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Lignin Oxidation by Laccase Isozymes from *Trametes versicolor* and Role of the Mediator 2,2′-Azinobis(3-Ethylbenzthiazoline-6-Sulfonate) in Kraft Lignin Depolymerization

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Two laccase isozymes (I and II) produced by the white-rot fungus *Trametes versicolor* were purified, and their reactivities towards various substrates and lignins were studied. The N-terminal amino acid sequences of these enzymes were determined and compared to other known laccase sequences. Laccase II showed a very high sequence similarity to a laccase which was previously reported to depolymerize lignin. The reactivities of the two isozymes on most of the substrates tested were similar, but there were some differences in the oxidation rate of polymeric substrates. We found that the two laccases produced similar qualitative effects on kraft lignin and residual lignin in kraft pulp, with no evidence of a marked preference for depolymerization by either enzyme. However, the presence of the mediator 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) prevented and reversed the polymerization of kraft lignin by either laccase. The delignification of hardwood and softwood kraft pulps with the two isozymes and the mediator was compared; either laccase was able to reduce the kappa number of pulp, but only in the presence of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate).

The enzyme laccase is widely distributed in plants and fungi. The enzyme is particularly abundant in many lignin-degrading white-rot fungi, and this has led to speculation that laccase plays a role in wood and pulp delignification. *Trametes* (= *Coriolus* = *Polyporus*) *versicolor* is the most-studied laccase-producing fungus. Low concentrations of several laccases are produced constitutively on wood and in submerged fungal cultures, while higher concentrations are induced by addition of aromatic compounds such as xylidine and ferulic acid (25). Higher concentrations of laccase have also been observed in older, noninduced cultures (3). Laccase reduces dioxygen to two molecules of water and simultaneously performs one-electron oxidation of many aromatic substrates. The oxidizing reaction substrate range is fairly broad and includes polyphenols, methoxy-substituted monophenols, aromatic amines, and other easily oxidized aromatic compounds (28). The initial reaction products are oxygen-centered radicals or cation radicals, which usually react further through nonenzymic routes.

Oxidation of lignin by fungal laccase has been studied intensively since the early 1970s. Oxidation of milled wood lignin (15), demethylation (16), and formation of carboxyl groups (19) were observed. The early work is reviewed by Ishihara (14), and the extensive studies on model compounds by Higuchi's group have been summarized by Higuchi (11). Only phenolic subunits of lignin are attacked, leading to Cα oxidation, Cα-Cβ cleavage, and alkyl-aryl cleavage. Often, polymerization products are observed when laccase oxidizes phenolic substrates. For example, milled wood lignin (14) and soluble lignosulfonates (20) are both polymerized to some extent by laccase.

The substrate range of laccase can be extended to nonphenolic subunits of lignin by inclusion of a mediator such as 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) (2). Thus, veratryl alcohol is oxidized to veratraldehyde and nonphenolic β-1 and β-0-4 model compounds are cleaved or oxidized at the Cα position. Some of these reactions can be rationalized on the basis of hydrogen atom abstraction to give an initial Cα-ketyl radical intermediate (23). However, Scaiano et al. (27) have proposed that such intermediates will rapidly lead to β-0-4 cleavage, which was not observed with the laccase-ABTS couple. Whatever the mechanism, it was found that kraft pulp was delignified by the combination of laccase and ABTS but not by laccase alone (3). It seems likely that ABTS must function as a diffusible electron carrier, because laccase is a large molecule (molecular weight [MW], around 70,000) and therefore cannot enter the secondary wall to contact the lignin substrate directly. Recently, more than 50% delignification of kraft pulps has been reported with laccase and another mediator, 1-hydroxybenzotriazole (5), or by repeated treatment with laccase-ABTS followed by alkaline extraction (4). These observations have provoked considerable interest in enzyme-catalyzed oxidative bleaching of kraft pulps.

Morohoshi et al. (22) found that only one of three laccases isolated from *Coriolus versicolor* depolymerized certain lignin preparations, while the other two laccases gave mainly polymerized products. The depolymerizing laccase, named laccase IIIc, was subsequently characterized by gene sequencing. At least four other laccase-encoding gene sequences have also been reported from ligninolytic fungi, namely, *C. hirsutus* (18), *Phlebia radiata* (26), *Agaricus bisporus* (24), and the unidentified basidomycete PM1 (6). The protein sequences derived from these genes encode proteins of between 515 and 619 amino acid residues, and sequence comparisons indicate close phylogenetic proximity between them (6). They are all glycoproteins, and sites of N-glycosylation are evident from the sequences.

In this report, we compare the catalytic actions of two laccases isolated from *T. versicolor*, the structure of one of which resembles that of laccase IIIc. The reactivities of these two enzymes on most of the low-MW substrates assayed were similar, but significantly higher reactivity of laccase I over laccase II was shown with a polymeric substrate. The two isozymes had

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similar effects on kraft lignin and residual lignin in kraft pulp, with no evidence of a marked preference for depolymerization by either enzyme. However, kraft lignin polymerization by either laccase could be reversed by addition of the mediator ABTS.

MATERIALS AND METHODS

Laccase production and purification. A monokaryotic strain (52J) derived from *T. versicolor* (ATCC 20869) isolated at the Pulp and Paper Research Institute of Canada (1) was used for laccase preparation. Three disks (1 cm in diameter) from the growing edge of the mycelium on malt agar plates were transferred to 500-ml polypropylene flasks each containing 250 ml of a synthetic liquid medium described by Fahraeus and Reinhammar (8). A glass marble was added to each flask to break up mycelial disks and prevent pellet formation, and the flasks were shaken at 200 rpm and 20°C for 5 days. This liquid (500 ml) was used to inoculate a carboy containing 17 liters of the same medium, which was then shaken with continuous air bubbling. After 4 days, laccase production was induced with 2,5-xylidine (0.2 mM). The culture was grown for a further 3 days and then filtered through cheesecloth. The filtrate was concentrated by ultrafiltration on a Pellicon membrane, followed by concentration on an Amicon YM10 membrane to a final volume of 200 ml and dialysis against sodium phosphate (20 mM, pH 7). Enzyme concentrate (100 ml) was applied to a DEAE Bio-Gel A column (5 by 25 cm) equilibrated in the same buffer. The column was washed with 100 ml of the initial buffer and then eluted with a linear buffer gradient up to 0.2 M Na phosphate (pH 7) to a total volume of 1.6 liters. Two major peaks of laccase activity were pooled separately. Laccase I (defined by its order of elution on DEAE) was further purified by gel permeation chromatography on Superose 12 (Pharmacia) in sodium phosphate (20 mM, pH 7.0). Laccase II was chromatographed on Mono-Q (Pharmacia) in sodium phosphate (20 mM, pH 7.0) buffer with a gradient to 0.1 M. The purified enzymes were dialyzed against distilled water, frozen in liquid nitrogen, and stored at −80°C.

Amino acid sequence determination. Automated gas-phase sequence analysis was performed on a 475A protein sequencing system (Applied Biosystems Inc., Foster City, Calif.) with 0.1- to 0.5-nmol quantities of protein. The samples were dissolved in 0.1% trifluoroacetic acid and applied to a glass fiber disk containing 0.75 mg of Polybrene (Applied Biosystems). Alignments of the amino acid sequences of the laccases were determined with GeneWorks version 2.2.1 software by Intelligenetics Inc., Mountain View, Calif.

Enzyme assays. Laccase activity was determined by oxidation of ABTS (29). The assay mixture contained 0.5 mM ABTS, 0.1 M sodium acetate (pH 5.0), and a suitable amount of enzyme. Oxidation of ABTS was monitored by determining the increase in A_{420} (ε_{420}, $3.6 \times 10^4\ M^{-1} \cdot cm^{-1}$). Enzyme activity was expressed in units defined as follows: 1 U = 1 μmol of ABTS oxidized per min.

The relative activities of each laccase on various substrates were determined by spectrophotometry. We chose wavelengths at which the absorption difference between the oxidized and nonoxidized forms of each substrate was maximal. The reaction (3 ml) was performed at room temperature in sodium acetate buffer (0.1 M, pH 5.0) with 0.1 U (measured with ABTS) of laccase I or II. The concentration of each substrate and the wavelength used are described in Results.

All of the chemical substrates used were purchased from Aldrich or Sigma. Indulin AT was from Westvaco Corp. Hardwood kraft lignin was prepared by acidification of black liquor obtained from a mixed-hardwood kraft digester; the precipitated lignin was washed with acidified water and freeze-dried.

Kraft pulp treatments. Unbleached hardwood kraft pulp (mixture of various wood species) was obtained from an eastern Canadian mill. Softwood kraft pulp from black spruce was prepared in a pilot plant at the Pulp and Paper Research Institute of Canada. Washed pulp (4 g [oven-dried weight]) was suspended in sodium acetate buffer (0.05 M, pH 5.0) in a final liquid volume of 200 ml. Laccase and ABTS were added to final concentrations of 0.1 U/ml and 1 mM, respectively, and flasks were shaken at 200 rpm and 50°C for 24 h. After treatment, pulp was filtered and washed with water.

Handsheets (i.e., sheets of paper produced from pulp samples in the laboratory) were prepared with a British Standard Handsheet Machine (Noram) by using 4 g of pulp that had been homogenized for 30 s in a Brookfield counter-rotating mixer. Each handsheet was then air dried on a blotter before it was tested for kappa number. The kappa number is defined as the amount (in milliliters) of a 0.1 N $KMnO_4$ solution consumed by 1 g of moisture-free pulp under standard conditions (CPPA standard method G18). The kappa number is equivalent to approximately six times the weight percent of lignin.

Methanol generated from pulp treatments was determined by gas chromatography as described previously (3).

Treatment of ^{14}C-labelled kraft lignin. ^{14}C-labelled aspen wood lignin was prepared by feeding [U-^{14}C]cinnamic acid to aspen branches (7). The labelled wood was then kraft cooked in the laboratory to an H factor of 1,400 with 15% active alkali and 27% sulfidity at a liquor-to-wood ratio of 5:1. The resulting cooking liquor was separated from the pulp by filtration, and the ^{14}C-labelled kraft lignin was precipitated from the cooking liquor by lowering the pH to 2. The precipitate was washed with distilled water and lyophilized. The resulting ^{14}C-labelled kraft lignin had a specific activity of 2,960 Bq/mg. For enzymatic treatment, the complete reaction mixture contained 0.4 mg of ^{14}C-labelled kraft lignin (1,183 Bq), 1 mM ABTS, and 0.1 U of laccase I or II per ml in sodium acetate (0.05 M, pH 5.0) to a final volume of 1 ml. After incubation at room temperature for a designated time period, the reaction was stopped by addition of 5 μl of 5 N NaOH and 125 μl of ethanol to 0.5-ml aliquots and filtered on a 0.45-μm-pore-size microfilter before gel permeation chromatography.

Gel permeation chromatography of ^{14}C-labelled kraft lignin. Gel permeation chromatography of kraft lignin was performed on a Superose 12 HR 10/30 fast protein liquid chromatography column (Pharmacia) with aqueous solvent containing NaOH (0.02 M), NaCl (0.05 M), and ethanol (20%) at a flow rate of 0.3 ml/min. The column was calibrated with the following MW standards: blue dextran, human gamma globulin, bovine serum albumin, horseradish peroxidase, chymotrypsinogen, cytochrome *c*, vitamin B_{12}, and tryptophan. For radiolabelled samples, 0.5 ml was applied on the column, 1-ml fractions were collected and mixed with 5 ml of OptiPhase (LKB) scintillation cocktail, and the radioactivity was measured in an LS 6800 liquid scintillation counter (Beckman).

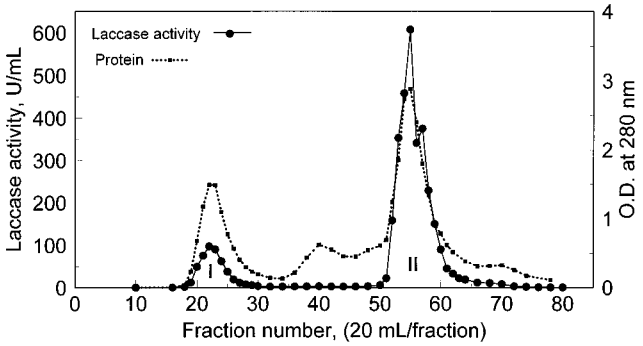

FIG. 1. Elution profile of laccase activities on DEAE–Bio-Gel. Concentrated initial enzyme (58,000 U) was applied on a DEAE column (5 by 25 cm) in sodium phosphate (0.02 M, pH 7.0) and eluted with a linear buffer gradient up to 0.2 M sodium phosphate. O.D., optical density.

RESULTS

Enzyme isolation and characterization. Two laccases were purified from a 2,5-xylidine-induced culture of *T. versicolor* and named laccases I and II on the basis of elution order from DEAE ion-exchange gel chromatography (Fig. 1). Before induction, the total laccase activity in the culture supernatant was less than 2,000 U/liter, and 2 to 3 days after induction with 2,5-xylidine, the laccase activity peaked at about 10,000 U/liter, with a net predominance of laccase II over laccase I. Table 1 presents a summary of the purification of the two laccases. Both laccases were glycoproteins with MWs of 67,000 and 70,000 for laccases I and II, respectively, as determined by Schiff staining of sodium dodecyl sulfate-polyacrylamide gels (data not shown). The two enzymes had similar A_{280}/A_{600} ratios of around 18. The specific activity of laccase II measured with ABTS was three to four times higher (135 to 160 U/mg) than that of laccase I (40 to 45 U/mg). The N-terminal amino acid sequences of laccases I and II from *T. versicolor* are compared to that of laccase IIIc from *C. versicolor* (13) in Table 2.

TABLE 1. Summary of laccase isozyme purification

Purification step (isozyme)	Total activity (μmol of ABTS/min)	Yield (%)
Initial enzyme	155,000	100
Concentrated ultrafiltrate	126,000	81
DEAE–Bio-Gel (laccase I)	16,700	10.8
DEAE–Bio-Gel (laccase II)	99,500	64.2
Superose 12 (laccase I)	12,525	8.1
Mono Q (laccase II)	22,880	14.8

TABLE 2. N-terminal amino acid sequences of laccases I and II from *T. versicolor* and laccase IIIc from *C. versicolor*

Laccase	Amino acid[a] at position:																													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
I	Ala	Ile	Gly	Pro	Val	Ala	Ser	Leu	Val	Val	Ala	Asn	Ala	Pro	Val	Ser	Pro	Asp	Gly	Phe	Leu	Arg	Asp	Ala	Ile	Val	Val	Asn	Gly	Val
II	Gly	Ile	Gly	Pro	Val	Ala	Asp	Leu	Thr	Ile	Thr	Asn	Ala	Ala	Val	Ser	Pro	Asp	Gly	Phe	Ser	Arg	Gln	Ala	Val	Val	Val	Asn	Gly	Gly
IIIc[b]	Gly	Ile	Gly	Pro	Val	Ala	Asp	Leu	Thr	Ile	Thr	Asn	Ala	Glu	Val	Ser	Pro	Asp	Gly	Leu	Ser	Arg	Gln	Ala	Val	Val	Val	Asn	Gly	Gly

[a] The underlined amino acids differ from those in the laccase II sequences.
[b] From Iimura et al. (13).

The amino acid residues from laccases I and IIIc are underlined where they differ from those of laccase II. The laccase I and II sequences show enough difference (10 of 30 residues) to originate from different genes. However, laccase IIIc from *C. versicolor*, which was reported to depolymerize lignin (22), is almost identical to laccase II (28 of 30 amino acid residues).

Relative activities with various substrates. The relative rates of oxidation of various monomeric and polymeric substrates by laccases I and II are listed in Table 3. For each substrate tested, equal amounts of laccases I and II, based on their activity with ABTS, were used. Each determination were performed in duplicate by using two different preparations of each laccase isozyme. The activities are expressed as the change in optical density per minute and were measured within the first minute of the reaction. A negative sign before the activity means decolorization of the substrate following the action of laccase. For all of the monomeric or dimeric substrates tested, the activity of laccase II was similar to or slightly less than that of laccase I, with laccase II/I activity ratios ranging between 0.73 and 0.97. With higher-MW substrates such as kraft lignins and Poly B-411 (Sigma), a polymer of sulfonated diaminoanthraquinone, the rate of oxidation tended to be higher with laccase I than with laccase II. Decolorization of Poly B-411 and other dyes of this type has been reported to correlate with lignin degradation by *Phanerochaete chrysosporium* (9). If this relationship were true for *T. versicolor*, it would imply that laccase I is more involved in lignin depolymerization than laccase II. However, the relative reactivities of the two laccases during kraft pulp delignification and kraft lignin depolymerization do not support this hypothesis (see below).

Kraft pulp treatment with laccase isozymes and ABTS. We found previously that laccase can effectively demethylate and delignify hardwood kraft pulp when the mediator ABTS is present (3). The laccase preparation used in that study was laccase I produced by a noninduced culture of *T. versicolor*. Here, we compared the activities of induced laccases I and II, with or without ABTS, on two different kraft pulps, one from hardwood and the other from softwood. The results, summarized in Table 4, show that in the absence of ABTS, the two purified laccases were not able to reduce the kappa number of either pulp, but both produced small amounts of methanol with the hardwood pulp only. When ABTS was present, the two enzymes were equally effective in delignifying and demethylating either pulp. The methanol produced and the decrease in kappa number were consistently less with hardwood pulp. This could be explained by the fact that the lignin content of hardwood pulp is only about 60% of that of softwood pulp. The results show that without the mediator, neither laccase I nor II can degrade lignin in pulp and that the two isozymes are equally effective when they are coupled to ABTS.

^{14}C-labelled kraft lignin oxidation by the laccase-ABTS couple. The oxidizing activities of laccases I and II on kraft lignin and the effect of ABTS on these reactions were measured by determination of the apparent MW distributions of ^{14}C-labelled kraft lignin by gel permeation chromatography. Figures 2 and 3 show the elution profiles of radiolabelled lignin after, respectively, 3 and 6 days of incubation with laccase I in the presence or absence of ABTS. The apparent average MW of the lignin samples was measured at the maximum of their elution peak by comparison with protein MW standards. The apparent average MW of unreacted radiolabelled lignin was estimated to be 3,500. After 3 days of incubation with laccase I, the lignin polymerized to an apparent average MW of 8,600 and went up to 11,500 after 6 days (Fig. 4). The addition of ABTS at the beginning of the incubation completely prevented polymerization by laccase and even produced some depolymerization of the labelled lignin (apparent average MW of 2,800 after 6 days). However, the most striking effect was shown when ABTS was added after 3 days of preincubation with laccase. Within 3 h of addition of ABTS, the condensed

TABLE 3. Relative rates of oxidation of various substrates by laccases I and II

Substrate	Concn	λ (nm)	Mean ΔOD[a]/min ± SD		Laccase II/I ratio
			Laccase I	Laccase II	
ABTS	1 mM	420	1.30 ± 0.13	1.30 ± 0.06	1.00
Guaiacol	1 mM	470	0.15 ± 0.03	0.12 ± 0.01	0.80
Syringaldazine	0.1 mM	530	1.37 ± 0.15	1.00 ± 0.1	0.73
Syringaldehyde	1 mM	370	0.026 ± 0.004	0.023 ± 0.001	0.88
Vanillyl alcohol	1 mM	295	0.078 ± 0.006	0.059 ± 0.003	0.76
Promazine	1 mM	512	0.28 ± 0.02	0.27 ± 0.01	0.96
4,5-Dihydroxy-2,7-naphthalene disulfonate	1 mM	400	0.047 ± 0.003	0.040 ± 0.001	0.87
8-Anilino-1-naphthalene sulfonate	1 mM	430	0.076 ± 0.008	0.068 ± 0.002	0.89
4,4′-Diamino-stilbene-2,2′-disulfonate	0.1 mM	360	−0.062 ± 0.004	−0.049 ± 0.002	0.79
Indulin	0.5 mg/ml	400	0.032 ± 0.002	0.021 ± 0.001	0.66
Hardwood kraft lignin	0.2 mg/ml	400	0.028 ± 0.002	0.012 ± 0.001	0.43
Poly B-411	0.1 mg/ml	600	−0.02 ± 0.001	−0.007 ± 0.001	0.35

[a] ΔOD, change in optical density.

TABLE 4. Kraft pulp treatment with laccases I and II and ABTS

Treatment	Methanol concn (mg/liter), kappa no.	
	Hardwood pulp	Softwood pulp
Control	3.8, 14.8	2.4, 25.9
Laccase I	6.1, 14.7	2.5, 25.9
Laccase II	5.1, 14.9	2.5, 26.0
Laccase I + ABTS	25.7, 12.1	33.9, 22.4
Laccase II + ABTS	24.4, 12.5	34.7, 22.2

lignin was effectively depolymerized to an average MW of 5,300. This depolymerization continued further, and after 3 days, the MW distribution was practically the same as that of the initial radiolabelled lignin. The results obtained with laccase II were similar, except that the rate and extent of polymerization of lignin were slightly less than with laccase I. The lignin MW averages were, respectively, 7,800 and 10,500 after 3 and 6 days of treatment with laccase II (data not shown). However, the extent of the depolymerization caused by ABTS was the same as with laccase I.

DISCUSSION

The above results confirm that laccase, like other phenol-oxidizing enzymes, such as peroxidases (10, 12, 17), preferentially polymerizes lignin by coupling of the phenoxy radicals produced from oxidation of lignin phenolic groups. It has been suggested that laccase possesses both polymerization and depolymerization activities on some preparations of lignosulfonates (20). However, our results show that when laccase is used alone, the only reaction that can be observed on kraft lignin is polymerization. The fact that ABTS prevents polymerization of kraft lignin by laccase cannot be explained only by inhibition or reduction of the lignin phenoxy radicals produced by laccase, because when ABTS was added after lignin polymerization by laccase, the lignin was effectively depolymerized. Recent reports have shown that laccase is able to oxidize and cleave nonphenolic lignin compounds when ABTS is present (2) and that the action of the laccase-ABTS couple proceeds via Cα-hydrogen abstraction (23). A similar mechanism involving Cα-Cβ bond cleavage in condensed lignins could explain the depolymerizing activity of laccase-ABTS. Another possibility is that the radicals produced by laccase on phenolic

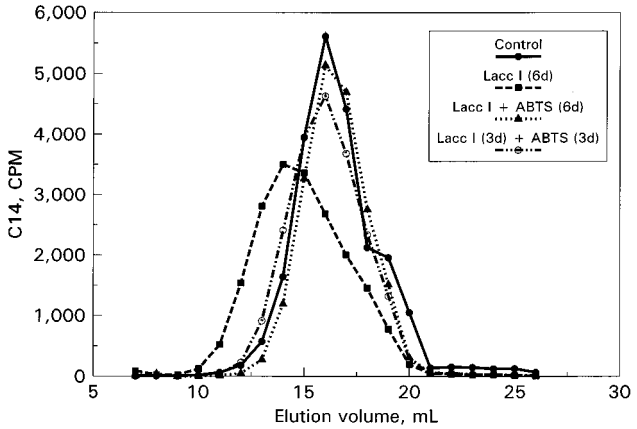

FIG. 3. Gel permeation chromatograph of ^{14}C-labelled kraft lignin after 6 days (d) of treatment with laccase (Lacc) I and ABTS.

groups of lignin would preferentially couple with ABTS instead of coupling with other lignin-derived radicals. Matsumura et al. (21) have reported that laccase catalyzes the coupling of ABTS with various phenolic derivatives to produce colored compounds. This lignin-ABTS coupling reaction would explain the complete inhibition of lignin polymerization when laccase and ABTS were simultaneously present. Furthermore, the higher solubility of lignin-ABTS complexes, due to the sulfonated group on ABTS, would also explain, to some extent, the delignifying activity of laccase-ABTS on kraft pulp. We are investigating further the mechanism of laccase-ABTS delignification and testing other possible mediators for laccase with the objective of improved oxidative bleaching of kraft pulp.

In conclusion, our results indicate that induced cultures of the monokaryon *T. versicolor* produce mainly two isozymes, laccases I and II, from two distinct genes. Their reactivities on many substrates are very similar, although with polymeric substrates, laccase I was more active than laccase II. Even though the N-terminal sequence of laccase II is almost identical to that of the reported lignin-depolymerizing laccase IIIc (13), we found no evidence of depolymerization of kraft lignin by laccase II. In fact, without the help of ABTS as a mediator, both laccases I and II polymerized kraft lignin and failed to delignify kraft pulps. The presence of ABTS prevents and even reverses kraft lignin polymerization and promotes delignification of

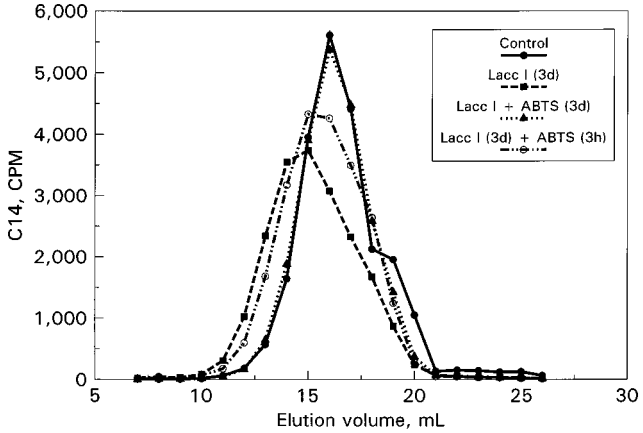

FIG. 2. Gel permeation chromatograph of ^{14}C-labelled kraft lignin after 3 days (d) of treatment with laccase (Lacc) I and ABTS.

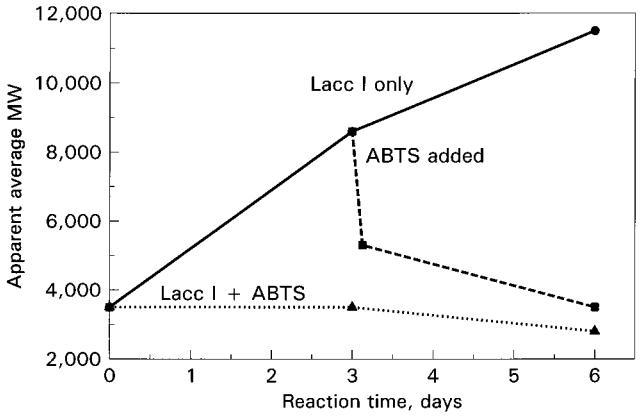

FIG. 4. Apparent average MW of ^{14}C-labelled kraft lignin following treatment with laccase (Lacc) I and ABTS.

kraft pulp by either laccase. On the basis of earlier model compound studies (2), delignification of pulp and kraft lignin depolymerization with laccase-ABTS are likely to proceed by a combination of two actions: first, Cα-Cβ cleavage of nonphenolic sites in lignin, and second, solubilization of the lignin fragments by formation of hydrophilic lignin-ABTS complexes.

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REFERENCES

1. **Addleman, K., and F. S. Archibald.** 1993. Kraft pulp bleaching and delignification by dikaryons and monokaryons of *Trametes versicolor*. Appl. Environ. Microbiol. **59:**266–273.
2. **Bourbonnais, R., and M. G. Paice.** 1990. Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. FEBS Lett. **267:**99–102.
3. **Bourbonnais, R., and M. G. Paice.** 1992. Demethylation and delignification of kraft pulp by *Trametes versicolor* laccase in the presence of 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate). Appl. Microbiol. Biotechnol. **36:**823–827.
4. **Bourbonnais, R., and M. G. Paice.** Optimization of kraft pulp delignification by laccase and mediator. Submitted for publication.
5. **Call, H. P.** December 1994. Process for modifying, breaking down or bleaching lignin, materials containing lignin or like substances. World patent application WO 94/29510.
6. **Coll, P. M., C. Tabernero, R. Santamaria, and P. Parez.** 1993. Characterization and structural analysis of the laccase I gene from the newly isolated ligninolytic basidiomycete PM1. Appl. Environ. Microbiol. **59:**4129–4135.
7. **Crawford, R. L., and D. L. Crawford.** 1988. [^{14}C]lignin-labelled lignocelluloses and ^{14}C-labelled milled wood lignins: preparation, characterization and uses. Methods Enzymol. **161:**18–31.
8. **Fahraeus, G., and B. Reinhammar.** 1967. Large-scale production and purification of laccase from cultures of the fungus *Polyporus versicolor* and some properties of laccase A. Acta Chem. Scand. **21:**2367–2378.
9. **Glenn, J. K., and M. H. Gold.** 1983. Decolorization of several polymeric dyes by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **45:**1741–1757.
10. **Haemmerli, S. D., M. S. A. Leisola, and A. Fiechter.** 1986. Polymerization of lignins by ligninases from *Phanerochaete chrysosporium*. FEMS Microbiol. Lett. **35:**33–36.
11. **Higuchi, T.** 1989. Mechanisms of lignin degradation by lignin peroxidase and laccase of white-rot fungi. ACS Symp. Ser. **399:**482–502.
12. **Huttermann, A., C. Herche, and A. Haars.** 1980. Polymerization of water-insoluble lignins by *Fomes annosus*. Holzforschung **34:**64–66.
13. **Iimura, Y., K. Takenouchi, M. Nakamura, S. Kawai, Y. Katayama, and N. Morohoshi.** 1992. Cloning and sequence analysis of laccase genes and its use for an expression vector in *Coriolus versicolor*, p. 427–431. *In* M. Kuwahara and M. Shimada (ed.), Biotechnology in pulp and paper industry. Uni Publishers Co., Ltd., Tokyo, Japan.
14. **Ishihara, T.** 1980. The role of laccase in lignin biodegradation, p. 17–31. *In* T. K. Kirk, T. Higuchi, and H. M. Chang (ed.), Lignin biodegradation: microbiology, chemistry and potential applications, vol. 2. CRC Press, Inc., Boca Raton, Fla.
15. **Ishihara, T., and M. Miyazaki.** 1972. Oxidation of milled wood lignin by fungal laccase. Mokuzai Gakkaishi **18:**415–419.
16. **Ishihara, T., and M. Miyazaki.** 1974. Demethylation of lignin and lignin models by fungal laccase. Mokuzai Gakkaishi **20:**39–41.
17. **Kern, H. W., and T. K. Kirk.** 1987. Influence of molecular size and ligninase pretreatment on degradation of lignins by *Xanthomonas* sp. Appl. Environ. Microbiol. **53:**2242–2246.
18. **Kojima, Y., Y. Tsukuda, Y. Kawai, A. Tsukamoto, J. Sugiura, M. Sakaimo, and Y. Kita.** 1990. Cloning, sequence analysis, and expression of lignolytic phenoloxidase genes of the white-rot basidiomycete *Coriolus hirsutus*. J. Biol. Chem. **265:**15224–15230.
19. **Konishi, K., Y. Inoue, and T. Higuchi.** 1974. Decomposition of lignin by *Coriolus versicolor*. V. Formation of carboxyl groups in the lignin treated with fungal laccase. Mokuzai Gakkaishi **20:**26–30.
20. **Leonowicz, A., G. Sklarz, and M. Wojtas-Wasilewska.** 1985. The effect of fungal laccase on fractionated lignosulphonates (Peritan Na). Phytochemistry **24:**393–396.
21. **Matsumura, E., E. Yamamoto, A. Numata, T. Kawano, T. Shin, and S. Murao.** 1986. Structures of the laccase-catalyzed oxidation products of hydroxybenzoic acids in the presence of ABTS. Agric. Biol. Chem. **50:**1355–1357.
22. **Morohoshi, N., H. Wariishi, C. Murasiso, T. Nagai, and T. Haraguchi.** 1987. Degradation of lignin by the extracellular enzymes of *Coriolus versicolor*. IV. Properties of three laccases fractionated from the extracellular enzymes. Mokuzai Gakkaishi **33:**218–225.
23. **Muheim, A., A. Fiechter, P. J. Harvey, and H. E. Schoemaker.** 1992. On the mechanism of oxidation of non-phenolic lignin model compounds by the laccase-ABTS couple. Holzforschung **46:**121–126.
24. **Perry, C. R., M. Smith, C. H. Britnell, D. A. Wood, and C. F. Thurston.** 1993. Identification of two laccase genes in the cultivated mushroom *Agaricus bisporus*. J. Gen. Microbiol. **139:**1209–1218.
25. **Reinhammar, B.** 1984. Laccase, p. 1–35. *In* R. Lontie (ed.), Copper proteins and copper enzymes, vol. 3. CRC Press, Inc., Boca Raton, Fla.
26. **Saloheimo, M., M.-L. Nikku-Paavola, and J. K. C. Knowles.** 1991. Isolation and structural analysis of the laccase gene from the lignin-degrading fungus *Phlebia radiata*. J. Gen. Microbiol. **137:**1537–1544.
27. **Scaiano, J. C., J. C. Netto-Ferreira, and V. Wintgens.** 1991. Fragmentation of ketyl radicals derived from α-phenoxyacetophenone: an important mode of decay for lignin-related radicals. J. Photochem. Photobiol. A Chem. **59:**265–268.
28. **Thurston, C. F.** 1994. The structure and function of fungal laccases. Microbiology **140:**19–26.
29. **Wolfenden, B. S., and R. L. Willson.** 1982. Radical-cations as reference chromogens in kinetic studies of one-electron transfer reactions. J. Chem. Soc. Perkin Trans. **II:**805–812.