measurement is at 651 nm, permitting the analysis of low levels of LP in samples containing lignin, aromatic, phenolic, or other species absorbing heavily in the 310-nm region. (ii) Quartz cuvettes and a UV spectrophotometer are not needed. On the other hand, an inexpensive visible-light spectrophotometer employing barrier filters instead of a monochromator may be unsuitable because its broad bandpass may prevent detection of the 647- to 617-nm shift. (iii) The visible brown color present in many lignin, mill effluent, polyphenol, and quinone-containing samples usually absorbs (interferes) very little at 651 nm. (iv) Azure B is a relatively refractory synthetic compound that is not decolorized under usual biological or physical conditions. Thus, there is much less chance of non-LP-mediated substrate oxidation or concomitant reduction, as often happens with veratryl radicals or veratraldehyde. Such reductions will give falsely low (or will mask) LP activity. (v) Since the disappearance of the substrate dye is monitored, variations in the nature of the products formed will probably not affect assay values. (vi) Unlike VA, Azure B is not oxidized by the nonperoxidase alcohol oxidases of *P. sajor-caju*. (vii) The strong preference of the enzyme for Azure B over VA means that the Azure B assay can be run even in the presence of VA, although some correction might be necessary.

At present, the only obvious shortcomings of the assay are the need to produce a standard curve and to avoid dye-

adsorbing particulates in the samples. More widespread use of this assay in various systems will be needed to determine what other flaws and advantages it possesses.

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