APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1993, p. 4010–4016 Vol. 59, No. 12
0099-2240/93/124010-07$02.00/0

Decolorization of Azo, Triphenyl Methane, Heterocyclic, and Polymeric Dyes by Lignin Peroxidase Isoenzymes from *Phanerochaete chrysosporium*

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Received 7 July 1993/Accepted 1 October 1993

The ligninolytic enzyme system of *Phanerochaete chrysosporium* decolorizes several recalcitrant dyes. Three isolated lignin peroxidase isoenzymes (LiP 4.65, LiP 4.15, and LiP 3.85) were compared as decolorizers with the crude enzyme system from the culture medium. LiP 4.65 (H2), LiP 4.15 (H7), and LiP 3.85 (H8) were purified by chromatofocusing, and their kinetic parameters were found to be similar. Ten different types of dyes, including azo, triphenyl methane, heterocyclic, and polymeric dyes, were treated by the crude enzyme preparation. Most of the dyes lost over 75% of their color; only Congo red, Poly R-478, and Poly T-128 were decolorized less than the others, 54, 46, and 48%, respectively. Five different dyes were tested for decolorization by the three purified isoenzymes. The ability of the isoenzymes to decolorize the dyes in the presence of veratryl alcohol was generally comparable to that of the crude enzyme preparation, suggesting that lignin peroxidase plays a major role in the decolorization and that manganese peroxidase is not required to start the degradation of these dyes. In the absence of veratryl alcohol, the decolorization activity of the isoenzymes was in most cases dramatically reduced. However, LiP 3.85 was still able to decolorize 20% of methylene blue and methyl orange and as much as 60% of toluidine blue O, suggesting that at least some dyes can function as substrates for isoenzyme LiP 3.85 but not to the same extent for LiP 4.15 or LiP 4.65. Thus, the isoenzymes have different specificities towards dyes as substrates.

The white rot fungus *Phanerochaete chrysosporium* produces several lignin peroxidase isoenzymes after depletion of an essential nutrient, usually carbon or nitrogen. Up to 15 veratryl alcohol-oxidizing species have been resolved by isoelectric focusing (24). Although at least some of these species obviously result from differential posttranslational modifications, several genes coding for different isoenzymes have been isolated and sequenced (1, 3, 5, 11, 23, 35, 40, 41). The reason for such a wide variety of slightly different isoenzymes is not fully understood. The isoenzyme genes have been reported to be differently regulated in response to nutrient starvation (22). It is also possible that, because of the very complex and heterogeneous structure of lignin, the organism is provided with isoenzymes having slightly different oxidation potential towards different lignin substructures. However, very little is known about the enzymological differences of the isoenzymes (16).

In addition to lignin, *P. chrysosporium* is also capable of oxidizing various recalcitrant xenobiotics released to the environment by human activity (6, 8, 12, 18–20). Among such compounds are many synthetic dyes. Various heterocyclic, azo, and triphenyl methane dyes used widely by, e.g., the textile and dyestuff industry are often resistant to biological wastewater treatment, and thus they are released into our aqueous environment.

The majority of the experiments on degradation of dyestuffs by *P. chrysosporium* has been carried out with either whole cultures, crude enzyme preparations containing most or all extracellular enzymes of the ligninolytic system of the fungus, or a single isolated lignin peroxidase isoenzyme (7, 10, 14, 27, 28, 30, 31, 37). We have here studied the possibility that different lignin peroxidase isoenzymes from *P. chrysosporium* may have different specificities and/or efficiencies toward dyes. We have isolated the three major lignin peroxidase isoenzymes from carbon-limited cultures of *P. chrysosporium* and studied decolorization of several dyes by these purified isoenzymes. The dyes belong to four structurally different groups: (i) triphenyl methane dyes, (ii) heterocyclic dyes, (iii) azo dyes, and (iv) polymeric dyes. The ability of the isoenzymes to decolorize the dyes was compared with that of the crude enzyme preparation obtained from the culture medium.

MATERIALS AND METHODS

Chemicals. The common names of all dyes have been used for convenience. The following names of these dyes are those recognized by the Chemical Abstracts Service (CAS): bromophenol blue, 4,4′-(3*H*-2,1-benzoxathiol-3-ylidene)bis [2,6-dibromophenol]-*S*,*S*-dioxide (E. Merck, Darmstadt, Germany); Congo red, 3,3′-{[1,1′-biphenyl]-4-4′-diylbis(azo)} bis[4-amino]-1-naphthalenesulfonic acid (disodium salt) (Sigma Chemical Co., St. Louis, Mo.); methylene blue, 3,7-bis(dimethylamino)-phenothiazin-5-ium chloride (E. Merck); methyl green, 4{[4-(dimethylamino)-phenyl][4-(dimethylimino)-2,5-cyclohexadien-1-ylidene]methyl}-*N*-ethyl-*N*,*N*-dimethylbenzenaminium bromide chloride (E. Merck); methyl orange, 4-{[(dimethylamino)phenyl]azo}benzenesulfonic acid (sodium salt) (E. Merck); Remazol brilliant blue R, 1-amino-9,10-dihydro-9,10-dioxo-4-[(3-{[2-(sulfooxy)ethyl]sulfonyl} phenyl)amino]-2-anthracenesulfonic acid (disodium salt) (Sigma Chemical Co.); toluidine blue O, 3-amino-7-(dimethylamino)-2-methylphenothiazin-5-ium chloride (Sigma Chemi-

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cal Co.); Poly R-478, CAS registry no. 68550-77-6 (Sigma Chemical Co.); Poly S-119, CAS registry no. 68550-82-3 (Sigma Chemical Co.); and Poly T-128, CAS registry no. 68550-83-4 (Sigma Chemical Co.).

Veratryl (3,4-dimethoxybenzyl) alcohol was purchased from Fluka Chemie AG, Buchs, Switzerland. All other chemicals were of analytical grade and used as such.

Microorganism and culture conditions. *P. chrysosporium* BKM-F-1767 (ATCC 24725) was obtained from Jakob Reiser (Institut für Biotechnologie, Zurich, Switzerland) and was maintained on 2% malt agar slants.

P. chrysosporium was grown immobilized at 37°C in the carbon-limited liquid culture medium previously described (15). For immobilization (25), 200 g of nylon web (Bear-Tex; Visella Oy, Valkeakoski, Finland) similar to Scotch Brite was used as a carrier. Nylon web cubes were washed three times with boiling water and sterilized in a fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) in 6 liters of the growth medium. After sterilization, vitamins and minerals were added and the medium was inoculated with 4×10^8 spores.

The enzyme production was activated when the glucose concentration had decreased from 2.0 to 0.2 g/liter by adding veratryl alcohol to a final concentration of 2.5 mM (25). At the same time, 200 ml of minerals (15) and Tween 80 were added to a final concentration of 0.05%, and the aeration was switched from air to pure oxygen. The culture fluid (crude lignin peroxidase) was harvested when the maximal lignin peroxidase activity was obtained. Very little manganese peroxidase activity has been reported to be present under these conditions (25).

Purification of lignin peroxidase. The extracellular fluid was separated from the mycelia by filtration through glass-wool and concentrated by using a Minitan concentrator (Millipore Corp., Bedford, Mass.) with a molecular exclusion limit of 10 kDa. The fluid was further concentrated by using Amicon centrifugal concentrators (Amicon Inc., Beverly, Mass.) having a molecular weight cutoff of 10 kDa.

Individual isoenzymes were isolated by preparative isoelectric focusing as previously described (16). Pharmalytes 2.5-5 and 4-6.5 (Pharmacia, Uppsala, Sweden) were used. About 25 mg of crude lignin peroxidase was loaded onto the gel, and the gel was run in a Multiphor II (Pharmacia) at 15°C for 5 to 7 h with 400 V of constant voltage. After focusing, gel slices containing the protein bands were cut out, and the protein was eluted with water. Eluates were freed from ampholytes by diafiltration and concentrated by using Amicon concentrators. The isoelectric points of the proteins were determined as previously described (16).

For kinetic assays, three major lignin peroxidase isoenzymes, LiP 4.65 (H2), LiP 4.15 (H7), and LiP 3.85 (H8) were isolated by chromatofocusing with Polybuffer exchanger 94 and Polybuffer 74 (Pharmacia). The column (0.7 by 27 cm) was equilibrated with 25 mM piperazine-HCl, and the sample was loaded and then eluted with the Polybuffer (pH gradient, 5.0 to 3.3). All steps were performed at 4°C.

Enzyme and protein assays. Lignin peroxidase activity was determined by the method of Tien and Kirk (38), and manganese peroxidase activity was determined as described previously (29). For kinetic measurements, 40 mM phosphate (pH 2.75) was used instead of tartrate buffer. Kinetic assays (0.18 to 0.34 μM lignin peroxidase depending on the isoenzyme) were performed with increasing concentrations of veratryl alcohol or H_2O_2 for the calculation of K_m and catalytic rate constant (k_{cat}) (9).

Protein concentrations were estimated by the method of Bradford (4) or by using bicinchoninic acid (36). Bovine serum albumin was used as a standard. The concentrations of the purified isoenzymes were also determined at 408 nm with the extinction coefficient of 133 mM^{-1} cm^{-1} (17). For lignin peroxidase an M_r of 40,000 was used for calculation.

Diafiltration of the crude lignin peroxidase. To remove the low-molecular-weight compounds from the crude lignin peroxidase the culture fluid was diafiltrated by using Millipore concentrators having a molecular weight cutoff of 10 kDa. The crude lignin peroxidase was first concentrated and then reconstituted to the original volume by adding 2 mM sodium tartrate (pH 4). The permeate from this first concentration was collected for further use. Concentration and reconstitution were repeated until a dilution factor of approximately 250 for the low-molecular-weight compounds was achieved.

Determination of veratryl alcohol. The veratryl alcohol concentration of the culture fluid was determined enzymatically and by using capillary electrophoresis. By the enzymatic method, different dilutions of pure veratryl alcohol were used. The slopes of the oxidation velocity of veratryl alcohol to veratraldehyde by the diafiltrated lignin peroxidase in the presence of H_2O_2 were measured. A double-reciprocal plot of 1/slope against 1/[VA], where VA is veratryl alcohol, was made and was used as a standard curve. The veratryl alcohol concentration in the culture fluid was determined by replacing veratryl alcohol with the permeate fraction from diafiltration.

Capillary electrophoretic experiments were carried out with an ISCO (Lincoln, Nebr.) model 3850 electropherograph equipped with an on-column UV detector connected to Hewlett-Packard (Avondale, Pa.) model 3393 A integrator. The dimensions of the fused silica capillary (Composite Metal Services Ltd., Hallow, Worchestershire, United Kingdom) were 75 μm (inner diameter) by 60 cm (total length), with a distance of 40 cm to the detector. Running conditions consisted of 17 kV of applied voltage with 20 mM sodium tetraborate (pH 9.7)–100 mM sodium dodecyl sulfate as the buffer. Vacuum injection was used for sample introduction, and the detection wavelength was 215 nm. Peak areas of known veratryl alcohol samples were measured and plotted against the veratryl alcohol concentrations. A sample of the permeate fraction from diafiltration was run in the same conditions.

Dye decolorization. Decolorization of dyes was monitored at the visible absorbance maximum of each dye. Unless otherwise stated in the text, the reaction mixture consisted of 10 to 80 μM dye (see Table 2), 0.1 U of lignin peroxidase, and 0.4 mM H_2O_2 in 50 mM sodium tartrate in a total volume of 1 ml. The reaction was initiated by the addition of H_2O_2, and absorbance was measured 15 min after initiation. The spectra of the dyes and decolorization ability of the crude lignin peroxidase (extracellular fluid) and the isolated isoenzymes were measured at five different pHs (2.5, 3.0, 3.5, 4.0, and 4.5). The molar extinction coefficient ($E^{1\ M}_{1\ cm}$) for each dye was measured at the absorbance maximum of each dye. For those dyes for which the visible absorption spectrum was affected by the pH, $E^{1\ M}_{1\ cm}$ was measured at each pH used. The molar extinction coefficient was used for calculation of the concentration of the dye in the reaction mixture after the decolorization reaction.

RESULTS

Three major lignin peroxidase isoenzymes were purified from the carbon limited cultures of *P. chrysosporium*. They differ from each other with respect to their N-terminal amino

TABLE 1. Kinetic parameters of purified lignin peroxidase isoenzymes

Isoenzyme	k_{cat} (s^{-1})	Veratryl alcohol		H_2O_2	
		K_m (μM)	k_{cat}/K_m ($M^{-1}s^{-1}$)	K_m (μM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
LiP 4.65	58.6	325	18×10^4	235	24.9×10^4
LiP 4.15	21.4	333	6.4×10^4	198	11×10^4
LiP 3.85	28.9	123	24×10^4	127	23×10^4

acid sequence and degree of glycosylation (unpublished results and reference 16). The three-dimensional crystal structure for lignin peroxidase has recently been reported (13, 32, 34).

To assess the purity of the isolated isoenzymes, protein concentrations were estimated both by bicinchoninic acid and by A_{408}, the latter being specific to heme proteins. Both methods gave equal results, indicating that there were no major impurities in the protein preparations. The specific activities of LiP 4.65, LiP 4.15, and LiP 3.85 were 26, 39, and 31 U mg^{-1}, respectively. Glumoff et al. (16) have reported that LiP 4.15 is especially sensitive to environmental factors. The difference in the specific activities (7.5 U mg^{-1} from the study by Glumoff et al. versus 39 U mg^{-1} in this study) is probably due to the lability of this particular isoenzyme. LiP 4.15 is also more sensitive to low pH than the other isoenzymes and particularly prone to peroxide-induced inhibition (16).

Table 1 shows the kinetic constants of the three isoenzymes. The values were determined by a two-substrate plotting method as described previously (9). The concentration of veratryl alcohol was varied in the presence of several different fixed concentrations of H_2O_2. The slopes of the primary plots of veratryl alcohol concentration over initial velocity, $[VA]/v_0$, against [VA] are equal to $1/V_{max}$(apparent), and they were used in a secondary replot of $[H_2O_2]/V_{max}$(apparent) against H_2O_2 concentration which gave a straight line of slope $1/V_{max}$ and intercept $K_m(H_2O_2)/V_{max}$ on the $[H_2O_2]/V_{max}$(apparent) axis. Major differences in K_m and k_{cat} were not observed. LiP 3.85 has the lowest K_m for veratryl alcohol and H_2O_2, but LiP 4.65 has the highest turnover. LiP 4.15, having the lowest k_{cat}/K_m, appears to be catalytically slightly less efficient than the other two isoenzymes. Comparison of the kinetic parameters for lignin peroxidase isoenzymes between different laboratories with enzymes purified by different methods is extremely difficult because of the intrinsic sensitivity of the assay for interference and variations in enzyme sensitivity to environmental factors (16, 39). Taken together, our results did not indicate major differences in the kinetic parameters between the three isoenzymes purified by chromatofocusing.

Dye decolorization by lignin peroxidase. It has been reported that lignin peroxidase isoenzymes vary with respect to their potential to oxidize other substrates besides veratryl alcohol (16). In this study we wanted to characterize the ability of the three isoenzymes to use different dye compounds as substrates. For decolorization of the dyes, isoenzymes were purified by preparative isoelectric focusing as described in Materials and Methods. Analytical isoelectric focusing of the purified proteins indicated that the isoenzymes were completely resolved (data not shown).

The dyes used in this study represent four distinct classes: (i) triphenylmethane dyes (bromophenol blue and methyl green), (ii) heterocyclic dyes (methylene blue and toluidine blue O), (iii) azo dyes (Congo red and methyl orange), and (iv) polymeric dyes (Poly R-478, Poly S-119, and Poly T-128). Remazol brilliant blue R is frequently used as a starting material for polymeric dyes. Table 2 shows the initial concentration and the absorbance maximum of each dye.

Decolorization of the dyes by crude lignin peroxidase and three purified isoenzymes from *P. chrysosporium* was examined at five different pHs. Table 2 also shows the pH at which decolorization of the dye proceeded furthest. For some dyes, the pH range for decolorization was very narrow. For example, decolorization of Poly S-119 by crude lignin peroxidase at pH 3.5 was only 10% compared with 79% at pH 4.0. All subsequent measurements were done at the optimum pH for decolorization of each dye. However, the rate of decolorization of the dyes was the highest at pH 2.5 to 3.0 despite the fact that for some dyes decolorization proceeded further at a higher pH. This is probably due to the instability of lignin peroxidase especially LiP 4.15, below pH 3.

In the presence of 2 mM veratryl alcohol, the crude lignin peroxidase was able to partially decolorize all 10 dyes (Fig. 1). Over 75% of most of the dyes was decolorized. The best decolorization (93%) was obtained for bromophenol blue.

TABLE 2. Conditions for dye decolorization by lignin peroxidase

Dye	Initial concn (μM)[a]	Absorbance maximum (nm)	Optimum pH for decolorization			
			Crude lignin peroxidase	LiP 4.65	LiP 4.15	LiP 3.85
Bromophenol blue	44.8	590, 434	4.0	NA[b]	NA	NA
Congo red	28.7	490	4.0	NA	NA	NA
Methylene blue	10.3	662	2.5	2.5–3.0	2.5–3.0	2.5–3.0
Methyl green	29.0	630	2.5–3.5	2.5–3.5	2.5–3.5	2.5–3.5
Methyl orange	21.3	502	2.5	2.5–3.0	2.5–3.0	2.5–3.0
RBBR[c]	79.8	590	3.5–4.0	NA	NA	NA
Toluidine blue O	16.3	625	2.5	2.5	3.0	2.5
Poly R-478	0.002%	519	3.5	NA	NA	NA
Poly S-119	0.002%	470	4.0	4.5	4.5	4.0
Poly T-128	0.002%	420	4.0	NA	NA	NA

[a] The concentrations of Poly R-478, Poly S-119, and Poly T-128 are expressed as percentages.
[b] NA, not assayed.
[c] RBBR, Remazol brilliant blue R.

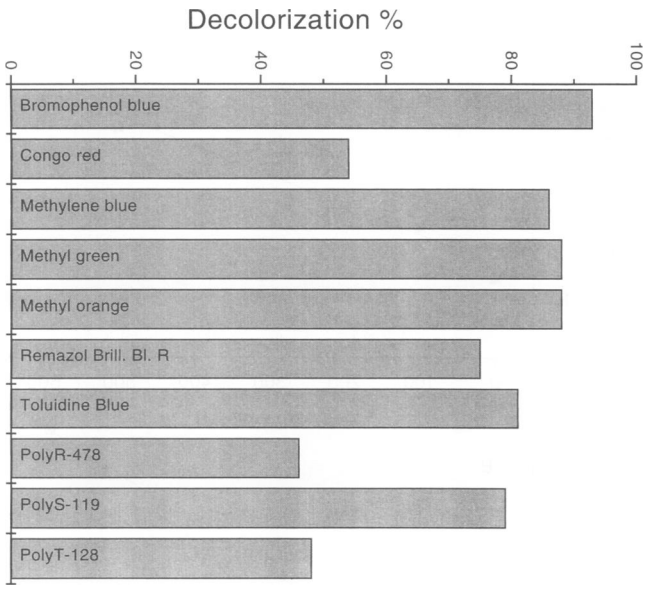

FIG. 1. Decolorization of 10 different dye compounds by crude lignin peroxidase in the presence of 2 mM veratryl alcohol after 15 min of incubation. Reactions were performed at the optimum pH for decolorization of each dye as shown in Table 2. Initial dye concentrations and monitored wavelengths are also shown in Table 2. The dye remaining after each reaction was calculated as described in Materials and Methods. Remazol Brill. Bl. R, Remazol brilliant blue R.

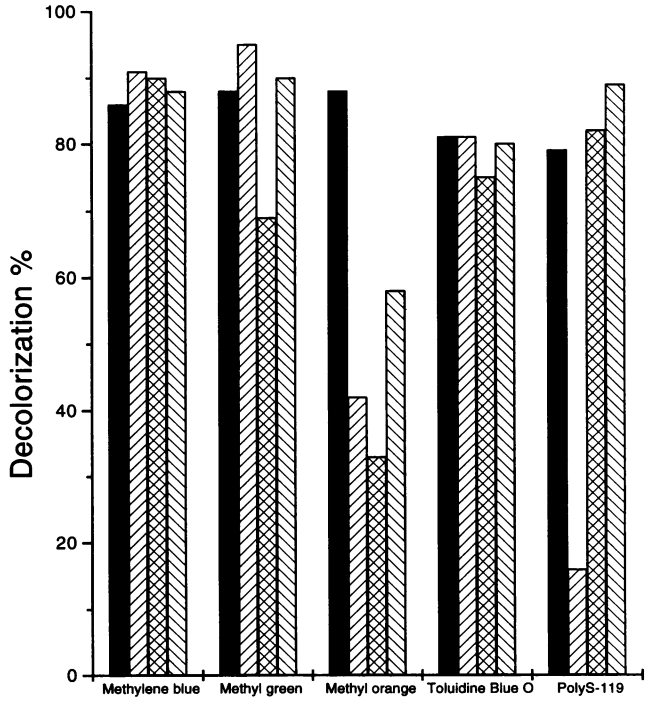

FIG. 2. Comparison of decolorization of five different dye compounds by crude lignin peroxidase and by the three purified lignin peroxidase isoenzymes in the presence of 2 mM veratryl alcohol. Assay conditions as described in the legend to Fig. 1. ■, crude lignin peroxidase; ▩, LiP 4.15; ▨, LiP 4.65; ▧, LiP 3.85.

Three dyes, Congo red, Poly R-478, and Poly T-128, were decolorized less than the others (54, 46, and 48%, respectively).

Five of the 10 dyes were then studied with isolated isoenzymes, LiP 4.65, LiP 4.15, and LiP 3.85, of lignin peroxidase. The dye decolorization in the presence of 2 mM veratryl alcohol with the isoenzymes was similar to that with the crude lignin peroxidase (Fig. 2). Only methyl orange was decolorized less efficiently by the isoenzymes than by the crude lignin peroxidase. Also, Poly S-119 was decolorized much less by LiP 4.65 than by LiP 4.15 and LiP 3.85.

To exclude the possibility that the decolorization of the dyes was due to a nonbiological oxidation, the dyes were incubated with 400 μM H_2O_2 in the absence of enzyme. None of the dyes showed any change in absorption after a 15-min incubation with H_2O_2.

The ability of the purified isoenzymes of lignin peroxidase to decolorize the dyes was decreased greatly when veratryl alcohol was omitted from the reaction mixture (Fig. 3). The decolorization ability of LiP 3.85 was the best. Only Poly S-119 was decolorized better by LiP 4.15 than by LiP 3.85.

The decolorization ability of the crude lignin peroxidase was not decreased when veratryl alcohol was omitted from the reaction mixture. To study the possibility that this was due to veratryl alcohol already present in the culture fluid, low-molecular-weight compounds were removed from the crude lignin peroxidase by diafiltration. The diafiltrated crude lignin peroxidase was used to decolorize methyl green. Because of the small amount of the diafiltrated crude lignin peroxidase needed in the reaction mixture, the low-molecular-weight compounds originally present in the culture fluid were diluted over 2,000-fold. Figure 4A shows that the ability of the diafiltrated crude lignin peroxidase to decolorize methyl green was decreased to the level of the purified isoenzymes (see Fig. 3). When the permeate of the diafiltrated culture fluid was added to the reaction mixture, the decolorization ability of the crude lignin peroxidase was restored proportionally (Fig. 4A). Pure veratryl alcohol was also added to the reaction mixture with the diafiltrated crude lignin peroxidase. The final concentrations of the added veratryl alcohol were 0.25, 0.5, 0.75, and 1.0 mM, and the rate of decolorization of the methyl green increased respectively. Figure 4B shows that in the presence of 1 mM veratryl alcohol the crude lignin peroxidase was able to decolorize methyl green almost completely.

The reconstitution experiment described above strongly suggested the presence of veratryl alcohol in the culture fluid. To confirm the identity of the electron donor as veratryl alcohol and to measure its concentration, the two independent methods described in Materials and Methods were used. Figure 5 shows the capillary electropherograms of pure veratryl alcohol and of the permeate of the diafiltrated crude lignin peroxidase. The peaks eluted reproducibly exactly at the same positions, indicating that the compound present in the culture fluid is veratryl alcohol. The concentration calculated from the peak areas was 150 μM. The same concentration was obtained by measuring veratraldehyde production at 310 nm from the permeate of the diafiltrated crude lignin peroxidase.

DISCUSSION

In this study we examined decolorization of several dyes by crude lignin peroxidase and three purified lignin peroxidase isoenzymes. While the kinetic parameters of the puri-

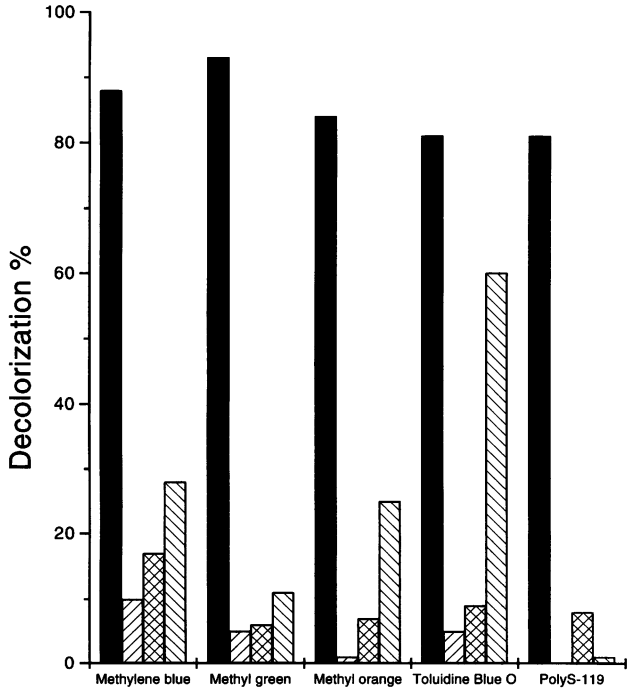

FIG. 3. Decolorization of dyes without added veratryl alcohol by crude lignin peroxidase and by the three purified lignin peroxidase isoenzymes. Assay conditions are described in the legend to Fig. 1, except that veratryl alcohol was omitted from the reaction mixture. Symbols are described in the legend to Fig. 2.

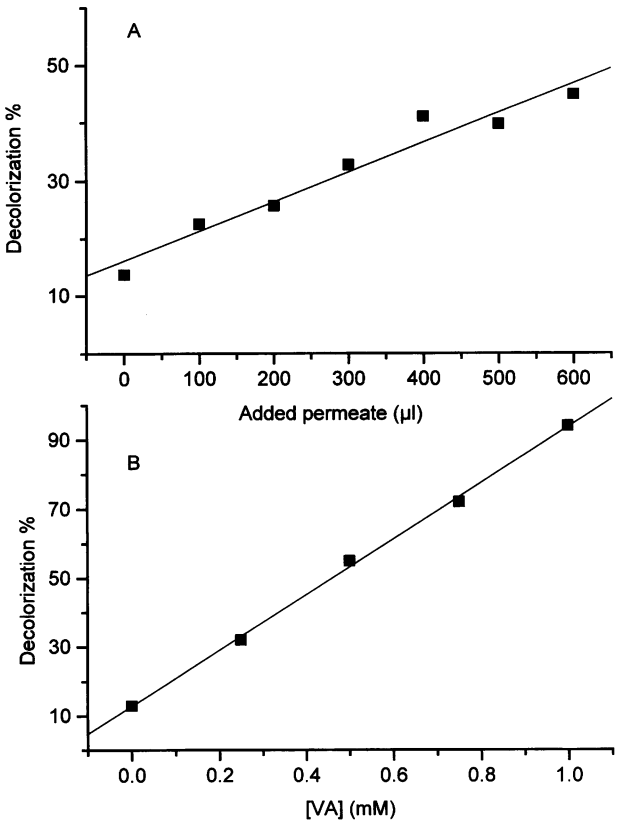

FIG. 4. Decolorization of methyl green by diafiltrated crude lignin peroxidase. The culture fluid freed from mycelia was fractionated by diafiltration as described in Materials and Methods. Diafiltrated crude lignin peroxidase (8 µl) was used as the source of the enzyme, and the indicated amounts of the permeate (A) or pure veratryl alcohol (B) were added to the reaction mixture.

fied enzymes for oxidation of veratryl alcohol to veratraldehyde were similar, some differences were observed for decolorization of the dye compounds.

The decolorization ability of the purified isoenzymes was greatly decreased when veratryl alcohol was not present in the reaction mixtures, suggesting that veratryl alcohol acts as a mediator in the reaction (21). On the other hand, it has been suggested that the azo dyes Biebrich Scarlet and Tetrazine can act as substrates for compound I of purified lignin peroxidase but not for compound II and that, when present, veratryl alcohol is able to reduce compound II to the native state (28). However, we found that LiP 3.85 was able to decolorize over 20% of methylene blue (a heterocyclic dye) and methyl orange (an azo dye) and up to 60% toluidine blue O (a heterocyclic dye) when veratryl alcohol was not present. Methylene blue was also decolorized to some extent by LiP 4.65 and LiP 4.15. This suggests that some dyes may act as substrates for lignin peroxidase compound II, while others are unable to do so, and also that the lignin peroxidase isoenzymes have different specificities for dyes as substrates.

In contrast, omission of veratryl alcohol from the reaction mixture had almost no effect on the ability of the crude lignin peroxidase to decolorize the dyes. This is probably due to the fact that the fungus itself synthesizes veratryl alcohol as a product of secondary metabolism (26). Veratryl alcohol was also added in the culture medium to a final concentration of 2.5 mM when the enzyme production had been activated and some of it had actually been carried over into the crude lignin peroxidase preparation. This was indicated by two lines of evidence. First, the increase in A_{310} in the lignin peroxidase assay with the permeate from diafiltration added instead of veratryl alcohol suggested that veratraldehyde was generated in the reaction. Second, the peaks in capillary electrophoresis of both pure veratryl alcohol and of the enzyme-free culture fluid eluted exactly at the same position. Moreover, quantitation with both methods gave similar results. The effect of other enzymes present in the crude enzyme on decolorization cannot be excluded, but the effect of manganese peroxidase should be minor because the assay conditions for lignin peroxidase do not favor the enzymatic activity of manganese peroxidase (29, 38).

Cripps et al. (10) did not observe decolorization of Congo red at pH 4.5 with crude lignin peroxidase from a nitrogen-limited culture of *P. chrysosporium*. In contrast, we show here that crude lignin peroxidase from a carbon-limited culture is able to decolorize up to 54% of Congo red in 15 min at pH 4.0. We have also observed that omission of veratryl alcohol from the reaction mixture does not significantly decrease the decolorization of Congo red (data not shown). This apparent contradiction is probably due to the fact that the crude lignin peroxidase used by Cripps et al. was dialyzed and there was little or no veratryl alcohol left. Our crude lignin peroxidase was untreated culture fluid, and it did contain veratryl alcohol, which is, as we have shown, important for the decolorization ability of lignin peroxidase. However, we have found that even purified isoenzymes are able to slightly decolorize Congo red without veratryl alco-

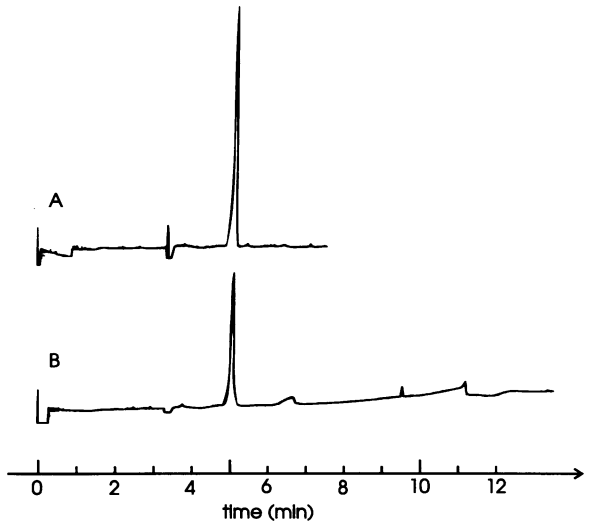

FIG. 5. Capillary electropherograms of 50 μM veratryl alcohol (A) and a sample of permeate of diafiltrated crude lignin peroxidase diluted 1:5 (B).

hol (unpublished results). Our results for the decolorization of methyl orange (Tropaeolin O) with isolated isoenzymes are in accordance with those of Cripps et al. (10).

Decolorization of Poly-B, a polymeric dye and azure B, a heterocyclic dye, have been utilized in lignin peroxidase detection and assay, respectively (2, 33). Archibald (2) showed that azure B can be used as a substrate in a lignin peroxidase assay that has significant advantages over the conventional veratryl alcohol assay. However, different lignin peroxidase isoenzymes were not tested, but rather isoenzyme mixtures from different sources were used. Toluidine blue O is structurally very close to azure B. We found that the presence of veratryl alcohol significantly enhances decolorization of toluidine blue O for all isoenzymes tested and that only LiP 3.85 was capable of decolorizing toluidine blue O significantly in the absence of veratryl alcohol.

ACKNOWLEDGMENTS

This research was supported by the Maj and Tor Nessling Foundation.

We thank Jukka Heinonen for critically reading the manuscript.

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