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Comparative Studies of Extracellular Fungal Laccases

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Various basidiomycetes, ascomycetes, and deuteromycetes, grown in a sugar-rich liquid medium, were compared for laccase-producing ability and for the inducing effect of 2,5-xylidine on laccase production. Clear stimulation of the extracellular enzyme formation by xylidine was obtained in the cultures of *Fomes annosus*, *Pholiota mutabilis*, *Pleurotus ostreatus*, and *Trametes versicolor*, whereas *Rhizoctonia praticola* and *Botrytis cinerea* were not affected by the xylidine, and in the case of *Podospora anserina* a decrease in laccase activity was observed. The laccases were purified, and electrophoresis on polyacrylamide gels indicated a particular pattern for each laccase. The bands of the induced forms appeared only with basidiomycetes. The optimal pH of *R. praticola* laccase was in the neutral region, whereas the optima of all the other exolaccases were significantly lower (between pH 3.0 and 5.7). All laccases oxidized the methoxyphenolic acids under investigation, but there existed quantitative differences in oxidation efficiencies which depended on pH and on the nature (noninduced or induced) of the enzyme. The sensitivity of all enzymes to inhibitors did not differ considerably.

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) have been found in fungal strains belonging to various classes. Laccases are produced by the ascomycetes *Aspergillus nidulans* (22), *Neurospora crassa* (12), and *Podospora anserina* (8, 9), by the deuteromycete wine fungus *Botrytis cinerea* (7, 15), and by several genera of basidiomycetes. The last class in particular has several important laccase-producing fungi. Schanel reported that among 130 wood-rotting basidiomycetes tested, 87 gave a positive polyphenoloxidase reaction (36). Laccase-rich basidiomycetes such as *Collybia velutipes* (19), *Fomes annosus* (16, 18), *Fomes fomentarius* (18, 19), *Lentinus edodes* (23, 35), *Phanerochaete chrysosporium* (20), *Pholiota mutabilis* (25), *Pleurotus ostreatus* (26), *Poria subacida* (13, 18), *Sporotrichum pulverulentum* (1), *Trametes sanguinea* (13) and *Trametes versicolor* (5, 10) are known as lignin degraders. The laccase-producing soil fungus *Rhizoctonia praticola* also belongs to the basidiomycetes class (6).

Exolaccases of *B. cinerea*, *N. crassa*, and *T. versicolor* have been induced by gallic acid (15), cycloheximide (12), and 2,5-xylidine (10), respectively. The induction of an endolaccase by ferulic acid was observed for three genera of basidiomycetes (26). This effect was preceded by the biosynthesis of a specific mRNA that codes for the synthesis of an inducible form of endolaccase (27). Blaich and Esser (4) tested two fungal strains (*Leptoporus litschaueri* and *Polyporus brumalis*) for their substrate specificities and found only quantitative differences in their ability to oxidize phenolic substrates. Similarly, it has been found that the inducible laccase of *T. versicolor* and the noninducible laccase of *R. praticola* transformed vanillic and syringic acids to the same respective products at a particular pH (24). These studies indicated that fungal laccases are similar in their activities, regardless of their origin and induced or noninduced form. To obtain more information about laccases from fungi of various environments, such enzymes were compared with respect to their physiological and metabolic characteristics.

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MATERIALS AND METHODS

The cultures of *B. cinerea* strains A235, BC, and C77.4 were obtained from B. Doneche. *P. anserina* strains (+) and (−) were obtained from K. Esser. *F. annosus* strain 215 (ATCC 28222), *L. edodes* (Berk), *P. chrysosporium* (Burds), and *S. pulverulentum* (Novobranova) were received from A. Hüttermann, H. Ishikawa, T. K. Kirk, and K.-E. Eriksson, respectively. *P. mutabilis* (Schaeff. ex Fr.) Quel no. 1, *P. ostreatus* (Jacqu) Fr. no. 13, *R. praticola* (Vaartija no. 1347), and *T. versicolor* (L. ex Fr.) Pil no. 7 came from our own laboratories.

All organisms were grown in a sugar-rich liquid medium prepared as described earlier (24). The pH values of the media (adjusted with Na_2HPO_4) were 6.8 for *R. praticola* and 5.5 for the other fungi. The cultures were grown in Roux flasks (under stationary conditions) at 24°C. In some experiments *R. praticola* was grown in Erlenmeyer flasks at 24°C on a rotary shaker (120 oscillations per min). During growth of the cultures (when the mycelium covered about half of the medium surface or, in shaking culture, about half of the volume), attempts were made to stimulate laccase production by adding 2,5-xylidine by the method of Fåhraeus and Reinhammar (10).

Laccase activity was measured on a Bausch & Lomb Spectronic 2000 spectrophotometer with syringaldazine as substrate (25), but with morpholineethanesulfonic acid buffer replaced by 0.1 M citrate-phosphate buffer (33). One unit of the enzyme was defined as that amount which at optimal pH causes a change in optical density of 1.0 unit min^{-1} at 525 nm in 3.5 ml of 0.1 M citrate-phosphate buffer containing 0.1 ml of enzyme and 0.3 ml of 0.1 mM (150 pmol) syringaldazine in ethanol at 24°C. Optimum pH values for this laccase reaction were 5.4 for *B. cinerea*, 5.4 for *P. anserina*, 5.0 for *F. annosus*, 5.0 for *L. edodes*, 5.0 for *P. chrysosporium*, 5.0 for *S. pulverulentum*, 5.2 for *P. mutabilis*, 5.7 for P. ostreatus, 7.4 for *R. praticola*, and 5.2 for *T. versicolor*. Protein concentration was determined by the method of Lowry et al. (29).

The extracellular enzymes were isolated from the culture filtrates at the peak of laccase activity as previously de-

scribed for *R. praticola* laccase (6), but instead of Tris-hydrochloride buffer, all laccases were eluted from a DEAE-cellulose column with 0.1 M NaCl in 0.01 M citrate-phosphate buffer at the pH that was found to be optimal for syringaldazine. The enzymes were usually stored under nitrogen at −10°C.

Polyacrylamide gel electrophoresis of laccase preparations was performed in a Tris-borate system at pH 8.45. About 20 μl of the preparations (15 μg of protein) was applied to the gel; electrophoresis was run at 2 μA per column, and laccases were visualized with *p*-phenylenediamine.

Determination of molecular mass of laccases was performed by cationic detergent polyacrylamide gel electrophoresis with cetyltrimethyl ammonium bromide by the method of Marjanen and Ryrie (30). The following protein reference standards (daltons) were used: phosphorylase, a monomer unit (94,000); bovine serum albumin, cross linked (66,000); catalase, monomer unit (60,000); pyruvate kinase (57,000); egg albumin (45,000); and pepsin (4,700).

The influence of pH on enzyme activity with syringaldazine and 2,6-dimethoxyphenol was assayed colorimetrically at 24°C and with other substrates by means of a Clark-type oxygen electrode provided with a linear EZ-10 recorder (Prague, Czechoslovakia). For the colorimetric reaction, a total of 3.5 ml of 0.1 M citrate-phosphate buffer with either 150 pmol of syringaldazine or 3.24 μmol of 2,6-dimethoxyphenol and 0.4 U of the laccase was used. The activity on either substrate was expressed by the increase of absorption per minute as described above for the determination of laccase activity, but absorption for the product of 2,6-dimethoxyphenol was measured at 468 nm. For measuring oxygen consumption, the incubation mixture contained in 0.1 M citrate-phosphate buffer (0.6 μmol per liter of a given substrate) and the quantity of laccase necessary to utilize 1 μmol of O_2 per min at optimal pH with syringaldazine as the substrate. The activity in this case was expressed as nanomoles of oxygen consumed per minute by each substrate.

The effect of inhibitors on laccase activity was assayed at the optimal pH with syringaldazine with 0.1 M citrate-phosphate buffer. The activity on syringaldazine was measured before and after the addition of inhibitor, followed by incubation for 15 min at 24°C.

Chemicals. Vanillic acid, syringaldazine, and 2,6-dimethoxyphenol were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). Hydroxylamine hydrochloride, *N′,N′*-methylene-bis-acrylamide, phosphorylase, and EDTA were from J. T. Baker Chemical Co. (Phillipsburg, N.J.), BDH Chemicals Ltd. (Poole, England), Boehringer Mannheim GmbH (Mannheim, Federal Republic of Germany) and Fisher Scientific Co. (Fair Lawn, N.J.), respectively. Caffeic, ferulic, protocatechuic, sinapic, and syringic acids and cetyltrimethylammonium bromide and *N,N,N′,N′*-tetramethylethylenediamine were purchased from Fluka A.G. (Buchs, Switzerland). Acrylamide, bovine serum albumin (cross linked), catalase, DEAE-cellulose anion exchanger (0.88 meq/g), diethyldithiocarbamic acid sodium salt, egg albumin, pepsin, pyruvate kinase, sodium azide, and thioglycolic acid were obtained from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Among the fungi investigated *B. cinerea* (all strains), *P. anserina* ([+] and [−]), *F. annosus*, *P. mutabilis*, *P. ostreatus*, *R. praticola*, and *T. versicolor* released considerable amounts of laccase into the growth medium. Release of the enzyme by other fungi was insignificant.

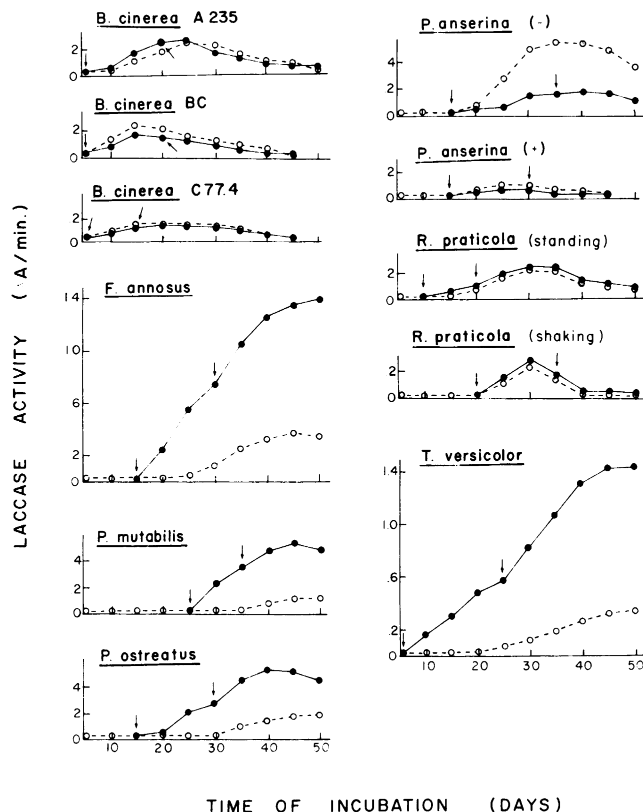

FIG. 1. Extracellular laccase production during growth of fungi in noninduced (○) or 2,5-xylidine-induced (●) cultures. The arrows show the time when xylidine was added to the growth medium.

The enzymatic activity of the medium was studied during growth of the fungi in noninduced and 2,5-xylidine-induced cultures (Fig. 1). Shaken and stationary cultures of *R. praticola* gave similar results. Other fungi in shaken cultures grew very slowly, producing few mycelial beads and extremely low laccase activity. The enzyme activities of *F. annosus*, *P. mutabilis*, *P. ostreatus*, and *T. versicolor* reached much higher levels in the induced cultures than in the noninduced ones; whereas those of *B. cinerea* and *R. praticola* remained almost the same, and *P. anserina* activity decreased with addition of an inducer. In comparison with other cultures, *F. annosus* and *T. versicolor* attained the highest levels of laccase activity in both forms. All measurements were the averages of six flasks.

The effect of 2,5-xylidine on laccase formation by the various fungi and the respective activities are summarized in Table 1. The enzyme preparations contained from 33 to 457 U of enzyme per ml. In comparison with other strains, the induction of laccase of *F. annosus* and *T. versicolor* was the most effective, yielding over 9,200 and 10,900 U of enzyme, respectively, in a culture medium of 1,000 ml. Other cultures produced not more than 4,000 U in an equal volume of medium. All of the enzyme preparations were relatively stable. Different preparations retained 70 to 85% of the original activity after storage under nitrogen for 90 days at −10°C.

The effect of induction on the acrylamide gel electrophoretic patterns of laccases is shown in Fig. 2. In the cases of

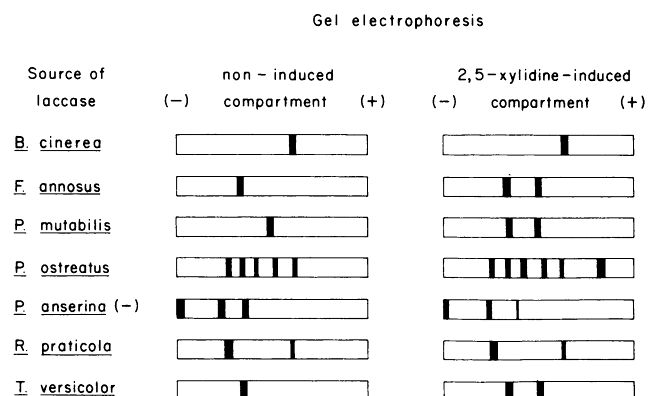

FIG. 2. Polyacrylamide gel electrophoresis of extracellular laccases isolated from 2,5-xylidine-induced and noninduced fungal cultures.

F. annosus, *P. mutabilis*, *P. ostreatus*, and *T. versicolor*, the laccases isolated from the xylidine-induced cultures varied markedly from those not induced. In each induced culture of the four fungi only one form of induced enzyme appeared, irrespective of the number of constitutive enzymes. The induced forms of laccase from *F. annosus*, *P. ostreatus*, and *T. versicolor* were indicated by an extra band that migrated on the gel faster than the constitutive forms, whereas the one from *P. mutabilis* migrated more slowly than the constitutive forms. The other fungi gave the same electrophoretic patterns for both laccase forms.

The DEAE-cellulose-purified preparations of the laccase forms were homogeneous on cetyltrimethylammonium bromide-polyacrylamide gel electrophoresis. In noninduced fungi the following values of molecular mass (daltons) were obtained: in *B. cinerea*, 56,000; in *P. anserina*, 70,000 and 80,000; in *F. annosus*, 73,000; in *P. mutabilis*, 64,000; in *P. ostreatus*, 55,000, 58,000, 66,000, 72,000, and 76,000; in *R. praticola*, 56,000 and 78,000; and in *T. versicolor*, 70,000. The molecular masses (daltons) of the induced forms were as follows: 62,000 in *F. annosus*, 72,000 in *P. mutabilis*, 47,000 in *P. ostreatus*, and 61,000 in *T. versicolor*.

The optimal pH values of laccase activity on 2,6-dimethoxyphenol, ferulic acid, sinapic acid, syringaldazine, syringic acid, and vanillic acid are indicated in Table 2. The optimal activities of the *R. praticola* laccase were at neutral pH (6.5 to 7.5), whereas those of the other laccases differed considerably (pH optima from 3.0 to 5.7). Enzymes incubated with syringaldazine had a higher pH optimum than did those incubated with other phenolic acids.

The activity data in Table 2 show quantitative differences in the ability of different laccases to oxidize various methoxyphenolic substrates. To obtain a reference value for each substrate, we used an enzyme concentration that required 1,000 nmol of O_2 per min to oxidize 2,6-dimethoxyphenol in each assay. Dimethoxyphenols such as syringic and sinapic acids were oxidized by the same laccase form more effectively than the monomethoxyphenols (ferulic and vanillic acids). Furthermore, the phenols containing longer side chains such as ferulic or sinapic acid were better substrates for laccase then their respective homologous compounds, vanillic and syringic acids. In addition, all inducible forms of enzyme showed two- to fivefold higher activity than did the constitutive enzymes. The greatest difference in the apparent activity of the two forms was observed in the case of *T. versicolor* toward ferulic acid.

The inactivation of laccases by various concentrations of potential inhibitors is shown in Table 3. The most effective inhibition was obtained with sodium azide, diethyldithiocarbamic acid, and thioglycolic acid, whereas the effect of EDTA was much lower, and hydroxylamine did not inhibit the enzyme. All laccase preparations showed similar responses to the inhibitors.

DISCUSSION

Laccases from various fungal cultures differed markedly in their inducibility, number of enzyme forms, molecular weight, pH optimum, and substrate specificity with methoxyphenolic acids. Their responses to several inhibitors, however, were similar.

An excess of saccharose or glucose in the liquid medium eliminated the induction of laccase. These media allowed the constitutive production of laccase by fungi, whereas the biosynthesis of the induced enzyme form is repressed by

TABLE 1. Effect of 2,5-xylidine on the formation of extracellular fungal laccases

Fungus	Culture filtrate			Enzyme prepn		
	2,5-Xylidine added	Dry mass of mycelium (g)	Sp act (U/mg of protein)	Sp act (U/mg of protein)	Yield (%)	Purification (fold)
B. cinerea A235	−	10.7	42	4,364	45.2	104
B. cinerea A235	+	11.2	33	3,923	72.0	119
F. annosus	−	11.2	63	7,846	46.1	124
F. annosus	+	10.4	284	39,909	63.6	140
P. mutabilis	−	4.3	28	2,972	76.9	106
P. mutabilis	+	2.9	76	12,853	78.6	169
P. ostreatus	−	6.1	38	4,500	55.6	118
P. ostreatus	+	5.0	90	13,307	78.1	148
P. anserina (−)	−	7.1	121	13,917	77.1	115
P. anserina (−)	+	6.3	40	4,000	54.1	100
R. praticola	−	5.4	35	6,909	68.5	197
R. praticola	+	5.6	44	5,833	68.2	132
R. praticola[a]	−	44.2	42	6,083	43.4	145
R. praticola[a]	+	47.0	44	7,364	64.8	167
T. versicolor	−	12.2	61	10,450	63.6	171
T. versicolor	+	11.0	300	41,134	75.5	137

[a] Shaken culture.

TABLE 2. Substrate specificity and pH optima of the constitutive and inducible forms of laccases isolated from fungal cultures[a]

Source of laccase	Form of laccase	2,6-Dimethoxyphenol		Ferulic acid		Sinapic acid		Syringic acid		Vanillic acid	
		Activity	pH optimum	Activity	pH optimum	Activity	pH optimum	Activity	pH optimum	Activity	pH optimum
B. cinerea A235	Constitutive	1,000	4.2	570	4.0	1,097	3.8	694	4.0	92	4.1
F. annosus	Constitutive	1,000	4.2	650	4.6	1,112	3.0	812	4.3	170	4.9
F. annosus	Inducible	1,000	4.2	1,570	4.6	3,430	3.0	2,115	4.3	501	4.9
P. mutabilis	Constitutive	1,000	3.4	420	4.1	1,211	3.9	970	4.1	104	4.3
P. mutabilis	Inducible	1,000	3.4	980	4.1	2,870	3.9	2,164	4.1	460	4.3
P. ostreatus	Constitutive	1,000	4.2	514	4.2	1,127	3.8	890	4.0	120	4.8
P. ostreatus	Inducible	1,000	4.2	1,374	4.7	3,210	3.8	2,970	4.0	630	4.8
P. anserina (−)	Constitutive	1,000	4.4	1,460	4.2	2,115	4.0	1,860	4.2	616	4.5
R. praticola	Constitutive	1,000	6.8	910	7.2	1,970	6.5	1,215	7.0	170	7.5
T. versicolor	Constitutive	1,000	3.8	190	4.0	987	3.6	420	4.0	97	5.0
T. versicolor	Inducible	1,000	3.8	1,028	4.0	2,126	3.6	1,270	4.0	420	5.0

[a] Activity is expressed in nanomoles of O_2 per minute. To obtain a reference value for all substrates, we used an enzyme concentration that required 1,000 nmol of O_2 per min to oxidize 2,6-dimethoxyphenol.

either sugar (17). In some species, the introduction of an inducing substance into the growth culture initiated the biosynthesis of a new intracellular laccase form, whereas the constitutive forms are synthesized continuously (26).

In our experiments, the enzyme was released into the medium during growth of the fungi in liquid cultures. An increase of extracellular laccase activity was observed after the addition of 2,5-xylidine to *F. annosus*, *P. mutabilis*, *P. ostreatus*, and *T. versicolor* (Fig. 1). In other fungal cultures 2,5-xylidine either did not affect or markedly inhibited enzyme production. Xylidine treatment was repeated when the activity started to decrease to maintain laccase production.

This phenomenon of selective laccase inducibility had been observed earlier in a study in which 3 of 13 fungi investigated (*P. mutabilis*, *P. ostreatus*, and *T. versicolor*) could be induced (26). Leonowicz and Trojanowski investigated basidiomycetes exclusively, used ferulic acid as an inducer, and assayed intracellular laccase only (26). We obtained similar results in our work with extracellular laccases, suggesting that the differentiation between intracellular and extracellular laccases is insignificant with respect to their inducibility. As a result of our investigations with 2,5-xylidine as an inducer, we found that only basidiomycetes were inducible, whereas other fungi were not affected by xylidine. Haars et al. (16) reported that an extracellular laccase of *F. annosus* was induced with dihydroxybenzenoic acids, gallic acid, hydroxyquinone, and protocatechuic acid; thus, a similar effect with 2,5-xylidine is not surprising.

2,5-Xylidine as an effective inducer of *T. versicolor* laccase was described for the first time by Fåhraeus et al. (11). They selected it from various isomers of xylidine and found

TABLE 3. Effect of inhibitors on the oxidation of syringaldazine by extracellular laccases

Inhibitor	Concn (mM)	% Inhibition of laccase from the following source:						
		B. cinerea A235	*F. annosus*	*P. mutabilis*	*P. ostreatus*	*P. anserina* (−)	*R. praticola*	*T. versicolor*
Sodium azide	0.01	51	59	37	58	61	62	55
	0.02	94	96	87	98	97	91	96
	0.05	100	100	100	100	100	100	100
	0.10	100	100	100	100	100	100	100
Diethyldithiocarbamic acid	0.02	6	3	4	7	5	4	5
	0.10	51	46	35	39	42	50	44
	0.20	78	82	87	78	80	83	80
	1.00	100	100	100	100	100	100	100
EDTA	0.10	7	4	3	7	5	4	6
	1.00	17	20	24	25	22	22	19
	2.00	40	35	37	49	41	45	42
	3.00	55	51	46	52	53	60	54
Thioglycolic acid	0.01	25	33	28	33	27	21	30
	0.10	65	70	59	67	73	55	70
	1.00	98	98	95	100	97	90	95
	2.00	100	100	100	100	100	100	100
Hydroxylamine	1.00	2	1	2	3	2	2	2
	2.00	2	3	2	5	3	0	3
	3.00	5	6	5	6	2	7	5

over 160-fold stimulation of enzyme when the compound was introduced into growing cultures. In a separate work, Fåhraeus and Reinhammar (10) stimulated a culture of *T. versicolor* with 2,5-xylidine to obtain a higher yield of the laccase preparation. According to Fåhraeus and Reinhammar, after treatment of the culture with an inducer "the protein fraction of the medium is almost exclusively laccase." The induction of *T. versicolor* laccase by 2,5-xylidine was confirmed by other authors (2), but treatment of other fungi did not result in increased laccase production (14, 38).

In our experiments 2,5-xylidine induced *T. versicolor* laccase as well as *F. annosus*, *P. mutabilis*, and *P. ostreatus* laccases. Gel electrophoresis showed that new laccase forms appeared in xylidine-induced cultures of basidiomycetes, whereas new forms did not exist in induced cultures of *B. cinerea*, *R. praticola*, and *P. anserina* (Fig. 2). In the case of *P. anserina* laccase, the constitutive form was much weaker on the gel in the induced case than in the noninduced case (Fig. 2).

Some of these results are in agreement with those obtained earlier. For example, induced endolaccase forms were shown in the polyacrylamide gels during electrophoresis of *P. mutabilis*, *P. ostreatus*, and *T. versicolor* laccases (28). Haars et al. (16) ran electrofocusing polyacrylamide gels of induced and noninduced *F. annosus* laccase and showed that the induced endo- and exolaccase isoenzymes, which varied in location on the gel, depended on the compound used as an inducer. Gigi et al. (14) reported that *B. cinerea* laccase did not change after induction of the culture by 2,5-xylidine; our investigation (unpublished data) and Wood (41) found the same results in the case of *R. praticola* and *Agaricus bisporus* laccases, respectively.

Our molecular mass determinations confirmed the earlier data for laccases of *B. cinerea* (32), *P. anserina* (34), *P. mutabilis* and *P. ostreatus* (26), *R. praticola* (6), and *T. versicolor* (21, 26). The molecular weight of *F. annosus* laccase forms was determined for the first time.

Among the seven fungi investigated, six had pH optima for laccase activity in the acidic region (Table 2), which suggests that activity at low pH is physiologically more significant. The natural habitats of these six genera—forest wood, litter, and soil (four fungi); dung of herbivores (*P. anserina*); and fruits (*B. cinerea*)—are generally acidic. Fungi growing in such environments come in contact with various acidic plant phenols or pesticides, and the lower pH optima of the laccase allows a more effective oxidation of toxic compounds (28). On the other hand, the pH optimum of *R. praticola* laccase is neutral, an exception among fungal laccases, which are usually unstable at higher pH values (31).

Our pH optimum measurements for 2,6-dimethoxyphenol and syringaldazine as the substrates are in agreement with those obtained earlier for laccases of *R. praticola* (6) and *T. versicolor* (3, 25). The results concerning the methoxyphenolic acids were done first in our laboratories.

Our finding of differing degrees of phenolic compound oxidation by various fungal laccases was consistent with data reported elsewhere (4). In our experiments we did not determine whether the substrate concentrations were saturating; this could be a reason for the different activities. However, the relation between the noninduced and induced form of the various fungal laccases was unexpected (Table 2). As was reported earlier, considerable differences appeared among laccase forms of *P. anserina* (37) and *P. mutabilis*, *P. ostreatus*, and *T. versicolor* (28). Leonowicz et al. (28) found that the induced forms of laccase were usually much more active than the constitutive forms, and they proposed that the higher activity of induced laccase forms is necessary for the detoxification of an environment polluted with phenols (28). Our present results support this conclusion.

The response to inhibitors did not differ among the investigated laccases (Table 3). All enzymes were completely inhibited by azide, thioglycolic acid, and diethyldithiocarbamic acid, which are classical inhibitors of copper-containing oxidases, whereas EDTA affected laccase activity to a lesser extent, and hydroxylamine had almost no effect.

In earlier work we showed that laccases from various sources formed different products at different pH values, but all enzymes generated the same chemicals at a particular pH (24). In this work, evidence was obtained that laccases from various fungal sources differ in certain specifics, but that their main function, transformation of phenolic substances, is the same. The differing properties of enzymes appear to allow the activities of laccase-producing fungi in various natural environments.

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