APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1985, p. 1040–1045
0099-2240/85/051040-06$02.00/0

Vol. 49, No. 5

Transformation of Halogen-, Alkyl-, and Alkoxy-Substituted Anilines by a Laccase of *Trametes versicolor*†

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Received 26 November 1984/Accepted 29 January 1985

The laccase of the fungus *Trametes versicolor* was able to polymerize various halogen-, alkyl-, and alkoxy-substituted anilines, showing substrate specificity similar to that of horseradish peroxidase, whereas the laccase of *Rhizoctonia praticola* was active only with *p*-methoxyaniline. The substrate specificities of the enzymes were determined by using gas chromatography to measure the decrease in substrate concentration during incubation. With *p*-chloroaniline as the substrate, the peroxidase and the *Trametes* laccase showed maximum activity near pH 4.2. The transformation of this substrate gave rise to a number of oligomers, ranging from dimers to pentamers, as determined by mass spectrometry. The product profiles obtained by high-pressure liquid chromatography were similar for the two enzymes. A chemical reaction was observed between *p*-chloroaniline and an enzymatically formed dimer, resulting in the formation of a trimer. All three enzymes oxidized *p*-methoxyaniline to 2-amino-5-*p*-anisidinobenzoquinone di-*p*-methoxyphenylimine, but only the *T. versicolor* laccase and the peroxidase caused the formation of a pentamer (2,5-di-*p*-anisidinobenzoquinone di-*p*-methoxyphenylimine). Our results demonstrate that in addition to horseradish peroxidase, a *T. versicolor* laccase can also polymerize aniline derivatives.

Substituted anilines can enter the environment in different ways. They are formed from the microbial metabolism of numerous pesticides, such as phenylurea, phenylcarbamate, and acylanilide herbicides (11, 12, 14, 16) and nitroaniline fungicides (24). They are also used industrially in the manufacture of dyes and may be released as pollutants in waste material.

The activity of peroxidase on anilines was studied extensively by Saunders et al. (21), who used horseradishes and turnips as enzyme sources. Bartha and Bordeleau (1) developed a method to quantify peroxidase activity in soil by using a dimethoxylated aniline (*o*-dianisidine) as the substrate. Differences in the substrate specificities of peroxidases from different sources were reported by Lieb and Still (19). Peroxidases from barnyard grass (*Echinochloa crusgalli*) and rice plants showed greater substrate specificity on chlorinated anilines than horseradish peroxidase. A peroxidase released by the soil fungus *Geotrichum candidum* exhibited a wide range of activity when it was tested with 43 different substituted anilines (9).

Laccase activity is generally associated with the oxidation of phenolic substances rather than aromatic amines (8, 10). One exception to this is an amine containing two amino groups, *p*-phenylenediamine, which frequently has been used to assess laccase activity and to distinguish this enzyme from other polyphenoloxidases (18). Sjoblad and Bollag (22) obtained negative results with an extracellular laccase isolated from the soil fungus *Rhizoctonia praticola* for the transformation of substituted anilines (*o*-, *m*-, and *p*-chloroaniline, *o*-, *m*-, and *p*-bromoaniline, and 2,4- and 3,4-dichloroaniline); only *p*-methoxyaniline was oxidized. Subsequently, these results were confirmed for *p*-chloroaniline and 3,4-dichloroaniline, and inactivity of the enzyme on 2,6-diethylaniline was also observed (7).

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† Pennsylvania Agricultural Experiment Station Journal Series no. 7066.

However, inability to transform chlorinated and alkylated anilines does not seem to be inherent to laccases as a class of enzymes. The present study revealed that a laccase purified from the growth medium of the white rot fungus *Trametes versicolor* showed activity on a wide range of substituted anilines. We also compared laccase activity with the activity of a horseradish peroxidase.

MATERIALS AND METHODS

Enzymes and enzyme assays. The extracellular laccases of *T. versicolor* and *R. praticola* were isolated from growth media and purified to similar degrees by DEAE-cellulose column chromatography as previously described (8, 17). Laccase activity was determined spectrometrically with a model 2000 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.) by using 2,6-dimethoxyphenol as the substrate (8, 17) and is expressed below in DMP units; 1 DMP unit was defined as the amount of enzyme that caused a change in absorbance of 1.0 U/min at 468 nm at the pH optimum of each laccase (pH 3.8 and 6.8 for the laccases from *T. versicolor* and *R. praticola*, respectively) (17). Assays were run at 24°C in 3.5-ml portions of a standard mixture containing 3.24 μM 2,6-dimethoxy-phenol in citrate-phosphate buffer prepared by the method of McIlvaine (20).

Horseradish peroxidase with an RZ (Reinheitszahl) of 0.3 and an activity of 40 U/mg of solid was purchased from Sigma Chemical Co., St. Louis, Mo. Peroxidase activity is expressed in purpurogallin units, as defined by Sigma Chemical Co.

The substrates were incubated at a concentration of 1 mM in citrate-phosphate buffer with the specified amount of enzyme. Unless otherwise stated, the assay pH for the *T. versicolor* laccase and horseradish peroxidase was 4.2, and the assay pH for the *R. praticola* laccase was 5.4. When peroxidase was used, the assay mixture contained hydrogen peroxide at a concentration of 5.0 μmol/ml. After specified time intervals the pH of the assay mixture was adjusted to pH 8.0 with 1 N sodium hydroxide and extracted twice with

an equal volume of methylene chloride. A volume of 10 ml was used in each assay to determine enzyme activity; for the isolation of products a volume of 150 ml was used. Incubation took place at 28°C in a water bath shaker at 50 cycles per min unless otherwise stated. All enzyme assays were performed in triplicate, and boiled enzyme preparations served as controls.

TLC. For thin-layer chromatography (TLC), the methylene chloride extracts were evaporated to small volumes and applied to precoated Silica Gel 60 F-254 plates with a layer thickness of 0.25 mm (Merck, Cincinnati, Ohio). For separation of *p*-chloroaniline and methoxyaniline derivatives, the plates were developed in solutions containing hexane and ethyl acetate at ratios of 80:20 (vol/vol) and 60:40 (vol/vol), respectively. Enzyme products were isolated from 0.5-mm layers by extraction with ethyl acetate. The extracts were then passed through a sodium sulfate (anhydrous) column (0.55 by 40 mm) and subjected to liquid chromatography or mass spectrometry.

Gas chromatography. Gas chromatographic analyses were performed with a model 7424 gas chromatograph (Packard Instrument Co., Inc., Rockville, Md.) equipped with a flame ionization detector. A glass column (1.8 m by 2.0 mm [inside diameter]) packed with 10% SP-2100 on 80/100 Supelcoport (Supelco, Inc., Bellefonte, Pa.) was used. The N_2 carrier gas flow rate was 40 ml/min. The injection port and detector temperatures were 240 and 230°C, respectively. The column temperature was adjusted according to the volatility of the compounds and ranged from 100 to 150°C.

For quantitation, 2 μl of a methylene chloride extract was injected, and the peak area was measured with a Hewlett Packard model 3390A integrator, by using the external standard method. The linearity of the calibration curve was established for each of 16 compounds in the range from 0 to 1.25 μmol/ml.

HPLC. High-pressure liquid chromatography (HPLC) was performed with a liquid chromatograph (Waters Associates, Milford, Mass.) equipped with two model 6000A solvent delivery systems and a model U6K injector. Sample detection was based on UV absorption at 300 nm, as measured with a model 480LC spectrophotometer which was connected to a model 730 data module and a model 720 system controller. A normal phase Radial-Pak B cartridge (10 μm) with an RCSS Silica Guard-Pak precolumn (Waters Associates) was used in a model RCM-100 radial compression module. The samples were eluted with hexane-ethyl acetate at a flow rate of 1.5 ml/min; the concentration of hexane was changed linearly from 81 to 75% in 10 min.

The 10-ml reaction mixture of each enzyme assay was extracted twice with 8 ml of methylene chloride. After evaporation of the extract to a volume of 1.0 ml in a gentle nitrogen stream, the sample was passed through a Sep-Pak Florisil cartridge (Waters Associates) equilibrated with ethyl acetate. The sample was then eluted twice with 2 ml of ethyl acetate, and the eluate was evaporated to a volume of 2 ml. Peak identification was based on comparison with pure products isolated by TLC and analyzed by HPLC.

Mass spectrometry. The molecular weights of metabolites were determined by electron impact mass spectrometric analysis (70 eV) with an AEI model MS-902 or Kratos model MS-g/50 mass spectrometer. Samples were introduced by using a direct insertion probe at temperatures varying from 250 to 300°C.

Chemicals. *o*-, *m*-, and *p*-chloroaniline, 2,3-, 2,4-, and 3,4-dichloroaniline, *m*-methylaniline, 2,5- and 2,6-dimethylaniline, 2,6-diethylaniline, 4-chloro-2-methylaniline, and 2,6-dimethoxyphenol were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. *m*-Methoxyaniline and *o*- and *p*-methylaniline were obtained from Sigma Chemical Co. *o*- and *p*-methoxyaniline were purchased from J. T. Baker Chemical Co., Phillipsburg, N.J., and 2-ethyl-6-methylaniline was purchased from Fluka Chemical Co., Hauppauge, N.Y. *o*-, *m*-, and *p*-bromoaniline and 2,5-dichloroaniline were obtained from Eastman Kodak Co., Rochester, N.Y.

RESULTS

Our results confirmed the observations of Sjoblad and Bollag (22) and Bollag et al. (5) concerning the substrate specificity for substituted anilines of the laccase from *R. praticola*. We showed that this laccase does not cause transformation of halogenated and alkylated anilines; only *p*-methoxyaniline was oxidized by the *R. praticola* laccase.

However, when a *T. versicolor* laccase was incubated with different substituted anilines, the formation of intense colors indicated transformation. Therefore, we wanted to know more about the optimal conditions and substrate specificity of this laccase. We compared the *T. versicolor* laccase results with those obtained with horseradish peroxidase, which is known to be active on substituted anilines.

Influence of pH on the activities of *T. versicolor* and *R. praticola* laccases and peroxidase on substituted anilines. The pH-dependent activities of the *T. versicolor* laccase and the peroxidase were studied by using *p*-chloroaniline as the substrate. The decrease in substrate concentration was followed by gas chromatography. Figure 1 shows that both enzymes had their highest activity in the acid region, with pH optima close to pH 4.2. A comparison of different buffer solutions showed that the *T. versicolor* laccase usually had higher activity in citrate-phosphate buffer than in 0.1 M acetate or 0.1 M phosphate buffer. Therefore, all of our experiments were performed with citrate-phosphate buffer, except when a pH of 7.6 was required to determine the activity of the *R. praticola* laccase. In this case phosphate buffer was used.

Since the *R. praticola* laccase does not cause transformation of *p*-chloroaniline, the pH-dependent activity of this enzyme was determined by using *p*-methoxyaniline as the substrate. Figure 1 shows that the maximum activity of the enzyme was at pH 5.4, which was higher than the pH optimum for the *T. versicolor* laccase and the peroxidase.

The pH optima reported above were used in assays for quantitative determination of substrate specificity. However, it must be emphasized that each compound may be oxidized at a different pH value for maximum transformation.

Substrate specificity. The first indication of oxidative coupling of substituted anilines by the *T. versicolor* laccase was the formation of color when the enzyme was added to a 1 mM substrate solution. Similar colors for the reacting anilines were observed in assays with peroxidase.

The substrate specificities of the *T. versicolor* and *R. praticola* laccases and the peroxidase were determined and quantitatively evaluated by gas chromatography. As shown in Table 1, the activities of the *T. versicolor* laccase and the peroxidase were higher with methylated and methoxylated anilines than with halogenated anilines. It is particularly interesting that of the 16 substrates used, only *p*-methoxyaniline was transformed when it was incubated with the *R. praticola* laccase.

In addition to the substrates listed in Table 1, the *T. versicolor* laccase and the peroxidase were assayed with *o*-, *m*- and *p*-bromoaniline and 2,4- and 2,5-dichloroaniline. The

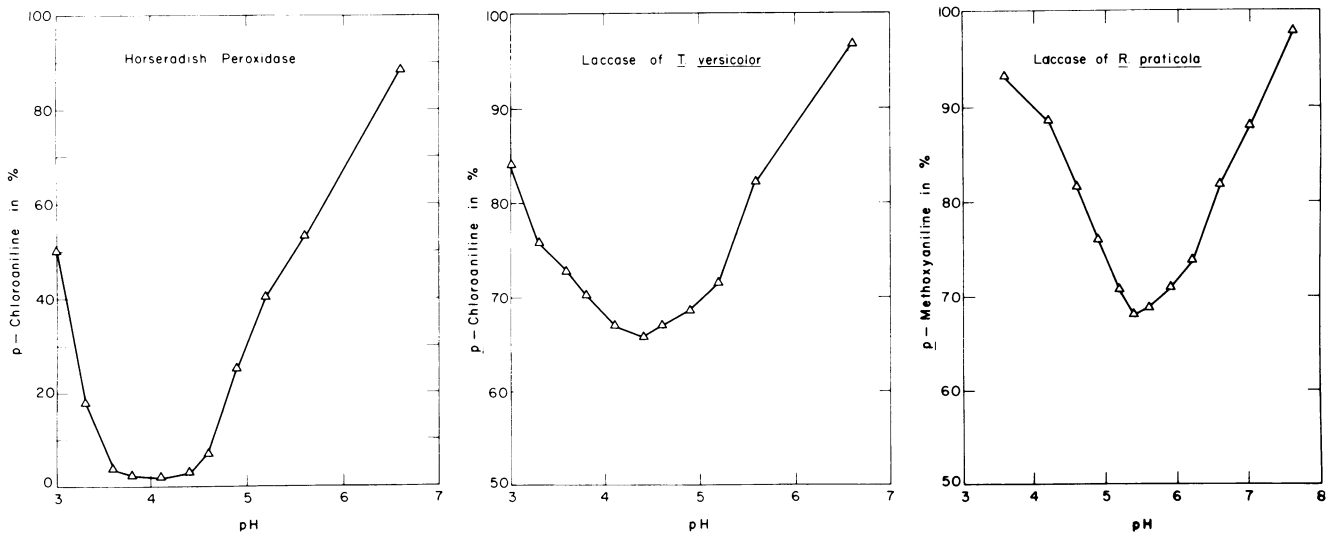

FIG. 1. Effect of pH on the activity of the *T. versicolor* laccase (2.5 DMP units per ml, 20°C) and the peroxidase (0.25 purpurogallin unit per ml, 20°C) on *p*-chloroaniline and of the *R. praticola* laccase (0.3 DMP unit per ml, 35°C) on *p*-methoxyaniline. Incubation times were 20 h for the two laccases and 20 min for the peroxidase.

bromo-substituted anilines and 2,4-dichloroaniline were also transformed, as indicated by a color change. Only 2,3-dichloroaniline and 2,5-dichloroaniline (not determined by gas chromatography) were not oxidized by the enzymes.

Oxidative oligomerization of *p*-methoxyaniline. When *p*-methoxyaniline was incubated with the *T. versicolor* or *R. praticola* laccase or with peroxidase, a number of oxidative coupling products were formed. Upon TLC analysis, all of the spots were distinguishable by their colors, with the exception of a spot at an R_f value of 0.38, which was detected under UV light and represented the unchanged substrate. All three enzymes caused the formation of high levels of compound R1 (R_f value, 0.53). This compound was green on thin-layer plates, but became dark red after extraction from the silica gel. Mass spectral analysis gave an *m/z* of 456. A comparison with the results of Daniels and Saunders (13) and Sjoblad and Bollag (22) indicated that compound R1 was the saturated form of 2-amino-5-*p*-anisidinobenzoquinone di-*p*-methoxyphenylimine (*m/z* 454).

A red spot with an R_f value of 0.59 was observed among the reaction products from an assay with *T. versicolor* laccase and the peroxidase. TLC analysis in hexane-ethyl acetate (80:20, vol/vol) resolved two components having R_f values of 0.19 and 0.21 (compound R2). A mass of 562 was determined for compound R2. This substance is thought to be the saturated form of 2,5-di-*p*-anisidinobenzoquinone di-*p*-methoxyphenylimine, a pentamer identified among the reaction products of *p*-methoxyaniline and peroxidase by Daniels and Saunders (13).

Although the *R. praticola* laccase was incubated with *p*-methoxyaniline under the same conditions as those used with the *T. versicolor* laccase, compound R2 was not formed. Incubation for a prolonged time at the optimal pH (pH 5.4) and with a 10-fold increase in enzyme units also did not lead to the formation of compound R2.

Additional spots with weak light yellow to orange colors were detected on the thin-layer plates, but since they were very unstable, no further characterization was attempted.

Oxidative oligomerization of *p*-chloroaniline. When incubated with the *T. versicolor* laccase or with the peroxidase, *p*-chloroaniline was oxidatively coupled. HPLC profiles of the reaction products at 50% substrate disappearance are shown in Fig. 2. They exhibited similar patterns, with small differences in the relative amounts of products formed.

The product characteristics as determined by HPLC, TLC, and mass spectrometry are given in Table 2. The peak

TABLE 1. Substrate specificities of different enzymes for substituted anilines[a]

Aniline substrate	% Substrate disappearance with:		
	T. versicolor laccase	*R. praticola* laccase	Horseradish peroxidase
o-Methoxy-	85	0	10
m-Methoxy-	37	0	0
p-Methoxy-	100	42	75
o-Methyl-	45	0	10
m-Methyl-	28	0	2
p-Methyl-	71	0	14
o-Chloro-	0	0	3
m-Chloro-	0	0	0
p-Chloro-	35	0	29
2,3-Dichloro-	0	0	0
3,4-Dichloro-	10	0	1
2,5-Dimethyl-	63	0	11
2,6-Dimethyl-	89	0	30
2,6-Diethyl-	100	0	14
2-Ethyl-6-methyl-	97	0	47
4-Chloro-2-methyl-	82	0	74

[a] Substrate (1 mM) in citrate-phosphate buffer at pH 4.2 was used for the *T. versicolor* laccase and the peroxidase; a pH value of 5.4 was used for the *R. praticola* laccase. Mixtures containing 5 DMP units of *Trametes* laccase per ml or 0.02 purpurogallin unit of peroxidase per ml were incubated for 6 h; mixtures containing DMP unit of *Rhizoctonia* laccase per ml were incubated for 10 h. The peroxidase reaction mixtures contained 5 μmol of H_2O_2 per ml. Assay mixtures were incubated at 28°C.

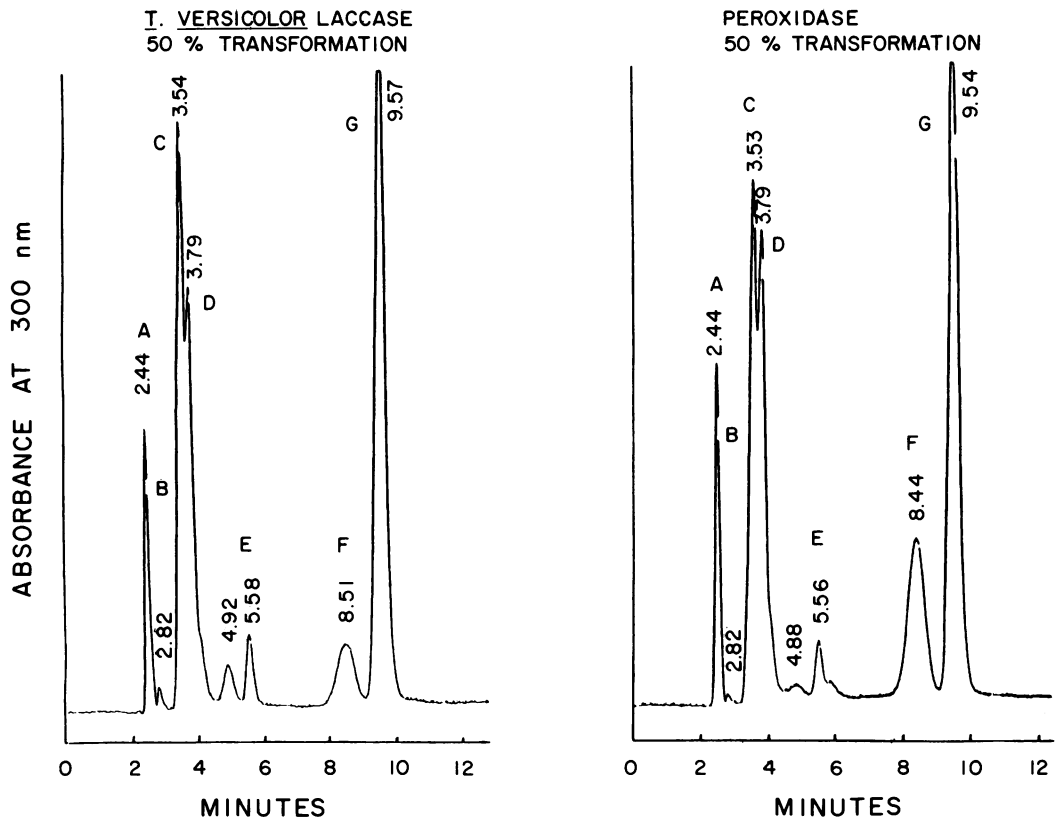

FIG. 2. HPLC chromatograms of products extracted with methylene chloride from enzyme assays with *p*-chloroaniline and *T. versicolor* laccase (15 DMP units per ml; incubation for 1.5 h) or peroxidase (0.04 purpurogallin unit per ml; incubation for 0.5 h) at 50% substrate transformation. A pH value of 4.2 was used.

occurring at a retention time of 4.9 min in the HPLC analysis could not be assigned to a product isolated by TLC and was not further characterized. The largest oligomer determined was compound B, a pentamer with a retention time of 2.82 min. The peak ratios of the mass spectrogram indicated that dechlorination had taken place, as the mass spectrogram showed a four-chlorine pattern for the ^{35}Cl isotope. Dechlorination was also determined to have occurred in compounds C, E, and F (a tetramer, dimer, and trimer, respectively; retention times, 3.54, 5.56, and 8.50 min, respectively). The tetramer, compound C, a red solid that was brownish green on thin-layer plates, was formed in high yields, as indicated by TLC. Both the tetramer and the pentamer were previously reported to have been formed in the reaction of *p*-chloroaniline with horseradish peroxidase by Holland and Saunders (15). Our molecular weight values of 468 and 578 for the tetramer and pentamer, respectively, differed from the values obtained by these authors (466 and 576, respectively). A two-unit difference was also observed with compound D, a trimer with a retention time of 3.79 min and molecular weight of 377. As previously suggested for *p*-methoxyaniline products, this difference is thought to be due to saturation of two nitrogen atoms of a benzoquinone structure, as proposed by Holland and Saunders (15) and Daniels and Saunders (13), which probably occurred during mass spectrometric analysis.

Compound A (*m/z* 250) and compound E (*m/z* 218) were previously described by Holland and Saunders (15). Both of these products were yellow solids, and the mass spectrum indicated that compound A was an azo compound and

TABLE 2. Oligomeric products formed during the incubation of *p*-chloroaniline with a *T. versicolor* laccase or peroxidase

Compound	Retention time in HPLC (min)	R_f in TLC	Color of spot on TLC Plate	Molecular ion (*m/z*)	State of oligomerization
A	2.44	0.85	Yellow	250	Dimer
B	2.82	0.73	Yellow	578	Pentamer (−Cl)
C	3.54	0.49	Brownish green	468	Tetramer (−Cl)
D	3.79	0.46	Orange	377	Trimer
E	5.56	0.34	Yellow	218	Dimer (−Cl)
F	8.50	0.20	Purple	343	Trimer (−Cl)
G (*p*-chloroaniline)	9.54	0.16	Detectable in UV light	127	Monomer

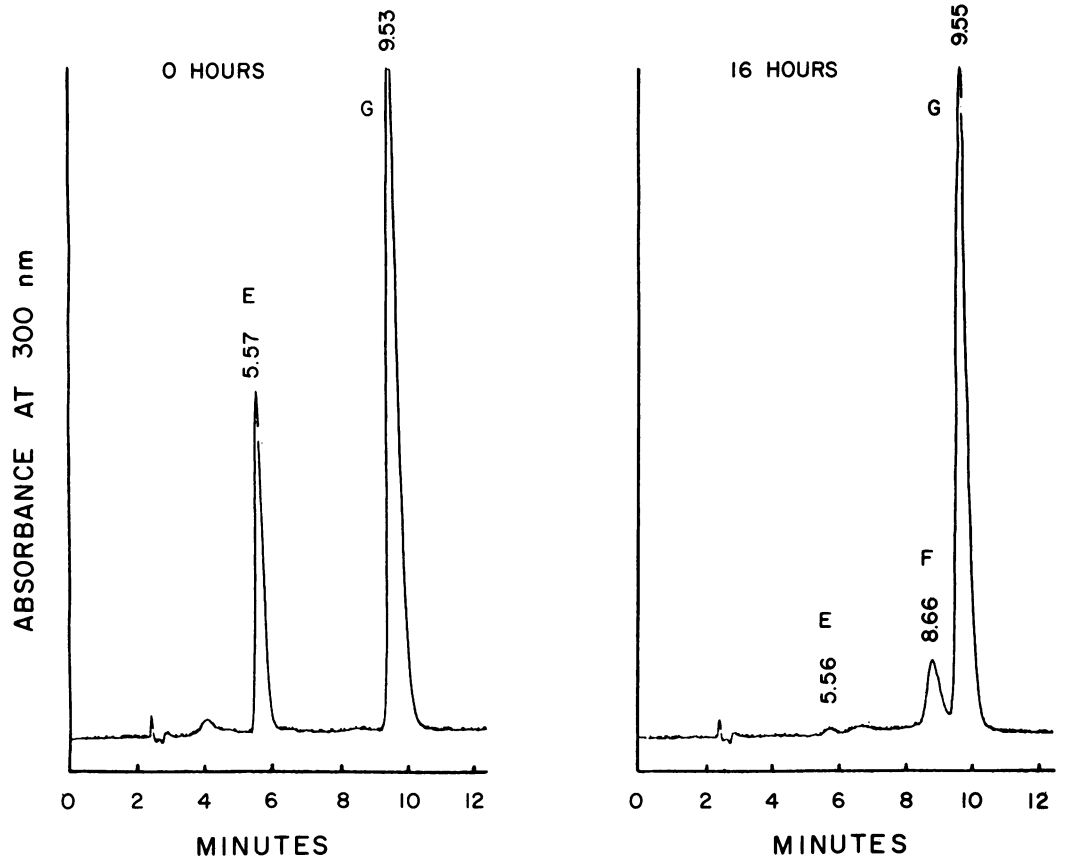

FIG. 3. HPLC chromatograms of a methylene chloride extract of a reaction mixture containing an intermediate dimer (retention time, 5.57 min) and *p*-chloroaniline (retention time, 9.54 min) without enzyme after 0 and 16 h of incubation.

compound E was a dehalogenated dimer. Compound E was present in higher concentrations at an earlier stage of the reaction catalyzed by peroxidase. This indicates that the dimer is a precursor for higher polymers. Holland and Saunders (15) confirmed this assumption when they added this dimer to a peroxidase assay mixture in which *p*-chloroaniline was the substrate. They found an increase in the yield of the tetramer (*m/z* 466) corresponding to the amount of dimer added.

To investigate whether such polymerization could also occur non-enzymatically, we incubated purified compound E in a 1 mM *p*-chloroaniline solution in citrate-phosphate buffer (pH 4.2) at 28°C for 16 h in the dark. A chromatographic profile of the reaction mixture showed the disappearance of compound E and the appearance of a new peak at a retention time of 8.66 min (Fig. 3). This new peak was compound F, a purple solid with a mass of 343, corresponding to a trimeric structure; the loss of one chlorine atom was indicated by the ^{35}Cl isotope pattern. A suggested scheme for the nonenzymatic reaction of compound E with *p*-chloroaniline is shown in Fig. 4.

DISCUSSION

Extracellular laccases of microorganisms are believed to cause detoxication by catalyzing the oxidative coupling of toxic phenolic substances that are produced during the normal metabolism of various organisms (4). Laccase activity on phenol derivatives has been the topic of numerous studies (10, 18, 23), and the possible importance of this enzyme for incorporation of pesticide intermediates into soil humus has been discussed previously (6, 7).

In this paper we describe a laccase which shows activity toward an entirely different group of substrates, namely, substituted anilines. This enzyme, which was isolated from the growth medium of the fungus *T. versicolor*, caused the transformation of numerous halogenated and alkylated anilines with a substrate specificity similar to that determined

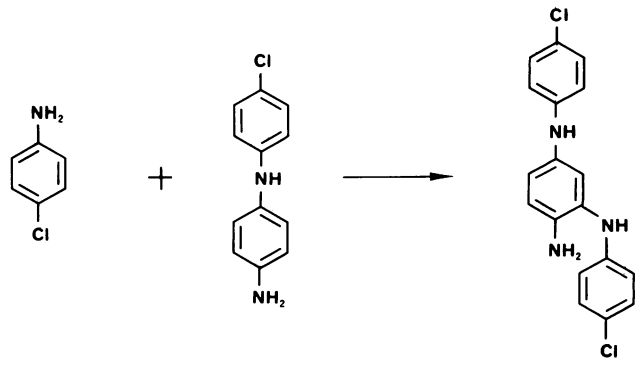

FIG. 4. Proposed pathway for the nonenzymatic coupling of the intermediate dimer (*m/z* 218), compound E, with *p*-chloroaniline to yield the trimeric compound (*m/z* 343), compound F.

for peroxidase. Neither enzyme caused the formation of color when it was incubated with 2,3- or 2,5-dichloroaniline, and gas chromatographic analysis showed that 2,3-dichloroaniline was not transformed by the enzymes. The inactivity of horseradish peroxidase on 2,5-dichloroaniline was reported previously by Bartha et al. (2). Although these authors reported that 2,3-dichloroaniline was transformed by the enzyme, the reaction rate was not determined. A peroxidase from the soil fungus *G. candidum* has been shown by Bordeleau and Bartha (9) to cause transformation of both substrates; however, its reactivity was low. Substrates on which laccase exhibited high activity were also transformed at high rates by the peroxidase, as in the case of the monosubstituted and dialkylated anilines (Table 1).

The observation that the *R. praticola* laccase does not react with halogenated and alkylated anilines, whereas the *T. versicolor* laccase does, casts doubt on the assumption that laccases are a homogenous group of enzymes. Differences in substrate specificity on phenols have been reported previously for laccases from different sources (3, 18). Indeed, Levine reached the conclusion "that laccase is not a single entity but rather represents a group of closely related enzymes" (18). This conclusion is consistent with our results.

An important difference between the laccases of *R. praticola* and *T. versicolor* is the range of pH values at which maximum activity is observed. Whereas the *R. praticola* laccase has been reported to have pH optima for numerous phenolic compounds in the neutral region, the pH optima for the *T. versicolor* laccase have been determined to be acidic (5, 17). When we performed assays with aniline substrates, we obtained similar results. *p*-Chloroaniline was transformed at the highest rate by the *T. versicolor* laccase at pH 4.4. The only substituted aniline to be acted on by the laccase of *R. praticola*, *p*-methoxyaniline, had its highest transformation rate at pH 5.4. The pH optimum determined for horseradish peroxidase on *p*-chloroaniline (pH 4.1) is close to the value obtained for the *T. versicolor* laccase (pH 4.4).

Nonenzymatic coupling was established by an experiment showing the reaction of an intermediate dimer formed by the enzymes with *p*-chloroaniline. The coupling yielded a trimeric compound that was also observed among the products of enzyme assays performed with *p*-chloroaniline.

Our study revealed more similarities in the oxidative oligomerization of substituted anilines between the *T. versicolor* laccase and the horseradish peroxidase than between the two laccases. The *T. versicolor* laccase and the peroxidase had similar substrate specificities and pH optima and gave rise to similar product profiles. However, the laccase of *R. praticola* was inactive on the alkylated and halogenated anilines tested. In addition, when assays were performed with *p*-methoxyaniline as the substrate, the product profile generated by the *R. praticola* laccase differed from the profiles which resulted from the actions of the *T. versicolor* laccase and the peroxidase on the compound.

We showed in this study that substituted anilines are transformed by a laccase of *T. versicolor*; this differs from our previous observation with a *R. praticola* laccase (7, 22). Evidence of this kind of laccase activity adds a further component to be considered in evaluating the environmental fate of substituted anilines.

ACKNOWLEDGMENTS

This work was supported in part by research grant R-811518 from the Environmental Protection Agency and by the Pennsylvania Agricultural Experiment Station.

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