Purification and Properties of *Neurospora crassa* Laccase

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Extracellular *Neurospora* laccase (*p*-diphenol:oxygen oxidoreductase; EC 1.10.3.2) has been purified to apparent homogeneity by classical purification techniques. The enzyme, which consists of mainly one form, has a molecular weight of 64,800 and contains 11% carbohydrate. The ultraviolet, visible, and electron paramagnetic resonance spectra indicate that both type I and type II copper are present, as described for the *Polyporus versicolor* enzyme. With the exception of phloroglucinol, only *para*- and *ortho*-diphenols serve as effective substrates for the enzyme. Like the extracellular form, intracellular laccase is a glycoprotein as shown by its ability to bind to Concanavalin A Sepharose. Other studies, including gel filtration and ion-exchange chromatography, revealed no differences between the intracellular and extracellular enzymes, suggesting that intracellular laccase is destined for excretion by the cell.

Laccase (*p*-diphenol:oxygen oxidoreductase; EC 1.10.3.2) has been isolated and purified from a number of different fungi, particularly *Podospora anserina* (6) and *Polyporus versicolor* (4, 9, 21). Physical characterization of the laccases produced by these two fungi has indicated differences in the molecular weight, carbohydrate content, and quaternary structure. In a previous paper, we showed that rapidly growing *Neurospora crassa* can be induced to produce laccase with low concentrations of protein synthesis inhibitors (11). Most of the enzyme is excreted into the medium. We have purified this extracellular enzyme to apparent homogeneity to compare its properties with those of other fungal laccases.

Similarities in the conditions of induction, plus studies with mutant strains, suggested that the synthesis of *Neurospora* laccase and tyrosinase (*o*-diphenol:oxygen oxidoreductase; EC 1.10.3.1) is regulated by the same mechanism, possibly at the level of transcription (11). Because of its extracellular location, laccase can also be controlled by regulating its transport through the cell membrane. As a basis for continued studies on the mechanism and control of the export of this phenol oxidase, we have performed limited investigations on the intracellular enzyme to determine if it is enzyme destined for excretion by the cell or if intracellular and extracellular laccase are totally different enzymes with different functions.

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MATERIALS AND METHODS

Organisms. *Neurospora crassa* wild-type strain 69-1113a maintained on agar slants (14) was used for enzyme production.

Culturing conditions for enzyme production. For the production of large amounts of laccase for purification, low-form culture flasks (2,000 ml) containing 500 ml of Fries minimal media (15; 20% normal sulfur concentration) were inoculated with 1.0 ml of a conidial suspension (absorbancy at 600 nm [A_{600}] $\simeq$ 0.1) and incubated for 2.5 to 3 days at 35 C without shaking. After the addition of cycloheximide to a final concentration of 2.8 μM (0.20 ml of a 2.0-mg per ml solution in 70% ethanol), incubations were continued for 6 days at 30 C on a rotary shaker at 150 rpm with a stroke diameter of 4 cm. Twenty-two flasks cultivated simultaneously produced 400 to 600 U of enzyme (see below for definition of an enzyme unit).

Extracellular laccase purification procedure. All steps were performed at 5 C unless otherwise noted. The culture fluid from 22 flasks (~11 liters) was collected by filtration at room temperature and concentrated by ultrafiltration (Amicon TC ultrafiltration system, model 1 B; PM-30 membrane) to about 1.5 liters. Concentrates were stored at −20 C (stable for at least 6 months) until several batches were collected.

Three or four batches of concentrated culture filtrate were pooled and 243 g of crystalline $(NH_4)_2SO_4$ per liter of solution (40% saturation) were added with stirring (no pH adjustment). After 2 to 3 h, the solutions were centrifuged at 23,300 × *g* for 20 min. The supernatant fluid was then increased to 80% saturation by the addition of 285 g of $(NH_4)_2SO_4$ per liter of solution and stored overnight. The precipitate was collected by centrifugation (23,300 × *g*, 20 min) and redissolved in 25 to 40 ml of 0.10 M sodium phosphate buffer, pH 6.0. The dark brown solution

was clarified by centrifugation at 17,300 × *g* for 15 min. The sample was then chromatographed (upward flow) on a G-100 Sephadex column (5.0 by 92 cm) equilibrated with 0.10 M sodium phosphate buffer, pH 6.0. Samples (16 ml) were collected at a flow rate of 4 ml per h per cm². After determination of the activity and absorbancy profiles, the appropriate fractions were pooled, 561 g of $(NH_4)_2SO_4$ was added per liter of solution, and the mixture was allowed to precipitate overnight. After collection by centrifugation as above, the precipitate was dissolved in 15 to 25 ml of 0.02 M sodium phosphate buffer, pH 7.2, and dialyzed against the same buffer (2 × 1 liter for at least 2 h each). After clarification by centrifugation (if necessary), the sample was applied to a diethylamino-ethyl (DEAE)-Sephadex A-50 column (2.8 by 16 cm) equilibrated with 0.02 M sodium phosphate buffer, pH 7.2. The column was then washed with the equilibration buffer at a flow rate of 10 ml per h per cm², which eluted most of the laccase activity as a single band after about 3-column volumes of eluant. Although smaller amounts of enzyme were eluted with stepwise changes to 0.05 M and 0.15 M sodium phosphate buffer, pH 7.2, only the material eluted with 0.02 M buffer, which had a much higher specific activity, was purified further. After concentration to about 10 ml with a collodion bag (Sartorius membrane filter, Göttingen, Germany), the sample was dialyzed against 0.02 M sodium phosphate buffer, pH 7.2, and applied to a hydroxylapatite column (2.0 by 16 cm; Bio-Gel HT, Bio-Rad Laboratories Richmond, Calif.) equilibrated with the same buffer. After washing with the equilibration buffer for 2- to 3-column volumes, the column was developed with a linear gradient of 150 ml of equilibration buffer plus 150 ml of equilibration buffer containing 0.40 M $(NH_4)_2SO_4$. Fractions of 3.6 ml were collected at a flow rate of 4 ml per h per cm². Most of the activity eluted as a band coincident with an absorbancy band (see Fig. 3). These fractions were pooled and concentrated with a collodion bag to about 3 ml. The purified enzyme was stored frozen at −20 C after dialysis against 0.10 M sodium phosphate buffer, pH 6.0.

Isolation and purification of intracellular laccase. Washed mycelial pads (~50 g) were ground with sand in a mortar and pestle and extracted with 3 to 5 volumes of 0.10 M sodium phosphate buffer, pH 6.0, by stirring in the cold for 30 min. After centrifugation at 23,300 × *g* for 20 min, the supernatants were precipitated with $(NH_4)_2SO_4$ as described for the extracellular enzyme, except that the 20 (114 g per liter) to 60% (262 g per liter of 20% saturated solution) fraction was taken. After dissolution in 0.10 M sodium phosphate buffer, pH 6.0 (1 ml per gram of mycelium), the sample was applied to a Concanavalin A Sepharose column (1.0 by 8.0 cm; Pharmacia Fine Chemicals AB, Uppsala, Sweden) at a flow rate of 3 ml per h per cm². The column was washed until A_{280} was less than 0.10, and the enzyme was eluted with 10% α-methylmannoside in 0.10 M sodium phosphate buffer, pH 6.0. The appropriate fractions were pooled, dialyzed against 0.10 M sodium phosphate buffer, pH 6.0, concentrated with a collodion bag to about 2.0 ml,

and chromatographed on a G-100 Sephadex column (2.5 by 95 cm) as described for the extracellular enzyme. Some preparations were purified further on a DEAE-Sephadex A-50 column (1.5 by 15 cm) as described above. For large preparations, the enzyme was bound to Concanavalin-A Sepharose in a batch-wise manner, and the column was poured and developed as described above. This avoided clogging the column, which often occurred with concentrated samples.

Enzyme assays. For determination of column profiles, the colorimetric assay with 10 mM dihydroxyphenylalanine was performed with 10 to 100 µliters of enzyme as previously described (11). For quantitative determination of yields and substrate specificity, oxygen consumption was measured with an oxygen electrode (Rank Brothers, Cambridge, England). The reaction was initiated by the addition of 10 to 100 µliters of enzyme solution to 5.0 ml of 0.01 M guaiacol in 0.10 M sodium phosphate buffer, pH 6.0, which had been equilibrated to 25 C in the electrode chamber. Initial rates of oxygen consumption were noted, and the actual amount of oxygen consumed was calculated assuming an initial concentration of 8.5 mg of O_2 per liter at 25 C. One unit of laccase is defined as the amount of enzyme required to consume 1.0 µmol of oxygen per min at 25 C (pH 6.0) with 10 mM guaiacol as the electron donor. Tyrosinase activity was determined colorimetrically as described for laccase except with 2.0 mM tyrosine as the substrate.

Polyacrylamide gel electrophoresis. Native gel electrophoresis (nondenaturing conditions) was performed according to Gordon (13) with a 7.5% acrylamide gel (6 × 110 mm) at pH 4.5. Samples (100 µliters) were subjected to electrophoresis for 2.5 h at 6 mA per gel toward the cathode. For the detection of protein, gels were stained with 0.25% Coomasie brilliant blue in methanol-water-acetic acid (5:5:1) for 4 h at room temperature, and then were diffusion destained in the same solution without the stain. For the detection of laccase activity, gels were immersed in 0.01 M guaiacol and 0.10 M sodium phosphate buffer, pH 6.0, and were transferred to 7% acetic acid as soon as the color appeared. Sodium dodecyl sulfate gel electrophoresis was performed according to Laemmli (18) with 10% acrylamide gels. After destaining, the gels were photographed. The outlines were cut out and rephotographed on a white background. This procedure yields a more desirable illustration without affecting the information presented.

Molecular weight determination by sedimentation equilibrium. Purified laccase at a concentration of 0.5 mg/ml in 0.05 M sodium acetate, pH 5.0, was centrifuged at 20 C for 16 h at 20,000 rpm in a capillary-type synthetic boundary cell. Calculations were carried out according to Chervenka (3) with the partial specific volume calculated from the amino acid and carbohydrate compositions.

Carbohydrate determination. The amount of carbohydrate present in the purified enzyme was determined by the orcinol method (23). An enzyme sample of 0.30 ml containing about 100 µg of protein was mixed with 0.6 ml of orcinol (2.0 mg/ml in concen-

trated H_2SO_4) and heated at 80 C for 15 min. A_{540} was then compared to a standard curve determined with glucose.

Absorption coefficient determination. After extensive dialysis against deionized, distilled water, samples were freeze-dried and the A_{280} determined directly on weighed samples which had been redissolved in 0.10 M sodium phosphate buffer, pH 6.0.

Copper determination. To remove free copper present as contaminants in the buffer, samples were either passed through a small (0.5 by 5.0 cm) Chelex-100 column or dialyzed against 0.10 M sodium phosphate buffer, pH 6.0, which had been purified by Chelex-100 chromatography. Samples treated in either manner gave the same results. Copper content was determined by atomic absorption spectrometry.

Spectral analyses. The ultraviolet and visible spectra were determined at pH 6.0 on a Cary 118 recording spectrophotometer in a 1-cm path length cell at 25 C. Electron paramagnetic resonance (EPR) spectroscopy was performed with a JES-ME-1X instrument (Japan Electron Optics Co. Ltd., Tokyo) in liquid nitrogen on samples which had been passed through a Chelex-100 column to remove free copper.

Amino acid content determination. Samples of enzyme were hydrolyzed in 6 M HCl at 110 C for 24 and 72 h, and the hydrolysates were analyzed on a Biochrom automatic amino acid analyzer equipped with an Infotronic CRS-12 integrator. Only the 72-h values were used for histidine, proline, half-cysteine, and isoleucine, and zero-time values calculated by first order extrapolation were used for serine, threonine, and tyrosine. Tryptophan was determined by the spectrophotometric method of Bencze and Schmid (1).

RESULTS

Extracellular laccase purification and evidence of purity. The results of a typical purification procedure for the extracellular enzyme are shown in Table 1. Since several other proteins and large amounts of pigment were present in the media, the purification scheme was designed to remove both these contaminants. The ratio A_{280} to A_{250} is an indication of the amount of pigment present and should be greater than 2.0 for the purified protein (9). Large amounts of pigment were removed by ultrafiltration and ammonium sulfate precipi-

tation, and, after G-100 chromatography (Fig. 1), the active fractions were blue in color. The pigment was located in the small, low-molecular-weight region between fractions 100 and 120. Chromatography on DEAE-Sephadex A-50 (Fig. 2) resulted in severe losses of activity (<10% recovery) if larger columns were used. Though three fractions of activity were resolved by this step, only the first one (representing ~80% of the recovered activity) was purified further (see below). The elution of the enzyme from hydroxylapatite with a salt gradient showed coincidence of the protein and activity bands (fractions 70 to 80 in Fig. 3), suggesting that the enzyme was nearly pure. Indeed, analysis by polyacrylamide gel electrophoresis confirmed the purity of laccase purified by this scheme. Electrophoresis on native gels stained for protein showed one rather broad band (Fig. 4). That this band was, in fact, laccase was indicated by its coincidence with the band stained for activity in a gel run simultaneously. Sodium dodecyl sulfate gel electrophoresis, which separates proteins according to molecular weight, showed a major band which contained >90% of the protein plus a minor low-molecular-weight contaminant (Fig. 4). No attempt was made to determine the molecular weight of laccase on sodium dodecyl sulfate gels since
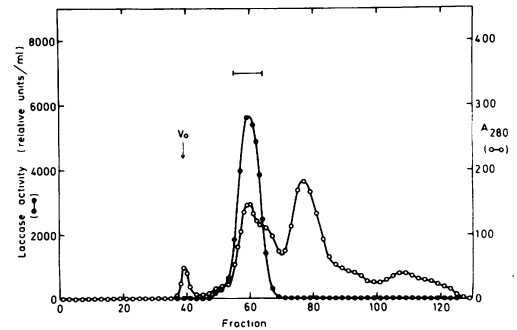

FIG. 1. *Gel filtration of* $(NH_4)_2SO_4$ *precipitate on G-100 Sephadex. Chromatography was performed as described. V_0 denotes the void volume of the column, and the bar indicates the fractions pooled.*

TABLE 1. *Purification steps for extracellular laccase*

| Purification step	Vol (ml)	Total activity (U)	Total A_{280}	Sp act (U/A_{280})	A_{280} to A_{250} ratio	Yield (%)	Fold purification
Culture filtrate	44,320	1,750	32,110	0.055	0.63	100	1
Ultrafiltrate	4,140	1,776	5,423	0.327	0.83	101	6
$(NH_4)_2SO_4$ precipitation	39	1,343	1,086	1.24	1.51	77	22
G-100 Sephadex chromatography	162	936	181	5.17	2.36	53	94
DEAE-Sephadex A-50 chromatography	122	565	38.2	14.8	2.13	32	269
Hydroxylapatite chromatography ...	43	337	16.8	20.1	2.21	19	365
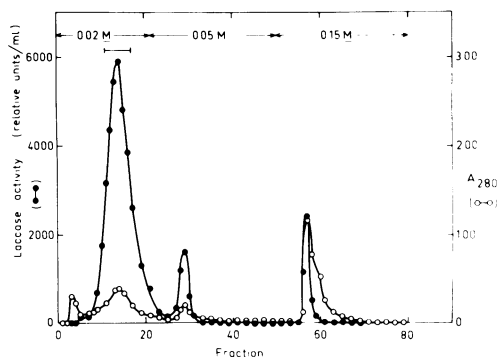
FIG. 2. *Chromatography of laccase on DEAE-Sephadex A-50. Chromatography was performed as described. The bar indicates the fractions pooled.*
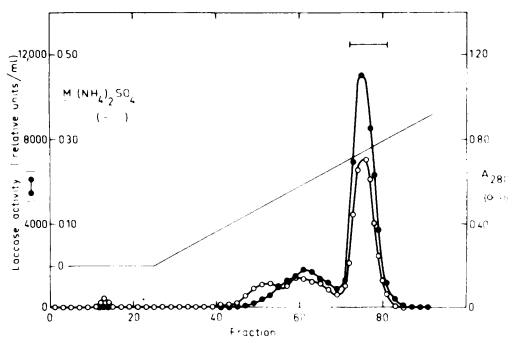

FIG. 3. *Adsorption chromatography on hydroxylapatite. Chromatography was performed as described. The bar indicates the fractions pooled.*

glycoproteins are known to migrate abnormally in this system.

The activity which eluted from DEAE-Sephadex with 0.05 M and 0.15 M buffer may represent laccase plus various amounts of tightly bound pigment since the A_{280} to A_{250} ratio was low (1.50 to 1.80). Several ultrafiltrates which contained large amounts of pigment also contained correspondingly larger amounts of these forms, sometimes as much as 40% of the total enzyme. Chromatography of the third form on hydroxylapatite resulted in an extremely broad elution pattern of activity rather than a sharp band as shown in Fig. 3. This point requires further study.

Molecular weight of extracellular laccase. As determined by sedimentation equilibrium, purified extracellular laccase has a molecular weight of 64,800. This value was obtained using a partial specific volume of 0.715, which was calculated from the amino acid composition (5) and the carbohydrate content. A partial specific volume for the carbohydrate of 0.613 was used (12). A plot of the log concentration versus R^2 (R

is the distance from the center of rotation; Fig. 5) was linear, indicating size homogeneity of the sample.

Amino acid composition. The amino acid composition (Table 2) showed no unusual features except for a relatively high amount of aromatic amino acids, particularly tryptophan. This was also reflected in the unusually high molar extinction coefficient at 280 nm (1 cm) for purified laccase (9.07×10^4).
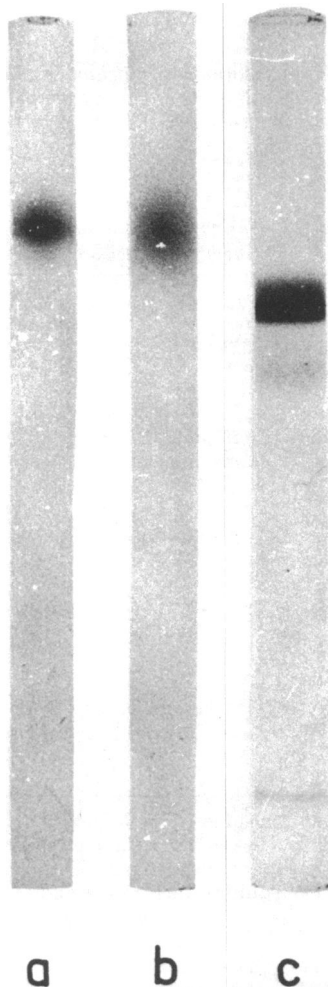
Isoelectric point. The ratio of the acidic-

FIG. 4. *Polyacrylamide gel electrophoresis of purified extracellular laccase. (a) Nondenaturing gel (pH 4.5) of 15 μg of purified laccase stained for protein. (b) Nondenaturing gel (pH 4.5) of 0.3 μg of purified laccase stained for activity. (c) Sodium dodecyl sulfate gel of 15 μg of purified laccase stained for protein. The first two were prepared simultaneously. The band near the bottom of the third gel is the tracking dye.*
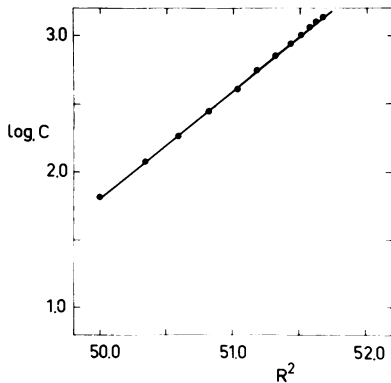
FIG. 5. *Sedimentation equilibrium of purified extracellular laccase. R is the distance from the center of rotation in centimeters and C is the protein concentration.*

TABLE 2. *Amino acid composition of extracellular laccase[a]*

Amino acid	No. of residues
Lysine	20
Histidine	16
Arginine	21
Aspartic acid + asparagine	77
Glutamic acid + glutamine	35
Threonine	34
Serine	27
Methionine	6
Half cysteine	2
Valine	32
Isoleucine	27
Leucine	38
Tyrosine	20
Phenylalanine	19
Tryptophan	26
Proline	33
Glycine	48
Alanine	35

[a] Σ number of residues, 516; Σ molecular weight of residues, 58,053; molecular weight of carbohydrates (calculated from molecular weight of enzyme [sedimentation equilibrium] and weight percent of hexose) 6,934.

basic residues suggests that the isoelectric point of laccase was acidic. Since laccase is bound poorly to DEAE-Sephadex at pH 7.2 and is bound to SP-Sephadex at pH 5.0, the isoelectric point must be between pH 5.0 and pH 7.2, at least at an ionic strength of ~0.02. Isoelectric focusing in polyacrylamide slab gels (24) or on a preparative scale indicated several bands (10 to 12) of laccase activity, especially if performed with partially purified preparations.

Carbohydrate composition. Approximately 11% by weight of extracellular laccase was carbohydrate, as determined by the orcinol method (76.6 ± 2.2 μg of hexose per A_{280} or 107 ± 14.5 μg of hexose per mg of enzyme; 1.40 ± 0.15 A_{280} per mg of enzyme). This carbohydrate was probably bound covalently since it was not removed from the enzyme during purification, by extensive dialysis or by chromatography on Bio-Gel P-30. Furthermore, extracellular laccase bound to Concanavalin A Sepharose at high ionic strength (1 M) and could be eluted with α-methyl-mannoside. Concanavalin A binds α-D-mannosyl, α-D-glucosyl, and sterically similar residues and, thus, absorbs polysaccharides and glycoproteins that contain such residues. α-Methyl-mannoside is a competitor of the binding reaction. Preliminary analysis of the hexoses present in laccase indicated large amounts of mannose plus small amounts of two sugars which were not readily identifiable. Small amounts of glucosamine were also detected in the amino acid determination. Such amino sugars often serve as the linkage between the carbohydrate chain and the protein via the side chain of threonine, serine, glutamine, or asparagine.

Spectral characteristics and copper content. The ultraviolet spectrum of extracellular laccase (Fig. 6) was typical for a protein, except for a shoulder at about 330 nm. As expected from the deep blue color, the visible spectrum contained a peak at about 595 nm. Since *Neurospora* laccase contained about 3 mol of copper per mol of enzyme (1.93 ± 0.12 μg of copper per A_{280} or 2.70 μg of copper per mg of enzyme; 1.40 ± 0.15 A_{280} per mg of enzyme), it was of interest to perform EPR spectroscopy to examine the state and environment of the copper. The EPR spectrum of one preparation is shown in Fig. 7. An interpretation of these spectral data is presented below.

Substrate specificity of extracellular laccase. A study of the substrates of *Neurospora* laccase was performed by using oxygen consumption as a measure of activity. Both para- and ortho-diphenols were effectively oxidized by *Neurospora* laccase, whereas meta-diphenols were oxidized very slowly if at all, with the notable exception of phloroglucinol (Table 3). The reason for this exception is unknown but was also reported by Schánel and Esser (22) for *Podospora* laccase. These results, plus the lack of activity with tyrosine, confirm that the enzyme under investigation was a laccase. No oxygen consumption could be detected with potassium ferrocyanide as a substrate.
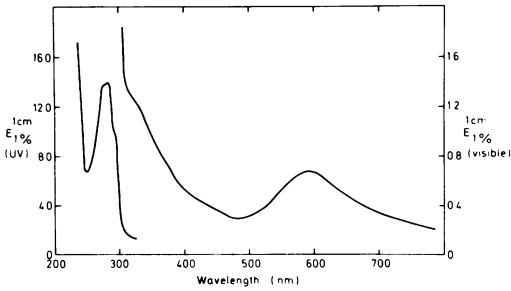
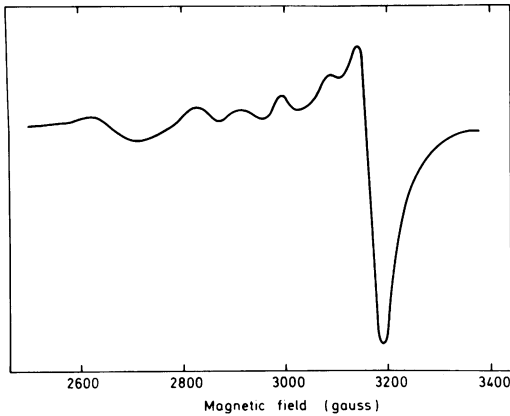FIG. 6. *Ultraviolet and visible spectrum of purified laccase.*

FIG. 7. *EPR spectrum of purified laccase. A solution of 3.85 mg of laccase per ml in 0.10 M sodium phosphate buffer, pH 6.0, was used.*

Partial purification and properties of intracellular laccase. Since 65 to 90% of the laccase of *Neurospora* is extracellular (11), only small amounts of intracellular laccase were obtainable. As a result, purification of the intracellular form to homogeneity has not been achieved, but several interesting properties were determined. Affinity chromatography on Concanavalin A Sepharose achieved a purification of nearly 100-fold with yields of 35 to 50%. Besides being a very useful purification step, this procedure indicated that intracellular laccase was a glycoprotein. Intracellular laccase bound to Concanavalin A Sepharose and could be eluted with α-methyl-mannoside (Fig. 8). Tyrosinase, which is not a glycoprotein, did not bind to the immobilized lectin. Laccase extracted from cultures which had been induced for only 3 days also bound completely to Concanavalin A Sepharose. Thus, no laccase devoid of carbohydrate could be detected, suggesting that the carbohydrate was attached to the protein during or immediately after synthesis.

Gel filtration of intracellular laccase on G-100 Sephadex revealed one component of activity. Since this activity co-chromatographed with the extracellular enzyme, it appears that the intracellular and extracellular laccases had very similar, if not identical, molecular weights. No indications of polymeric forms of laccase were noted.

Intracellular laccase, which had been purified by Concanavalin A Sepharose and gel filtration, was chromatographed on DEAE-Sephadex A-50 according to the procedure described for the extracellular enzyme. Essentially all of the

TABLE 3. *Substrate specificity of extracellular laccase*

Substrate	Activity[a]
Hydroquinone (1,4-dihydroxybenzene)	54.3
Gallic acid (3,4,5-trihydroxybenzoic acid)	72.7
p-cresol (p-hydroxytoluene)	17.6
Dopa (3,4-dihydroxyphenylalanine)	38.0
Protocatechuic acid (3,4-dihydroxybenzoic acid)	41.7
Guaiacol (1-hydroxy-2-methoxybenzene)	57.7
Pyrogallol (1,2,3-trihydroxybenzene)	73.6
Pyrocatechol (1,2-dihydroxybenzene)	35.0
Resorcinol (1,3-dihydroxybenzene)	1.8
m-cresol (m-hydroxytoluene)	0
Phloroglucinol (1,3,5-trihydroxybenzene) ...	37.0
Orcinol (3,5-dihydroxytoluene)	5.0
Tyrosine	0
Ascorbic acid	33.3
Potassium ferrocyanide	0

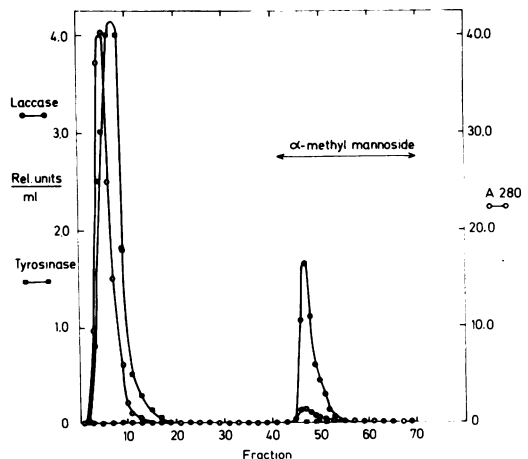[a] nmol of O_2 consumed per min.

FIG. 8. *Concanavalin A Sepharose chromatography of intracellular laccase. Chromatography was performed as described.*

laccase activity was eluted from the column after washing with 2- to 3-column volumes of 0.02 M sodium phosphate buffer, pH 7.2. Sodium dodecyl sulfate gel electrophoresis indicated that this enzyme was 50 to 60% pure, but further purification was not performed due to lack of material.

DISCUSSION

The existence of multiple forms of laccase in various fungi is well established. *Podospora anserina* produces three intracellular isoenzymes, one of which (laccase I) is a polymeric form of the other two (laccases II and III) (6). Laccase II, which has a molecular weight of about 70,000, contains 25% carbohydrate, whereas laccase III has a molecular weight of 80,000 and a carbohydrate content of 23%. The relative amounts of these forms present is dependent on the age of the culture (20). The extracellular laccase of *Polyporus versicolor* can be resolved into two or possibly three forms (4, 9, 21).

Neurospora laccase seems to consist of mainly one form, at least under the conditions of induction used. The apparent heterogeneity noted on DEAE-Sephadex chromatography may represent isoenzymic forms, but the possibility that this is due to artifactual binding of pigments seems more likely in view of our results. The induction conditions used here for the production of *Neurospora* laccase (low concentrations of cycloheximide) may preclude the appearance of other isoenzymes if, for example, they turnover more rapidly than the form studied here or if their regulation mechanism does not involve an unstable protein repressor (11).

The reason for the microheterogeneity of laccase when examined by isoelectric focusing is unknown. Such extensive heterogeneity could not be due to differences in amino acid composition alone. Similar results were reported for *Polyporus* laccase, and it was suggested that the heterogeneity was due to differences in carbohydrate composition (16). Indeed, molecular-weight microheterogeneity of *Podospora* laccase has been shown to be due to different amounts of carbohydrate (7). Other possibilities include contamination of the enzyme with various amounts of pigment or artifactual binding of the ampholytes to the enzyme possibly via the copper ions. Experiments that will hopefully resolve this question are in progress.

The *Neurospora* enzyme appears to be very similar in several respects to *Polyporus* laccase A (9). Both have similar molecular weights (64,800 for *Neurospora* and 64,400 for *Polyporus*) as well as carbohydrate contents (10.7 and 10.12%, respectively). A comparison of the amino acid contents of these enzymes also reveals similarities. In contrast, the ascomycetes *N. crassa* and *P. anserina* produce very different *p*-diphenol oxidases, both in molecular weight and carbohydrate content (6, 20). In view of this and their different physical localizations (intracellular versus extracellular), it is possible that these enzymes have different biological functions in these two fungi of the same genus.

The state and functional aspects of copper in laccase have been thoroughly studied for the *P. versicolor* enzyme (2, 19). To compare *Neurospora* and *Polyporus* laccase, we performed limited studies of the copper in the enzyme described here. *Polyporus* laccase contains four copper atoms, two of which (type I and II) are paramagentic but nonequivalent (19). Type I Cu was responsible for the blue color of the enzyme (A_{max} = 615 nm), whereas type II Cu absorbed in the ultraviolet giving rise to a shoulder at about 330 nm. Although type I Cu appeared to undergo a reversible valence change during the oxidation reaction, type II Cu may be involved in the binding of an intermediate in the reduction of oxygen. The remaining two copper atoms are diamagnetic and make no known contribution to the spectral properties of the enzyme (2).

The visible and ultraviolet spectra of *Neurospora* laccase present evidence for the existence of type I and II Cu in this enzyme also. A shoulder at about 330 nm suggested the presence of type II Cu, whereas a peak at 595 nm was probably due to type I Cu. The ratio A_{280} to A_{595} for *Neurospora* laccase, approximately 20, is somewhat larger than that for the *Polyporus* enzyme (2), but this is probably a reflection of the unusually high absorption at 280 nm.

A more sensitive indication of the state and environment of the copper in laccase was provided by EPR spectroscopy. The EPR x-band spectrum of *Neurospora* laccase, as recorded at 77 K (liquid nitrogen; ca. 196 C, is shown in Fig. 7. A comparison of this spectrum with that of *Polyporus* laccase (19) indicates that the two are identical. Thus, both type I and II Cu were present in *Neurospora* laccase and probably in environments similar to those in the *Polyporus* enzyme.

Analysis of the total copper present in *Neurospora* laccase indicates that there were about 3 mol per mol of enzyme. Whether this represented a real difference from the *Polyporus* enzyme or was due to error in the determination of the copper content or the absorption coefficient of the protein at 280 nm is unknown. It is noteworthy, however, that Esser and Minuth

(6) report a value of 3.3 ± 0.1 copper atoms per molecule of form II laccase from *Podospora anserina*.

A study of intracellular *Neurospora* laccase was performed not only to permit a comparison with the intracellular *Podospora* enzyme but also as the initial investigations of the mechanism and control of laccase secretion. No differences in the molecular weight, the chromatographic properties, or the ability to bind Concanavalin A of the intracellular and the main form of the extracellular enzymes were noted. It appears that the intracellular laccase was destined for secretion by the fungus and that some factor other than the availability of active enzyme was limiting the export process.

Eylar (8) has proposed that the carbohydrate moiety of a glycoprotein serves as a label and signals the cell that this protein is to be exported. This theory has been disputed partly on the grounds that intracellular glycoproteins also exist (25). If it is assumed that intracellular laccase is simply awaiting excretion, then the finding that both intracellular and extracellular laccase are glycoproteins (as indicated by their Concanavalin A-binding ability) is consistent with the Eylar hypothesis. Direct proof can be obtained by inhibiting the attachment of all or part of the carbohydrate moiety to the protein to examine its role in protein excretion (10, 17).

Neurospora laccase may be well suited as a model for investigations of the mechanism of enzyme secretion and the importance and function of the carbohydrate moiety in this respect. Its ability to bind to Concanavalin A Sepharose should be particularly useful as an assay for the absence of or at least a modification in the carbohydrate chain when devising methods for the prevention of attachment of the sugars to the polypeptide chain.

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