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Purification and Characterization of the Conidial Laccase of *Aspergillus nidulans*

MYRA BERMAN KURTZ* AND SEWELL P. CHAMPE

Waksman Institute of Microbiology, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854

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Conidial laccase of *Aspergillus nidulans* was purified by standard protein purification methods. Although the purified material showed a cluster of several protein bands on a nondenaturing gel, each of these protein bands had laccase activity. All bands of activity, however, were absent in a strain carrying a mutation in the structural gene for laccase. Concentrated solutions (greater than 1 mg/ml) were bright blue, suggesting that, like other laccases, this enzyme contains copper. The enzyme contained asparagine-linked carbohydrate (12% by weight) which could be removed by digestion with endo-β-N-acetylglucosaminidase H. The molecular weight of native enzyme as determined by gel filtration was 110,000, but the largest component in a sodium dodecyl sulfate gel was 80,000. Several smaller components (55,000 and 36,000 molecular weight) were also visible. We present evidence which suggests that the smaller components are in vivo cleavage products tightly associated with enzymatically active molecules. Comparison of the laccase from a white-spore (*wA*) and a green-spore (wA^+) strain showed, surprisingly, that the enzymes differed in electrophoretic pattern, in vitro heat stability, and in vivo metabolic stability. The difference was manifested for enzymes isolated from cultures after conidial pigmentation of the wA^+ strain had occurred. If examined earlier, before pigmentation, the enzymes were indistinguishable. Since *wA* strains lack the precursor of the wild-type green pigment, i.e., the laccase substrate, we suggest that the transformation of the enzyme of the *wA* strain is due to its failure to interact with its normal substrate.

The morphogenesis of the spores and spore-bearing structures of many fungi is often associated with the phenol oxidases tyrosinase and laccase (2, 4, 7–10, 12, 16, 17). In the ascomycete *Aspergillus nidulans*, laccase (*p*-diphenol:oxygen oxidoreductase; EC 1.10.3.2) is not detectable in the non-conidiating mycelium, but appears in coincidence with the first conidia and functions subsequently to convert a yellow precursor conidial pigment to the mature green form (4). Because the appearance of laccase activity is tightly coupled to developmental events, the mechanism of this coupling is of considerable interest for an understanding of the regulatory switches that operate during developmental processes. Law and Timberlake (15) recently showed that the accumulation of laccase activity during conidiation is prevented by an inhibitor of protein synthesis (cycloheximide) and several inhibitors of RNA synthesis or RNA function. These results indicate that the sudden appearance of laccase during conidiation is due to the transcriptional activation of the laccase gene.

Three known genetic loci influence the synthesis of laccase in *A. nidulans*. Recessive mutants that produce bright yellow conidia define a single locus, *yA*, which almost certainly codes for at least part of the enzyme since thermosensitive *yA* alleles produce a thermosensitive laccase (4). Yellow-green mutants (*ygA*) are also deficient in laccase but exhibit enzyme activity in vivo when cultures are grown at low pH and in vitro when extracts are supplemented with high levels of copper salts (4). We recently isolated and characterized a third class of laccase-deficient mutants (*yB*) which, like *yA* mutants, have a yellow-spore phenotype but are dominant to wild type (12). The properties of *yB* mutants suggest that, like *ygA* mutants, their phenotype is due to copper deficiency (12). White-spore mutants (*wA*) are not deficient in laccase (4) and are epistatic over yellow-spore mutants (20), which suggests that the probable function of the *wA* gene is to provide the yellow precursor pigment.

As a prerequisite for further studies on the regulation of laccase activity during conidiation we purified this enzyme, analyzed its subunit composition, and prepared antibody to the puri-

fied material to assay the laccase protein in unfractionated extracts. We found that laccase prepared from a white-spore strain exhibited a set of enzymatically active electrophoretic bands which differed from those exhibited by a green-spore strain. Furthermore, the activity in a white-spore strain had a greater metabolic stability than it did in a green-spore strain. These results suggest that the in vivo stability of laccase is influenced by the interaction of the enzyme with its substrate.

MATERIALS AND METHODS

Strains. The laccase-producing green-spore and white-spore strains used in this study were, respectively, WIM-70 (*pabaA1*) and R-153 (*wA3 pyroA4*). The strain WIM-70 was derived from a cross between R-153 and R-21 (*yA2 pabaA1*) as described previously (12). The parental strains R-153 and R-21 were obtained from C. F. Roberts, University of Leicester, and have been described by Armitt et al. (1).

Medium. The YGT growth medium was composed of 0.5% yeast extract (Difco), 2% glucose, and trace elements at concentrations described previously (12). The medium was solidified with 1.5% agar (Difco).

Mycelial growth and extraction. Laccase was obtained from confluent mycelial lawns grown at 37°C on YGT agar plates and harvested at 48 h as described previously (12). For large-scale preparations, 22- by 22-cm, square plastic culture dishes (Nunc) containing 200 ml of YGT agar were inoculated with 10^6 conidia in 40 ml of YGT broth. The mycelial pads were peeled from the agar and homogenized in a Waring blender with 25 ml of extraction buffer (10 mM Tris-hydrochloride, 1.0 mM EDTA, 10 mM *p*-tosyl-L-arginine methyl ester [TAME], and 1.0 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.4) per dish. Mycelial debris was removed by centrifugation for 5 min at 15,000 × *g*. Although this procedure does not result in complete breakage of hyphae, Law and Timberlake have reported that almost all of the laccase can be extracted by physically gentle procedures (15). For small-scale preparations, 100- by 5-mm standard plates containing 20 ml of YGT agar were inoculated with 10^5 conidia in 4 ml of YGT broth.

Laccase purification. In the following procedure the standard buffer was Tris-hydrochloride, pH 7.4 (of various molarities) containing additives as indicated. Column fractionations were performed at room temperature.

The mycelial homogenate was stirred with DEAE-cellulose (about 1 ml, packed volume, in 0.01 M buffer per 10 ml of homogenate) for 60 min at room temperature. The slurry, in which greater than 80% of the activity was bound to the adsorbant, was washed in a column, first with 0.01 M buffer and then with buffer containing 0.1 and 0.3 M NaCl. The activity was eluted from the column by a final wash with buffer containing 0.4 M NaCl. No further activity was eluted with higher salt concentrations. Pooled fractions containing laccase activity were brought to 4 M NaCl and applied to a phenyl-Sepharose column (1.5 by 20 cm) equilibrated with 0.01 M buffer. After the column was washed with several column volumes of 0.01 M buffer containing 2 M NaCl, the activity was eluted with buffer containing 1 M NaCl in 2-ml fractions. The pooled fractions containing activity were desalted and concentrated to 1.5 ml by ultrafiltration through an immersible CX-10 filter (Millipore Corp.), and the sample was applied to a Sephacryl S-200 column (90 by 1.6 cm). The activity was eluted from the column with 0.1 M buffer containing 0.1 M NaCl and 1 mM PMSF and collected in 2-ml fractions. The fractions with activity were pooled and applied to a DEAE-Sephadex column (2.5 by 20 cm) equilibrated in 0.1 M buffer containing 0.1 M NaCl. After the column had been washed with several column volumes of this buffer, the activity was eluted with a linear gradient of NaCl from 0.25 to 0.4 M. The active fractions were again concentrated by ultrafiltration and applied to a small concanavalin A-Sepharose column of 2 ml packed volume. The column was washed with three column volumes of 0.1 M buffer containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$ and eluted with 10% α-methyl mannoside, which was subsequently removed from the final purified material by ultrafiltration. Purified laccase stored at −20°C in 0.01 M buffer was stable for at least 3 months.

Assay of laccase activity. Quantitative laccase assays employed the chromogenic substrate *N,N*-dimethyl-*p*-phenylene diamine sulfate (DMP) and were performed as described by Clutterbuck (4). One unit of activity catalyzes the oxidation of 1.0 μmol of DMP per min. For specific activity (units per milligram of protein), protein was determined by the Lowry phenol method or by the Bio-Rad protein assay. For rapid identification of column fractions containing activity, samples of 10 to 25 μl were mixed in microtiter dishes with 0.1 ml of a solution containing 2.0 mg of DMP per ml in 37 mM citric acid–124 mM Na_2HPO_4, pH 6.0, and color development was scored visually.

Immunological methods. Antibody against purified laccase was induced in rabbits by intradermal injection of 50 μg of antigen with complete Freund adjuvant (11). Booster shots of 10 μg each were given at 3-week intervals. Gamma globulin was partially purified as described previously (12). Quantitative analysis of antigen was performed by rocket immunoelectrophoresis (14, 22). The immunoprecipitins in the gel corresponding to laccase were identified by staining the gel enzymatically with a buffer solution containing DMP (4).

One-dimensional peptide mapping. The polypeptides of purified laccase were separated on a 10% polyacrylamide gel containing sodium dodecyl sulfate (SDS) by the method of Laemmli (13). The gel was stained for 30 min as described by Cleveland et al. (3), and the components of interest were cut from the gel, soaked for 30 min in 0.125 M Tris-hydrochloride (pH 6.8) containing 0.1% SDS and 1 mM EDTA, and frozen until needed. The gel slices were placed in the wells of a second SDS gel (12% polyacrylamide), overlaid with 0.5 μg of *Staphylococcus aureus* protease V8 (Miles Laboratories), and digested for 30 min with the power off after the stacking gel had been entered, as described by Cleveland et al. (3). After electrophoresis, the digestion products were visualized by the sensitive silver staining technique of Merril et al. (19).

Digestion with endo H. Purified endo-β-*N*-acetylglucosaminidase H (endo H) was a gift of J. Tkacz, Squibb Institute for Medical Research. Pure or partially purified laccase preparations were digested with

TABLE 1. Purification procedure for conidial laccase

Purification step	Volume (ml)	Total activity (U)	Total protein (mg)	Sp act (U/mg of protein)	Yield (%)	Purification (×)
1. Crude homogenate	900	3,840	2,250	1.70	100	1
2. DEAE-cellulose	200	2,133	224	9.52	56	5.6
3. Phenyl-Sepharose	1.5	1,600	28.5	56.0	42	33
4. Sephacryl S-200	50	1,920	23.5	81.7	50	48
5. DEAE-Sephadex	45	825	9	91.0	21	53
6. Concanavalin A-Sepharose	1.64	612	3.7	165.8	16	97

endo H in the standard Tris buffer with 1 mM PMSF to prevent proteolysis. Digestion was allowed to continue for 16 to 24 h at 37°C at a concentration of 0.8 μg/ml. Complete digestion was assayed by the change of electrophoretic mobility on nondenaturing gels.

RESULTS

Purification and properties of conidial laccase. It has been reported (4), and we have confirmed, that laccase activity is greater in white-spore mutants of *A. nidulans* than in wild-type green-spore strains. We therefore chose the white-spore strain R-153 as the source of enzyme for the purification process. A successful purification scheme (Table 1) used five sequential column chromatographic fractionations, giving a 97-fold purification with a recovery of 16%. Concentrated solutions (greater than 1 mg/ml) were bright blue, suggesting that, like other laccases, this enzyme contains copper (18).

Trial procedures showed that enzyme activity was lost during lyophilization or conventional dialysis. The enzyme was concentrated and desalted by vacuum dialysis to avoid these losses. The order of the steps after the first DEAE fractionation did not affect the stability or composition of the final product and was chosen for convenience. The DEAE-cellulose and DEAE-Sephadex steps both resulted in considerable loss of activity but were included to remove pigments and a possible contaminant of a molecular weight similar to laccase. All of the detectable activity in the crude extracts was bound to concanavalin A-Sepharose and was eluted with 10% α-methyl mannoside, indicating that, like other laccases, the conidial enzyme is a glycoprotein. No activity was bound to unconjugated Sepharose.

The purity of the final product was assayed by polyacrylamide gel electrophoresis (Fig. 1) in which the components were visualized by staining for laccase activity (lane D) or for protein (lane B). The two staining methods gave coincident patterns, each consisting of a diffuse cluster of bands. Multiple bands were also seen in gels of freshly prepared crude homogenates to which PMSF and TAME had been added to reduce proteolysis. It is unlikely therefore that the observed heterogeneity of the purified enzyme arose in the purification procedure. All bands of activity were missing for a strain carrying the *yA2* mutation (data not shown), indicating that all were products of the *yA* locus, the structural gene for laccase. No other protein bands could be detected even when 50 μg of purified material was run on nondenaturing gels.

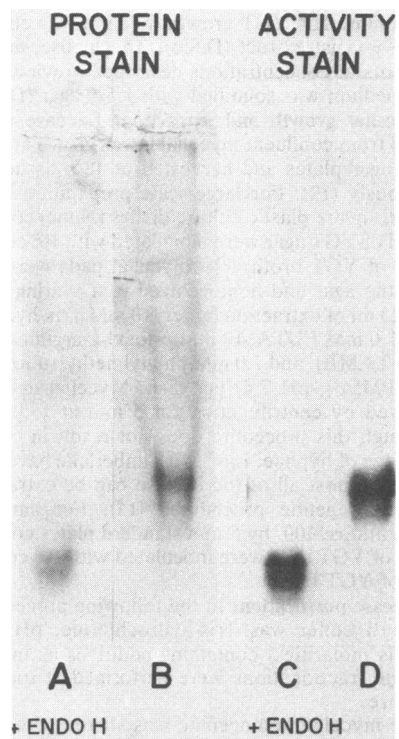

FIG. 1. Polyacrylamide gel electrophoresis of purified laccase digested with endo H. Purified laccase (16 μg) was digested for 16 h at 37°C with 0.4 μg of endo H in 50 μl of laccase assay buffer, diluted in gel sample buffer, and electrophoresed in 7.5% nondenaturing gels. Control samples were incubated without endo H. Lanes: (A) 12 μg of laccase treated with endo H and stained for protein with Coomassie Blue; (B) 12 μg of laccase incubated without endo H and stained with Coomassie Blue; (C) 4 μg of laccase treated with endo H and stained for laccase activity with DMP; (D) 4 μg of laccase incubated without endo H and stained for laccase activity with DMP.

The applied material was preincubated with endo H, which cleaves the β1-4 bond that links oligosaccharide chains to peptide chains via asparagine (Fig. 1, lanes A and C). The resulting material was changed in electrophoretic mobility but still exhibited multiple components whether stained for protein (lane A) or activity (lane C). Quantitative experiments showed that 85 to 90% of the laccase activity remained after endo H treatment, and immunodiffusion showed that antibody raised against the native enzyme was able to recognize the endo H-treated material. Using the orcinol method to assay carbohydrate content (21), we estimated that 12% by weight of laccase is carbohydrate.

The molecular weight of conidial laccase was determined by filtration through a column of Sephacryl S-200 which had been calibrated with proteins of known molecular weight. The value obtained by this method was 110,000, in good agreement with the value of 107,000 reported by Clutterbuck (4), who used filtration through Sephadex G-200. Both of these values are somewhat higher than the 85,000 value estimated by Law and Timberlake (15) from mobility in an SDS-polyacrylamide gel. However, as shown below, electrophoresis of our purified material in an SDS gel exhibited an 80,000-molecular-weight component as well as several smaller components.

Rocket immunoelectrophoresis was used to quantitate the amount of protein in a crude homogenate that reacted with antibody raised against purified laccase. This assay takes advantage of the fact that laccase retains activity even when immunoprecipitated (12, 15); thus, the rocket corresponding to laccase can be identified by staining the gel for laccase activity. Calculation of the specific activity (units per milligram of laccase protein) of crude homogenates by this method gave values of 190 to 200, which are close to the specific activities of the purified enzyme (165 to 198 for different preparations). Thus, if inactive laccase protein was present in the purified material, it was also present in the crude extract.

Subunit composition of purified laccase. Electrophoresis of purified laccase on an SDS gel gave three major components with apparent molecular weights of 80,000, 55,000, and 36,000, the latter often appearing as a doublet (Fig. 2, lane A). The mobility of each of these bands was increased by preincubation with endo H (data not shown), indicating that all three are glycoproteins and that the heterogeneity is not due simply to various amounts of endo H-removable carbohydrate.

To test the possibility that the three polypeptides have related amino acid sequences, we subjected the material in each band to limited proteolysis by *S. aureus* V8 protease, and the digests were compared by electrophoresis on an identical gel. The electrophoretic pattern generated by this treatment was reproducible and specific for a given protein. Almost all of the digestion products from the 55,000 and 36,000 bands were also present in the digest of the 80,000 band (Fig. 2, lanes B, C, and D). In addition, the 55,000 and 36,000 digests had several products in common.

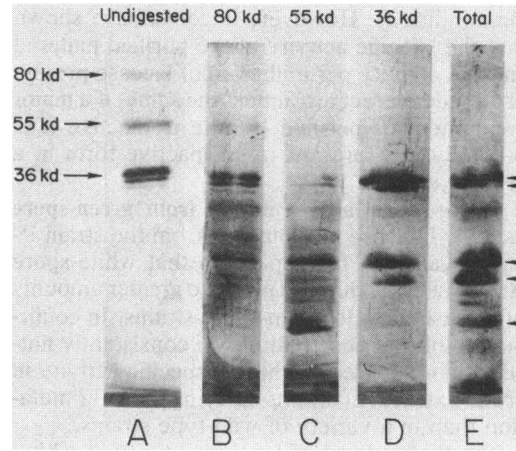

FIG. 2. Comparison of proteolytic fragments of purified laccase polypeptides. Polypeptides of laccase cut from a 10% SDS gel were digested by *S. aureus* V8 protease during electrophoresis as described in the text. Lanes: (A) unfractionated purified laccase run without protease to indicate the 80,000, 55,000, and 36,000 peptides; (B) digested 80,000 band; (C) digested 55,000 band; (D) digested 36,000 band; (E) unfractionated laccase, digested. Blue-colored peptides are indicated by arrows.

Although not visible in the black and white photograph, the silver stain used to visualize the peptides produced a blue color for several of the peptides rather than the usual brown color. Each of the three bands yielded blue-colored peptides, some of which were common to all three (Fig. 2, arrows). The digestion of bovine serum albumin gave a totally dissimilar pattern of bands, none of which stained blue. Although the reaction of silver with peptides is not understood, this color specificity could be useful for analytical purposes if it identifies peptides of unusual composition or those with bound carbohydrate. The congruence of the colored peptides (Fig. 2) is further evidence that the three components of purified laccase are related and that the smaller two may be cleavage products of the larger.

It was not possible to determine whether each of the components of purified laccase possessed phenol oxidase activity, because so far we have been able to separate them only under denatur-

ing conditions. However, since we have shown that the specific activity of the purified material and the activity per milligram of laccase protein in a crude extract are almost the same, if a major component of purified laccase is inactive it is probably also present in an inactive form in a crude extract.

White-spore laccase differs from green-spore laccase. Laccase was purified from the strain R-153 because of the report (4) that white-spore strains such as this one produce greater amounts of laccase than do green-spore strains. In confirmation of this observation, we consistently noted a two- to threefold higher specific activity in crude extracts of strains carrying the *wA2* mutation than in a variety of wild-type strains.

The higher level of laccase activity in white-spore strains could be due either to more laccase protein or to an altered enzyme with a higher turnover number. To distinguish between these possibilities, we measured laccase protein by rocket immunoelectrophoresis. The white-spore strain, which had twice as much laccase activity (units per milligram of protein in crude extracts) as the wild type did, also had twice as much laccase protein (Fig. 3).

Electrophoretic comparison, on the other hand, showed that the enzyme derived from a white-spore strain (WH enzyme) differed from that derived from a green-spore strain (GR enzyme). Whereas the WH enzyme exhibited a set of fairly distinct bands of activity (Fig. 4, lane F), the GR enzyme migrated principally as a single diffuse band (Fig. 4, lane E). This difference, though not striking, was more convincing when a mixture of the two extracts was run in the same lane (Fig. 4, lane D).

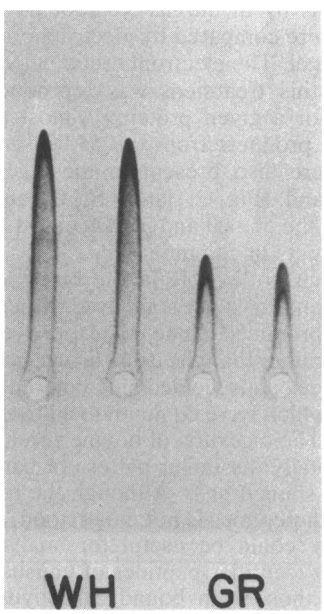

FIG. 3. Rocket immunoelectrophoresis of WH and GR laccases. Equal volumes of crude homogenates from a white-spore (WH) and a green-spore (GR) strain were subjected to immunoelectrophoresis for 6 h at 150 V. The rockets were visualized by enzymatic staining. The WH extract had 2.4-fold more laccase activity per ml than did the GR extract and produced rockets with twofold-greater areas.

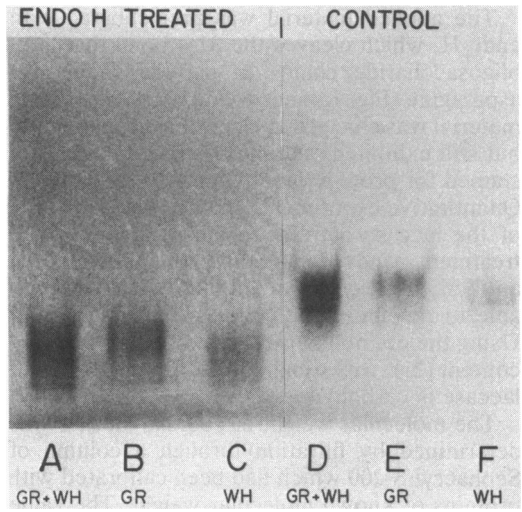

FIG. 4. Endo H-treated laccase from *wA* and *wA*$^+$ strains retains heterogeneity. Crude homogenates from a white-spore (WH) and a green-spore (GR) strain were prepared as described in the text. Laccase activity was partially purified by batch treatment with DEAE-cellulose in 0.1 M buffer to adsorb the enzyme. After the resin was washed with several volumes of 0.1 M buffer, the activity was eluted in buffer containing 0.4 M NaCl. The extract was diluted 1:3 with water containing 1 mM PMSF and incubated with endo H at a concentration of 0.8 μg/ml overnight at 37°C. The samples were diluted in gel sample buffer, run on 10% polyacrylamide gels, and stained for laccase activity with DMP. Lanes: (A) GR and WH, endo H treated; (B) GR, endo H treated; (C) WH, endo H treated; (D) GR and WH; (E) GR; (F) WH.

For determining whether the difference between the WH and GR enzymes resided in their carbohydrate moieties, crude extracts were partially purified by DEAE-cellulose chromatography and then digested with endo H. The digested material was run on a nondenaturing gel and stained for enzymatic activity (Fig. 4). Although endo H treatment produced components with greater migration rates, the difference between the WH and GR enzymes remained. Further comparison of the properties of the WH and GR enzymes showed that the WH enzyme was more heat sensitive than the GR enzyme when prepared at 45 h (Fig. 5). This experiment was

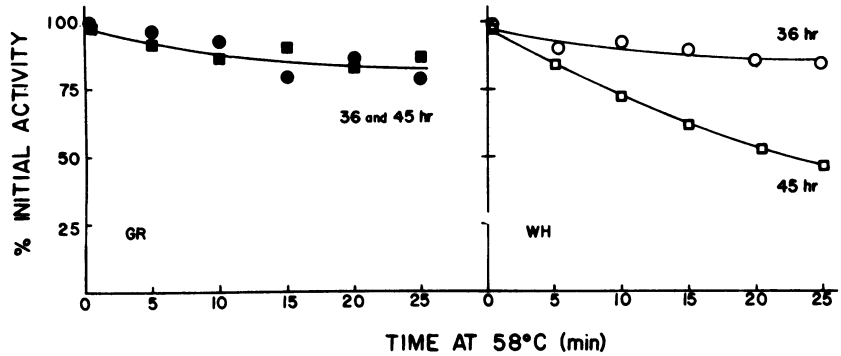

FIG. 5. Heat stability of laccase activity in crude extracts before and after conidial greening. Crude extracts from a green-spore (GR) and a white-spore (WH) strain were prepared before conidial greening (36 h; ●, ○) and after full conidial pigmentation (45 h; ■, □).

performed with crude extracts containing PMSF to inhibit proteases, but similar curves were obtained with purified WH enzyme, indicating that enhanced proteolysis in extracts of the white-spore strain is not the explanation for the difference in lability.

Although the greater lability of the WH enzyme substantiates the conclusion that the WH and GR enzymes are physically different, these results cannot explain the greater yield of laccase from white-spore strains. However, the inactivation kinetics (Fig. 5) were measured at 58°C and may be irrelevant to the in vivo turnover rate at the much lower growth temperatures. To measure the in vivo stability of laccase, we transferred the mycelial pads of conidiating cultures with active laccase to medium containing 100 μg of cycloheximide per ml to inhibit protein synthesis. At various times thereafter, the remaining activity in the inhibited samples was compared with a control sample transferred to fresh medium without cycloheximide. The results of this experiment (Fig. 6) showed that in the absence of protein synthesis, the green-spore strain lost laccase activity at a greater rate than did the white-spore strain. Thus, the greater yield of laccase from white-spore strains must be at least in part due to greater in vivo stability of the enzyme.

The WH and GR enzymes differed from each other in electrophoretic pattern, in vitro heat stability, and in vivo metabolic stability. If, as seems likely, the *yA* locus is the structural gene for a laccase subunit and if there is but one kind of subunit, how can the effect on laccase structure of a mutation at the *wA* locus be explained?

The fact that *wA* mutations are epistatic over *yA* mutations (20) (i.e., strains with both mutations produce white spores) suggests that the probable function of the *wA* gene product is to provide the yellow conidial pigment which then is converted to the terminal green pigment by laccase. The differences between the WH and GR enzymes could, therefore, be an indirect result of the interaction of laccase with its natural substrate in green-spore strains, the opportunity for which does not exist in white-spore strains. In the absence of the normal substrate interaction, laccase may undergo a conformational change that increases its metabolic stability (e.g., by a change in protease sensitivity) and at the same time alters its electrophoretic mobility and heat stability. This model predicts that laccase isolated from both green-spore and white-spore strains before appreciable conidial greening has occurred should be predominantly of the GR form.

To test this hypothesis, we isolated laccase both before (36 h) and after (45 h) greening and assayed it for heat stability and electrophoretic

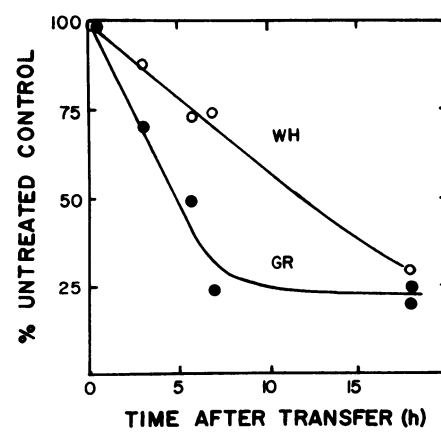

FIG. 6. Stability of laccase activity in vivo in a green-spore (GR) and a white-spore (WH) strain. The graph shows the loss of laccase activity in mycelial pads after they were transferred to medium containing cycloheximide as described in the text.

pattern. The enzymes isolated early from the white-and green-spore strains were indistinguishable in the two assays, as predicted by the model, and were the same as the GR enzyme isolated from older cultures (Fig. 5). The WH enzyme from the 45-h culture showed the characteristic decreased heat stability and altered electrophoretic mobility.

DISCUSSION

The purification procedure we have described yields laccase that is greater than 95% pure as judged by electrophoresis on nondenaturing gels. The product, however, is heterogeneous, giving several diffuse bands that stain positively for laccase activity. This heterogeneity could be due to a variable carbohydrate moiety, but digestion with endo H, which removes asparagine-linked polysaccharide, did not remove the heterogeneity.

Electrophoresis on SDS denaturing gels showed that the purified material was composed mainly of three glycopolypeptides of 80,000, 55,000, and 36,000 molecular weight. Whether each of the bands had laccase activity could not be determined, but comparisons of protease-produced peptide fragments suggested that all three species have amino acid sequences in common. Although we cannot rule out the possibility that the smaller polypeptides are produced by proteolysis during purification, the fact that the activity of the purified material per unit of immunoprecipitable protein was almost the same as that of a crude extract argues that any inactive laccase-related protein present in the purified material existed as such before purification. That laccase is, in fact, degraded in vivo is clear from the studies of Law and Timberlake (15).

Taken together, these findings suggest that the 55,000 and 36,000 components seen on SDS gels are in vivo cleavage products tightly associated with enzymatically active molecules. For example, enzymatically active forms could be 80,000, 80,000 + 36,000, and 80,000 + 55,000, in which the smaller subunits of the complexes are irrelevant for activity but may influence heat stability and in vivo degradation rate. A specific distribution of such complexes could account for the fact that the molecular weight of the active material determined by gel filtration (110,000) was higher than that of the largest component seen on denaturing gels (80,000). The heterogeneity of the major laccase activity in *Podospora anserina* may be due to a similar mechanism. Recently it was shown that the product of the single laccase structural gene can exist in three forms, two monomeric (80,000 molecular weight) and one oligomeric, which are interconvertable (6).

Our experiments were done with laccase purified from a white-spore mutant. Although we have not yet obtained pure laccase from a green-spore strain, the studies of Law and Timberlake (15) utilized such a strain. They reported that laccase antibody precipitates a polypeptide which migrates as an 86,000-molecular-weight component on denaturing gels (which we assume to be the same as our 80,000 component), and that this polypeptide is absent from a *yA* mutant. Unfortunately their immunoprecipitate had several major contaminants which would probably have obscured any other *yA*-specific components corresponding to our 36,000 and 55,000 polypeptides.

Our comparisons of the laccase from white-spore and green-spore strains, using crude or partially purified extracts, showed, surprisingly, that the WH and GR enzymes differ from each other in electrophoretic pattern, in vitro heat stability, and in vivo metabolic stability. We have shown that before conidial greening occurs, both the white- and green-spore strains produce the GR form of the enzyme, and we suggest that its failure to interact with the normal substrate (yellow pigment) allows the enzyme of the white-spore strain to be converted to the WH form. Conversion may be the result of a conformation change that decreases metabolic stability and changes the distribution of enzymatically active degradation intermediates. This change in distribution could, in turn, manifest itself as an alteration in the pattern of electrophoretic microheterogeneity and average heat stability of the set of components. The nature of the reaction which produces the WH form is not known. Incubation of mixed WH and GR crude extracts did not convert the GR form to the WH form (unpublished data).

Although the control of laccase degradation is an interesting question, the main impetus of the present study was to elucidate the regulation of its synthesis. Recent preliminary experiments indicate that transfer of conidiating cultures from copper-deficient to copper-rich medium results in an increase in laccase activity, but that the increase is prevented if protein synthesis is inhibited. This finding suggests that under conditions of copper limitation no inactive laccase protein is made that can later be activated by copper. Whether copper is required for transcription of the laccase gene, as is the case for mammalian metallothioneins (5), or merely for stabilization of laccase protein are questions under investigation.

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