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Production and Characterization of Laccase from *Botrytis cinerea* 61-34

D. SLOMCZYNSKI, J. P. NAKAS, AND S. W. TANENBAUM*

State University of New York College of Environmental Science and Forestry, Syracuse, New York 13210

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An isolate of *Botrytis cinerea* (strain 61-34) constitutively expresses substantial amounts of extracellular laccase on a defined growth medium. The enzyme has been purified to homogeneity by a facile operational sequence, the last stage of which involves hydrophobic interaction chromatography. By these means, over 80 mg of laccase liter^{-1} can be obtained from aerated fermentor reaction broths. The enzyme, with an estimated M_r of 74,000 and pI of 4.0, is a monomeric glycoprotein containing 49% carbohydrate predominantly as hexose. With 2,6-dimethoxyphenol, it exhibits a pH optimum of 3.5 and a temperature optimum of 60°C, and its K_m is 100 μM. The purified enzyme with this substrate has a specific activity of 9.1 mkat mg of protein^{-1}. Taken together with a broad substrate range and its stability in 4% sodium dodecyl sulfate or 2 M urea solutions, several biotechnology transfers are suggested.

Laccases (benzenediol:oxygen oxidoreductase; EC 1.10.3.2) have been investigated since 1883, when Yoshida (42) reported upon a "diastase-like" activity, requiring air, for polymerization of *Rhus vernicifera* extracts. In the intervening years, a large number of publications have dealt with this enzyme obtained from plant, fungal, and insect sources and even from one bacterium. The evolution, comparative biochemistry, and function of laccases in producing organisms have been the subject of at least four recent reviews (10, 23, 32, 37). In structural terms, these enzymes are either monomeric or multimeric copper-containing glycoproteins, which may exhibit additional heterogeneity because of variable carbohydrate content or differences in copper content (41) or because they are expressed as the products of multiple genes (25). Furthermore, the laccases represent an interesting family of enzymes in that they exhibit rather broad (or "relaxed") substrate specificities, which may be expanded by inclusion of redox mediators (33) into their reaction mixtures.

The implications for biotechnology of these oxidases follow from the foregoing characteristics, from their already-demonstrated effectiveness as agents for selected bioremediations (11), catalysts for regiospecific biotransformations (2, 33), and participants in biosensor constructs (40), and from their considerable retention of activity in organic solvents (22).

The objectives of the work reported here were to identify a microorganism which would reproducibly and constitutively form substantial amounts of extracellular laccase on an inexpensive and defined medium, to develop a facile process for its purification, and to detail its salient biochemical features in relation to biotechnology transfers. We hereby describe an isolate of *Botrytis cinerea* which possesses these attributes and compare the molecular properties of its laccase to those of laccases from taxonomically related (44) and unrelated sources. The data demonstrate that this purified preparation has a specific activity about 1,000-fold higher than those reported from other sources and that it exhibits intrinsic kinetic, stability, and substrate range patterns which could be favorable for future commercial applications.

* Corresponding author. Mailing address: Chemistry Faculty, 315 Baker Laboratory, SUNY College of Environmental Science & Forestry, Syracuse, NY 13210. Phone: (315) 470-6850. Fax: (315) 470-6856.

MATERIALS AND METHODS

Microorganism and cultivation conditions. A large number of isolates of *B. cinerea*, locally obtained, were screened for laccase activity on potato dextrose agar plates in the presence or absence of inducers. Experimental conditions included amendation with either 100 mg of 2,5-xylidine per liter or 50 mg of cycloheximide per liter followed by visualization with 5 mM guaiacol solution. One particular strain, 61-34, isolated from a contaminated onion proved to have favorable biochemical properties, including rapid constitutive formation of extracellular laccase on a defined medium. This strain was cultivated on our modification of liquid medium (14), which contained the following (in grams per liter): glucose, 40; glycerol, 7; L-histidine, 0.5; $CuSO_4$, 0.1; $NaNO_3$, 1.8; NaCl, 1.8; KCl, 0.5; $CaCl_2 \cdot H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.05; KH_2PO_4, 1.0; and $MgSO_4 \cdot 7H_2O$, 0.5. For routine enzyme production in shake flasks, 100 ml of this medium in 500-ml wide mouth Erlenmeyer flasks was inoculated with a 10% (vol/vol) fungal spore suspension and incubated for up to 19 days at 24°C on a shaker at 250 rpm. For scale-up, 4 to 5 liters of growth medium in a 7.5-liter fermentor was inoculated with a 10% (vol/vol) mycelial suspension and incubated for up to 8 days at 24°C. The impeller was agitated at 500 rpm with a 2.5-standard-liter/min air flow.

Enzyme assays and kinetics. Measurement of enzyme activity was based on the oxidative dimerization of 2,6-dimethoxyphenol (2,6-DMOP) (3). To 500 μl of 5 mM 2,6-DMOP was added 500 μl of 100 mM citrate-NaOH (pH 3.5), followed by a 10-μl aliquot of enzyme dilution. Reaction mixtures were monitored at 477 nm with a Spectronic 1001 (Milton Roy Co., Rochester, N.Y.) equipped with an external Citizen iDP-560RS printer (Japan CBM Corp.). Reaction temperatures were controlled with a jacketed quartz cuvette connected to an L-2/R water bath (MGW Lauda, Messgeräte, Germany) and a 50% ethylene glycol–50% water mixture. Enzyme activities are expressed as katals. For this purpose, periodate-formed 3,3′,5,5′-tetramethoxydiphenoquinone was isolated and purified (melting temperature, 250 to 252°C) and was determined to have an E_m of 14,800 at 477 nm. Therefore, specific laccase activities are herein recorded as microkatals per milligram of protein. For K_m and V_{max} determinations, stage IV enzyme was diluted 120-fold with buffer and 10 μl of this dilution was added to substrates in cuvettes to a final volume of 1.01 ml. The protein concentration in the initial dilution was assayed by the Lowry et al. assay (18). The effect of pH on laccase activity was measured over the range of 1.5 to 7.0 in 50 mM citrate–NaOH buffer. The effects of temperature on enzyme activity and stability were measured at pH 3.5 with 50 mM sodium citrate at a range of 0 to 85°C.

Enzyme purification. From either shake flask or fermentor experiments, liquid cultures were centrifuged for 20 min at 20,000 × *g* in a GSA rotor with a Sorvall RC-5B centrifuge (stage I). The resulting supernatants were then treated with 10% (vol/vol) acetone and refrigerated for at least 1 h. The material was then centrifuged at 20,000 × *g* for 20 min to remove the β-1,3-D-glucan exopolysaccharide (stage II). The supernatant was first concentrated in a DC-2 ultrafiltration unit (Amicon Corp., Danvers, Mass.) equipped with a 30,000-molecular-weight-cutoff hollow-fiber filter unit (stage IIIA), which concurrently removed considerable sticky material. Further desalting and reduction to 20 to 50 ml took place in a stirred pressure cell (Amicon Corp.) equipped with a 47-mm 10,000-molecular-weight-cutoff filter (stage IIIB). Ammonium sulfate (30%, wt/vol) was added to this concentrate, and the mixture was then filtered through an 8-μm-pore-size mixed-cellulose filter (SC type; Millipore Corp., Milford, Mass.) to further remove particulate matter. The filtrates were then resolved by hydrophobic interaction chromatography (stage IV) in one of two ways. The first method involved phenyl-Sepharose Cl-4B (Pharmacia LKB Biotech., Piscataway, N.J.)

TABLE 1. Purification of fermentor-produced laccase[a]

Resolution stage	Vol (ml)	Amt of protein (mg ml^{-1})	Activity (mkat ml^{-1})	Sp act (mkat mg^{-1})	Recovery (%)	Purification (fold)
I. Mycelium-free broth	2,750	1.71	0.82	0.480	100	
II. Acetone (10%) supernatant	3,250	1.40	0.52	0.372	75	
IIIA and IIIB. Ultrafiltered retentates	13.5	21.9	136	6.20	81	13.5
IV. Major HIC enzyme peak	10.8	21.1	192	9.08	92	19.0

[a] Results are the average of two experiments.

packed in a Pharmacia low-pressure column (1.5 by 30 cm). A step gradient system was used to elute laccase from the packing material. Each step of the gradient consisted of 2 column volumes. The steps were (i) 2.67 M ammonium sulfate with 66.7 mM citrate–NaOH (pH 3.9), (ii) 1.34 M ammonium sulfate with 66.7 mM citrate–NaOH (pH 3.9), (iii) 0.267 M ammonium sulfate with 66.7 mM citrate–NaOH (pH 3.9), and (iv) 66.7 mM citrate–NaOH (pH 3.9). In the second method, hydrophobic interaction chromatography (HIC)–high-pressure liquid chromatography (HPLC) was carried out on a phenyl-5PW column (22.5 by 300 mm; Bio-Rad Laboratories, Hercules, Calif.). The flow rate used was 3 to 5 ml min^{-1}. The gradient was from 2.67 M ammonium sulfate in 66.7 mM citrate–NaOH (pH 3.9) to 66.7 mM citrate–NaOH (pH 3.9), and the gradient time was 50 min. To achieve reproducible elution profiles, we used a beginning gradient delay at 5 min and a 15-min hold at 66.7 mM citrate–NaOH (pH 3.9) at the end of the chromatographic run. The HPLC apparatus consisted of a no. 2360 gradient controller, a no. 2350 pump, and a UV-4 variable-wavelength detector (all from Isco Inc., Lincoln, Nebr.), a no. 7125 injection valve (Rheodyne Inc., Cotati, Calif.), and a fraction collector (Gilson, Middleton, Wis.). The column eluate was monitored at 280 nm. Fractions were collected at 1-min intervals and concurrently assayed for laccase activity.

Enzyme electrophoresis and isoelectric focusing. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of laccase from *B. cinerea* was carried out by the Laemmli method (16) in a dual-minislab gel apparatus (Novex, San Diego, Calif.). Alternatively, 12% acrylamide gels or gradient (5 to 20%) gels were used. Samples were dialyzed against water and concentrated in volume, and dissolution buffer (Novex) containing 125 mM Tris-HCl, 20% glycerol, 0.2% SDS, and 0.004% bromophenol blue was added. Molecular mass markers in the range of 20,000 to 250,000 Da (Novex) were used as standards. Visualization was accomplished with Coomassie brilliant blue R-250 (0.1%) dissolved in 40% ethanol–10% acetic acid–water. The gel was placed into the stain and heated in a microwave oven (high setting) for 1 min. The gel was then agitated at 25 rpm for 15 min, the stain was decanted, and the gel was rinsed with distilled water. A gel-destaining solution (Gel-clear 10× concentrate; Novex) was subsequently used to remove excess stain. The gel was again placed in the microwave for 1 min and allowed to further destain overnight. Protein bands were stained with Coomassie brilliant blue R-250. The marker proteins for pI determination were test mixture no. 9 (Serva Biochemicals, Westbury, N.Y.). Glycoprotein staining was achieved with the Schiff stain (34).

Isoelectric focusing was performed by the method of Adams (1), with mixed ampholytes (Servalytes pH 3.5–10; Serva) and 5% glycerol. For gels focused from pH 2.0 to 6.0, the cathodic buffer was 10 mM L-histidine and the anodic buffer was 10 mM phosphoric acid. The gels were prefocused initially for 3.25 h at 200 V (constant) and 1.75 mA. The current was then increased to a constant 500 V at 5 mA for an additional 1 h. Zymogram analysis was performed by immersing the gels in a solution of 18 mM 2,5-dimethylaniline plus 9 mM 4-amino-2,6-dibromophenol in 75% 10 mM citrate–NaOH (pH 3.5) containing 25% dimethyl sulfoxide. The exposed gels were gently agitated (5 rpm) for 5 min and then decanted. A solution of 75% 10 mM citrate–NaOH (pH 3.5) plus 25% dimethyl sulfoxide (vol/vol) was then added with shaking. The resultant precipitates from oxidized substrate polymerization pinpointed the location of the enzyme.

Determination of enzyme molecular mass. The molecular mass of laccase was measured by gel permeation chromatography (GPC) on a Bio-Sil TSK-250 HPLC column (7.8 by 300 mm; Bio-Rad) run at room temperature. The eluent used was 6 M guanidine HCl at a flow rate of 1 ml/min, and the injection volume was 100 μl. The column eluate was monitored at 280 nm. The HPLC used was an isocratic system (Isco Inc.) fitted with a no. 7125 (Rheodyne Inc.) injection valve. Protein standards (wheat germ acid phosphatase, 100,000 Da; bovine serum albumin, 66,000 Da; aldolase, 44,000 Da; and cytochrome *c*, 14,000 Da) were used to estimate the molecular mass of laccase.

Enzyme carbohydrate composition. Purified lyophilized laccase (32 mg, type IV) was dissolved in 2 ml of 4 M trifluoroacetic acid and placed into a sealed glass tube. Hydrolysis took place at 100°C for 6 h. The reaction mixture was then dried in a vacuum desiccator over solid NaOH and anhydrous $CaCl_2$. Distilled water was added, the liquid was evaporated, and the process was repeated three times. This material was then dissolved in 0.5 ml of distilled water, and the solution was filtered through a 0.45-μm-pore-size filter. Analysis by thin-layer chromatography was performed with HPTLC LHP-K plates (Whatman, Maidstone, England). The plates were impregnated with 0.1 M KH_2PO_4 and developed with isopropanol–acetone–1 M lactic acid (2:2:1) (15, 20) with appropriate carbohydrate standards. The chromatograms were developed twice with an air dry interval, and the detection reagent was 4 ml of aniline–4 g of diphenylamine–30 ml of acetone–200 ml of phosphoric acid. To complete the identification of enzyme carbohydrate constituents, HPLC (Bio-Rad HP-87H carbohydrate analysis column; 7.8 by 300 mm) was used. The eluent used was either 0.0065 N H_2SO_4 at a flow rate of 0.6 ml/min at 35°C or 0.013 N H_2SO_4–acetonitrile (85%:5%, vol/vol) at a column temperature of 30°C and a flow rate of 0.6 ml/min with an LC-25 refractive index detector (Perkin-Elmer Corp., Norwalk, Conn.). Alternatively, hydrazinolysis was used to release protein-bound oligosaccharides (24) from laccase, and the oligosaccharides were subjected to hydrolysis with 4 M trifluoroacetic acid and qualitative analysis as described above.

Denaturing GPC of enzyme. Guanidine HCl (2 M) in 50 mM Na_2HPO_4 (pH 7.3) was used as the eluent for HPLC-GPC on a Bio-Sil TSK-250 column (7.8 by 300 mm; Bio-Rad) at a flow rate of 1 ml/min. An isocratic HPLC system (Isco, Inc.) with a 200-μl sample loop was used. The eluate was monitored at 280 nm. Carbohydrates were analyzed by the phenol-sulfuric acid assay (22). Fractions, diluted with 0.5 ml of water before the assay, were taken every 30 s. To these, 25 μl of 80% phenol and 2.5 ml of concentrated sulfuric acid were added. The tubes were incubated for 30 min, and their A_{480} values were read.

Substrate specificity probes. Test compounds were dissolved in dimethyl sulfoxide to concentrations of 500 mg/ml for stock solutions. To these were added 50 ml of 20 mM citrate buffer (pH 3.5) to provide final concentrations of 1 to 3 g/liter. The enzyme was then added (0.1 ml stage IV, 1:100 dilution; 13.7 μkat) to the flasks, which were shaken at 24°C at 250 rpm. After 24 h, 10-ml samples of these reaction mixtures were extracted with 5 ml of ethyl acetate. The ethyl acetate layers were dried over Na_2SO_4, further diluted to 10 ml with solvent, and filtered through a 0.45-μm-pore-size filter (Durapore filter; Millipore Corp.). The resulting solutions were chromatographed on a Pl gel 3μ mixed-E GPC column (Polymer Laboratories Inc., Amherst, Mass.) with ethyl acetate at a flow rate of 1 ml/min. Fraction peaks were monitored at both 254 and 280 nm.

RESULTS

Growth and enzyme production. When grown in shake flasks for 8 days at room temperature on modified Dan'Shina (9) medium, *B. cinerea* 61-34 approached 5 g/liter (mycelial dry weight) with concomitant utilization of 20% glucose. At this point, enzyme formation approached values in excess of 300 μkat/ml. A comparison of enzyme production with time for aerated fermentation versus shake flasks (data not shown) demonstrated that the laccase yields under the former conditions are at least doubled in half the time. For this reason, bulk enzyme production routinely followed a workup scheme after fungal growth in 7.5-liter fermentors (see Materials and Methods).

Purification of extracellular laccase. A facile four-step purification of the enzyme was developed (Table 1). The following purification stages are delineated. Stage I is defined as the medium after the removal of fungal mycelia. Stage II is the supernatant after the removal of 1,3-β-glucan exopolysaccharides (18) by 10% (vol/vol) acetone. Stages IIIA and IIIB reflect volume reduction and clarification after physical concentration. Qualitative analysis of residual material trapped on the filter discs from stages IIIA and IIIB indicated the presence, besides polysaccharide, of extraneous protein which was devoid of laccase activity. The filtrates contained ca. 25% of

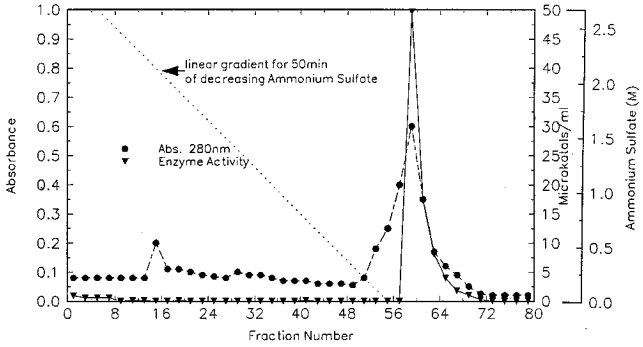

FIG. 1. Elution profile of stage III laccase subjected to HIC resolution on a Bio-Gel-5 PW column.

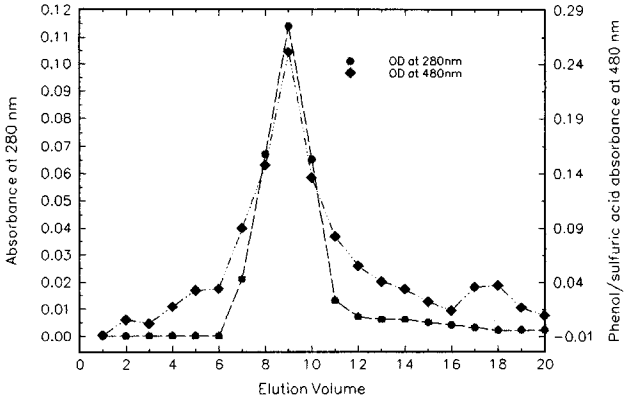

FIG. 3. Coincidence of carbohydrate and protein profiles of purified laccase following HPLC-GPC with 2 M guanidine in phosphate buffer (pH 7.3).

the initial mycelium-free broth "protein." Stage IV represents purified enzyme after HIC. These operations are tabulated in Table 1, which gives the average of two sequential fermentor runs. The pale-blue extracellular laccase (stage IV) was purified approximately 19-fold and had a specific activity of 9.1 mkat mg of protein^{-1}. Extrapolation of these data indicates that approximately 80 mg of homogeneous enzyme was obtained per liter of starting volume. Laccase purified to stage IV appears to be stable indefinitely when kept at −80°C in 50% glycerol–50% 50 mM citrate-phosphate buffer (pH 3.5).

The results of the final HIC purification step, on a Bio-Gel-5 PW column, are presented in Fig. 1. The major peak of enzyme activity appeared as a blue band whose progression was visible during the decreasing ammonium sulfate gradient. Fractions 56 to 63 were collected and combined to provide stage IV enzyme with a specific activity, using 2,6-DMOP as substrate, of ca. 9 mkat mg of protein^{-1}. SDS-PAGE resolution of this preparation revealed its homogeneity (Fig. 2), which was confirmed by isoelectric focusing. No other proteins or Schiff's-reactive glycoproteins were detected in these analyses. Comparison with protein standards gave an estimated pI of 4.0 for this laccase (data not shown). An additional zymogram demonstrated that protein, carbohydrate, and enzyme activities were coincident.

Stability and inhibitor parameters. Purified enzyme (stage IV) at 25°C exhibited a broad pH optimum range, centering at pH 3.5 (Fig. 2A). The temperature optimum for resolved laccase (stage IV) in 0.1 M citrate buffer (pH 3.5) is around 60°C (Fig. 2B). The inhibitory activities for a number of known and presumptive laccase antagonists, with a resolved stage IV enzyme preparation, were examined. In keeping with the general properties of fungal laccases (4), the enzyme from this isolate of *B. cinerea* was inhibited by 0.6 mM diethyldithiocarbamate (88%), 0.5 mM EDTA (73%), 0.6 mM sodium azide (29%), and 2.5 mM KCN (100%). Furthermore, tropolone, a compound which is known to inhibit the copper-containing tyrosinases (38), did not affect laccase from *B. cinerea* at up to 5.0 mM. Additionally, *B. cinerea* laccase was unaffected in its catalysis by SDS concentrations of up to 4% or by 2 M urea.

Carbohydrate content. Purified laccase, as analyzed by the phenol-sulfuric acid colorimetric assay (12), contains 49% carbohydrate. Laccase hydrolyzed with 4 M trifluoroacetic acid directly, or subjected to initial hydrazinolysis beforehand, was analyzed for monosaccharide composition by thin-layer chromatography as well as by two HPLC methods. The only carbohydrate monomers so far detected in these hydrolysates were glucose and mannose. To ensure that this result was not a manifestation of an association of the fungal 1,3-β-glucan with the enzyme, denaturing GPC in the presence of 2 M guanidine hydrochloride was carried out (Fig. 3). The results clearly indicate that the substantial reducing-sugar components of purified laccase represent an integral portion of this glycoprotein.

Molecular mass, subunit composition. The molecular mass of the purified stage IV laccase was determined by denaturing GPC-HPLC (Fig. 4A). Parallel analyses of the laccase from *B.*

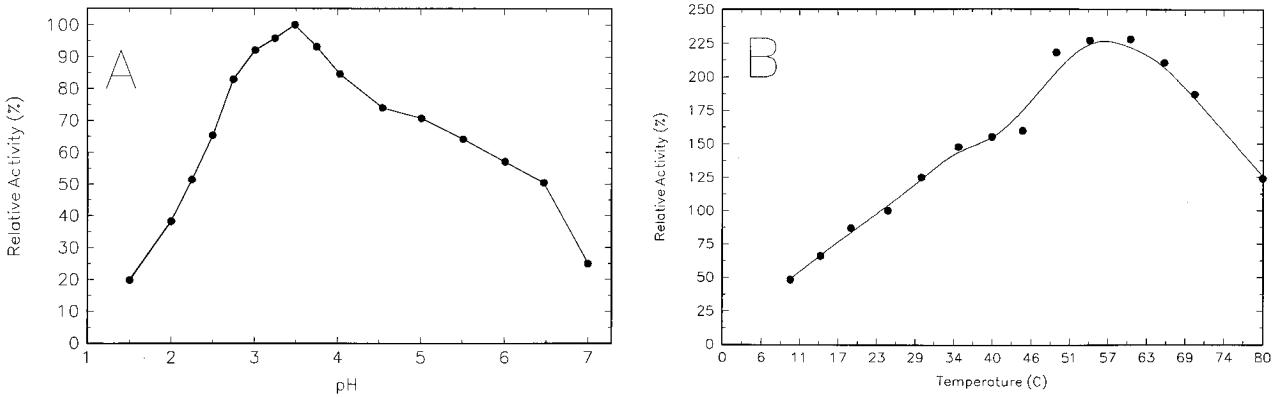

FIG. 2. Relative activity of purified (stage IV) laccase as a function of pH at 25°C (A) and as a function of temperature at pH 3.5 (B).

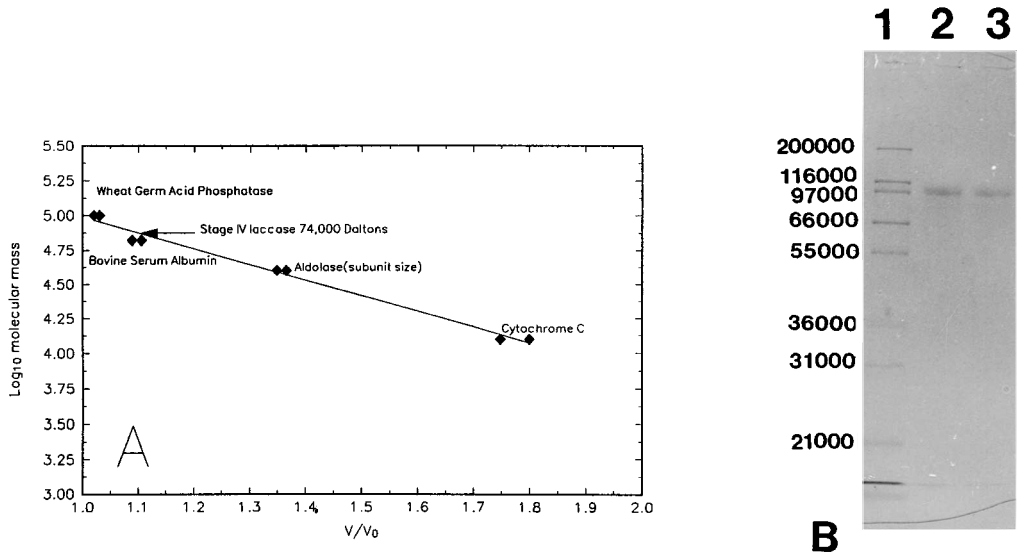

FIG. 4. Molecular mass determinations on stage IV laccase by gel filtration and by SDS-PAGE. (A) Enzyme and protein standards chromatographed on a Bio-Sil TSK column under denaturing conditions. (B) SDS-PAGE. Lanes: 1, molecular mass standards; 2 and 3, 10 and 5 μg of enzyme, respectively. V/V_0, eluted volume for each standard divided by the void volume.

cinerea by SDS-PAGE (Fig. 4B) as well as by isoelectric focusing had already indicated its monomeric nature. Denaturant size exclusion chromatography of the purified laccase with guanidine, along with glycoprotein standards, allowed for an extrapolated molecular mass of 74,000 Da for this preparation.

Enzyme kinetics. Enzyme activity versus substrate concentration for 2,6-DMOP, using stage IV laccase, is delineated in Fig. 5. The insert represents a Lineweaver-Burke plot of these data. By extrapolation, a K_m of 100 μM and a V_{max} of 10.5 mkat mg^{-1} were determined.

Substrate specificity. As evidenced by its oxidative transformation of a diverse number of phenols and chlorinated phenols, aliphatic and aromatic amines, phenylpropanoids, and heterocyclic (triarylimidazoles) and alicyclic compounds, *B. cinerea* 61-34 laccase exhibited a rather relaxed substrate specificity. Additionally, it was demonstrated (data not shown) that this enzyme oligomerizes and dechlorinates 4,5-dichloroguaiacol (30).

DISCUSSION

A screening of *B. cinerea* isolates yielded one clone, strain 61-34, which is a constitutive producer of extracellular laccase on a defined medium under either shake flask or fermentation conditions. The isolation of laccase-derepressed mutants of *Neurospora crassa* by UV irradiation was reported previously (36). Although the relative amounts of enzyme formed by the latter were higher than from the wild type induced with cycloheximide, these specific activities were significantly lower than those we have found for strain 61-34. On the basis of current

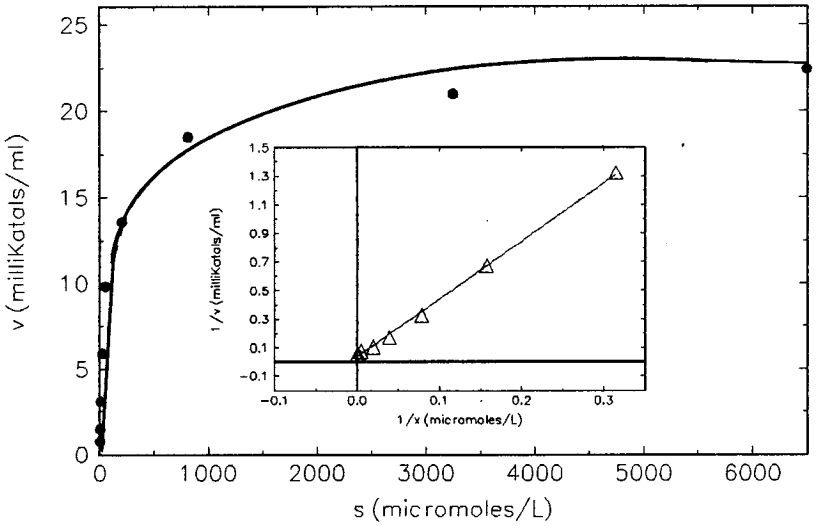

FIG. 5. Stage IV laccase kinetics in relation to substrate (2,6-DMOP) concentration. (Inset) Lineweaver-Burk plot of the data. Enzyme dilutions for this assay are given in the text.

knowledge about the catalytic mechanisms for copper-containing oxidases (21), the substitution of L-histidine for L-aspartic acid and the 10-fold increase in copper ion concentration were essential modifications to the medium of Dan'shina et al. (9) for optimizing enzyme production. Earlier, the induction of laccase in *B. cinerea* cultures by 2,5-xylidine (4), by combinations of gallic acid and pectin (39), and by phenolic constituents presumed to be present in muscat grape juice (44) was reported. Under the conditions specified in this article, *B. cinerea* can form over 350 μkat of crude enzyme ml^{-1}, in comparison with 26 μkat ml^{-1} for enzyme from *Basidiomycets* strain PM-1 (8), 240 μkat ml^{-1} for the *Cryphonectria* enzyme (28), or 8 nkat ml^{-1} for a recombinant *Trichoderma reesei* enzyme expressed in *Phlebia radiata* (31).

A facile purification scheme for *B. cinerea* 61-34 laccase, following initial precipitation of coproduced 1,3-β-glucan (35) with dilute acetone and then concentration and clarification by ultrafiltration, was completed by HIC on a phenyl-substituted column. Enzyme concentration by sequential physical procedures (stages IIIA and IIIB) resulted in a reproducible 15- to 20-fold purification by removal of nonspecific, lower polypeptides, as well as by protein entrapment on filters. Earlier, Coll et al. (8) showed a sixfold purification of laccase activity from a basidiomycete by the ultrafiltration process. By this means, homogeneous enzyme was resolved in 80% yield and provides over 80 mg of enzyme liter of initial mycelium-free $broth^{-1}$. Comparable-scale purification of a labile and lower-specific-activity laccase from *Agaricus bisporus* grown on compost has been reported (19). Further, the quantity of high-specific-activity material from *B. cinerea* 61-34 exceeds the yields of recombinant laccase (31) from *P. radiata* or that from *Lentinus tigris* (14) by a four- to sevenfold factor. Laccases from *B. cinerea* strains have previously been purified by ion-exchange chromatography (44) and by affinity chromatography (29). HIC on phenyl-substituted columns also appears to be the method of choice for the purification of other phenol oxidases (27).

As determined by denaturing size exclusion chromatography against marker glycoproteins (Fig. 4A), the molecular mass of the laccase purified in this study is approximately 74,000 Da. This value is considered more reliable than the higher molecular mass, which could have been extrapolated from the SDS-PAGE data (Fig. 4B), based on the known anomalous migrations of glycopeptides (17) when analyzed by this procedure. While this finding is generally in keeping with those for laccases obtained from strains within a wide variety of fungal genera (37), it is at variance in particular with the molecular masses (ca. 52,000 Da) for the *B. cinerea* enzyme found by Mayer et al. (20), who attributed differences in enzyme molecular masses to be a function of the inducers used; and with the molecular mass determinations (up to 102,000 Da) by Zouari et al. (44) for enzymes from several isolates of *B. cinerea*. Another apparent discrepancy between the laccase we have obtained from *B. cinerea* 61-34 and those reported in these earlier studies lies in the extent and composition of its glycosyl residues. Thus, while all laccases are intrinsically glycoproteins and may vary in carbohydrate content from ca. 15 to 80% (37), the *B. cinerea* enzymes were assessed as containing 86 to 91% reducing-sugar content (13), comprising heteropolysaccharide(s) with rhamnose, arabinose, xylose, galactose, glycose, and *N*-acetylglycose residues (14). The homogeneous laccase preparation which we describe in this article contains 49% carbohydrate, primarily as glucose and mannose. Because of this unanticipated finding, care was exercised to ensure that this result did not reflect association of fungal cell wall-associated glucan with the purified enzyme. This possibility was minimized by demonstration, under denaturing size exclusion chromatography, that the saccharide profile was coincident with that of enzyme activity (Fig. 3). At this point, aside from strain differences, no rational hypothesis for these differences in sugar composition of the various laccases from *B. cinerea* can be offered. It is assumed that these covalently linked sugar residues protect this laccase, as was postulated for laccase III from *Trametes* (*Coriolus*) *versicolor* (43), from high-temperature denaturation (see below) and perhaps also from proteolysis. It is also likely that the high concentrations of covalently bound carbohydrate moieties of this enzyme contribute to its resistance to denaturation and high residual activity in wet organic solvents (data not shown).

The temperature optimum for laccase from *B. cinerea* 61-34 is in fact 60°C, and the pH optimum is 3.5. The enzyme is, furthermore, tolerant to relatively high concentrations of urea and to SDS. It was subject to inhibition by concentrations of diethyldithiocarbamate, EDTA, and azide in the range that inhibited other fungal laccases (4), although it was refractory to the copper chelator tropolone. The specific activity of the stage IV enzyme, with 2,6-DMOP substrate, was 9.1 mkat mg of $protein^{-1}$. Extrapolation of Lineweaver-Burk plots gave a value of 10.5 mkat mg of $protein^{-1}$ and a K_m of 100 μM. The latter constants can be contrasted with a K_m of 178 μM for laccase I from *Armillaria mellea* (also with 2,6-DMOP) (26) and K_m values of ca. 500 μM for highly purified laccases from *Basidiomyces* PM-1 (with the substrate guaiacol) (8) and *L. tigris* (with the substrate pyrocatechol) (14).

A wide variety of phenols, aromatic and aliphatic amines, hetero- and alicyclic compounds, and redox mediators can serve as substrates for this enzyme. In contrast to laccases 1 and 2 from other *Botrytis* isolates (44), which do not attack chlorinated phenols, the enzyme described here can oxidize and dechlorinate several of these substrates, as was first demonstrated for the laccase from *T. versicolor* (30). It is of interest that although cholic acid and α-conidendrin are substrates for laccase oxidation, podophyllotoxin, for example, is biotransformed only in the presence of 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS). This observation relates to earlier studies of Sariaslani et al. (33) on redox mediator-assisted laccase oxidations of nonsubstrates and upon the expanded role for laccase biotransformations of phenylpropanoids (5) with electron relay compounds.

The foregoing characteristics which have been detailed for this laccase from *B. cinerea*, including a facile production and purification process, combined with the intrinsic kinetic attributes of this particular oxidase, would appear to lend themselves to a variety of industrial applications. Among such biotechnology transfers envisioned for both the free and immobilized laccase forms are utilization for effluent treatment in the pulp and paper industry, application in biobleaching and/or biopulping, use in stressful (i.e., organic) environments for substrate transformations (22), applications in the fabrication of amperometric or optical biosensors, as an agent in chemoenzymatic polymer synthesis, and applications in "dry-reagent" or film chemistries for beverage stabilization (6). Additionally, it is conceivable, by the use of site-directed mutagenesis (7), to alter the type 1 or type 2 copper site in this protein and hence its catalytic and specificity properties. If successful, the use of laccase biocatalysis in the foregoing technologies could save energy inputs and avoid by-product pollutions at the source as well as in downstream processing. We are currently exploring several of these facets.

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