Formation of an Extracellular Laccase by a *Schizophyllum commune* Dikaryon

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(Received 27 February 1986; revised 28 May 1986)

A dikaryon of the basidiomycete *Schizophyllum commune* growing in surface culture at 30 °C in the dark produced extracellular laccase (EC 1.10.3.2). Little extracellular laccase was formed in the light or at 24 °C. The co-isogenic monokaryons from which the dikaryon was generated generally failed to produce laccase. The activity in the medium of the dikaryon accumulated until the glucose was consumed and then declined steadily. At its peak level of activity the enzyme accounted for about 3% of the extracellular protein. The enzyme was purified by DEAE-Sephacel chromatography. Substrate specificity and inhibitor studies showed the enzyme to be a typical fungal laccase. Electrophoresis of a purified enzyme preparation showed one major band accounting for 98% of the laccase proteins, and two minor bands with laccase activity. The major laccase protein was used to raise specific antibodies. After denaturation of the major laccase two immuno-reactive protein forms of M_r 64×10^3 and 62×10^3 were produced, the former being convertible into the latter form. The intracellular extract contained one immuno-reactive protein of M_r 72×10^3 . The presence of laccase protein in the medium of various cultures was detected using Western blots. Accumulation of extracellular laccase protein only occurred in the dikaryon at 30 °C in the dark while the subsequent decrease in activity was not accompanied by a decrease in laccase protein.

INTRODUCTION

Fungal laccases are extracellular phenol oxidases specified by their ability to oxidize monophenols, *o*- and *p*-diphenols, aminophenols and diaminoaromatic compounds using molecular oxygen (cf. Reinhammar, 1984). These enzymes are commonly produced by the wood-destroying white-rot fungi and may play a role in lignin degradation and/or detoxification of lignin degradation products (Ishihara, 1980; cf. Reinhammar, 1984). Laccases have also been implicated in the pigmentation of fungal spores (Clutterbuck, 1972) and in the development of spore-bearing structures of fungi (Bu'lock, 1967; Harkin *et al.*, 1974; Hermann *et al.*, 1983; Leatham & Stahman, 1981; Wood & Goodenough, 1977; Wood, 1985), including *Schizophyllum commune* (Leonard & Phillips, 1973; Phillips & Leonard, 1976).

As a preliminary to further studies on the regulation and role of laccase in fruit-body morphogenesis in *S. commune* we have analysed the genetic and environmental conditions which permit the excretion of laccase into the medium. We have purified the extracellular laccase, prepared antibodies to assay the laccase protein, and investigated some of its properties.

METHODS

Organism. The *S. commune* monokaryons 4-39 (*A41B41*) and 4-40 (*A43B43*) (CBS 341.81 and CBS 340.81, respectively) were maintained co-isogenic by regularly backcrossing against strain 4-39. The dikaryon 4-39/4-40 was obtained by mating these monokaryons.

Abbreviations: KPB, potassium phosphate buffer; ADBP, 4-amino-2,6-dibromophenol; DMA, 2,5-dimethyl-aniline; DAB, diaminobenzidine.

Culture methods. Surface cultures were made on 9 cm Petri dishes using as an inoculum 2.5 ml of a mycelial macerate in minimal medium which was uniformly spread. The plates contained 25 ml of minimal medium (Dons *et al.*, 1979) with or without 0.7% (w/v) agar. In the latter case the mycelial macerate was spread over the bottom of an empty Petri dish and incubated at 30 °C in the dark for 46 h resulting in a thin but coherent mycelial mat. Then, at time zero, 40 ml of liquid minimal medium was carefully layered under the mat with a syringe equipped with a 2 mm gauge needle. In this way submerged growth of mycelial fragments was prevented. The cultures were incubated under the conditions indicated in the text. Harvested mycelial mats were stored at -70 °C.

Preparation of extracts. The frozen mycelial mats were fragmented in an X-press (LKB) at -25 °C. To analyse active laccases the pulverized material was thawed in 2 × concentrated SDS-sample buffer [final concn 2% (w/v) SDS, 0.05 M-Tris/HCl, pH 6.8, 10% (v/v) glycerol]. The suspension was then sonicated for 5 s (Branson sonifier) and the extract cleared by centrifugation in an Eppendorf microfuge for 3 min. Alternatively, to examine denatured proteins β -mercaptoethanol (final concn. 5%, v/v) was included in the SDS-sample buffer and the suspension was immediately boiled for 5 min before sonication and centrifugation as described for active proteins. The protein contents of the extracts were determined by the Lowry method on 5% TCA precipitates washed with cold acetone, using BSA as a standard.

Laccase assays. Reactions were followed at 24 °C using a colorimetric assay. The assay mixture consisted of 0.1 ml enzyme solution, 0.8 ml 0.1 M-potassium phosphate buffer (KPB) (pH 6.0) and 0.1 ml freshly prepared substrate solution in 96% ethanol in a 1 ml cuvette. Reactions were initiated by addition of the substrate. The linear oxidation rate of the substrate was then determined for a maximum of 1 h. In quantitative assays the substrate was 4-amino-2,6-dibromophenol (ADBP, Kodak) at a final concentration of 0.01% (w/v) (0.375 mM) in the presence of 0.02% (v/v) (1.65 mM) 2,5-dimethylaniline (DMA, Fluka); the progress of the reaction was monitored at 690 nm (Hermann *et al.*, 1983). One unit of enzyme activity is defined as a change in A_{690} of 0.001 per min. For specific activity (units per milligram of protein) protein was determined according to Bradford (1976). Other substrates and their final concentration in the reaction mixture are indicated under Results.

Laccase purification. Liquid medium (5400 ml) from surface cultures of the dikaryon grown for 44 h at 30 °C in the dark was filtered through Whatman 3MM paper and cooled on ice. All further operations were done at 5 °C. The medium was concentrated to 1/19 of its volume in an Amicon ultrafiltration cell using a PM 10 membrane (molecular cut-off 10 kDa). No laccase activity was detected in the filtrate. Further concentration to 1/190 of the original volume was done in dialysis tubing (diam. 2 cm) embedded in solid sucrose for 5 h. (Concentration by lyophilization or acetone precipitation resulted in losses of up to 98% of activity.) The concentrate was then dialysed overnight against two changes of 0.01 M-KPB (pH 6.0). The retentate (95 ml) was centrifuged (13000 g, 30 min) to remove solids. To the viscous supernatant 4 g DEAE-Sephacel (Pharmacia) was added. The DEAE-Sephacel had been pre-treated with 1 M-KPB (pH 6.0) followed by extensive washings with 0.01 M-KPB (pH 6.0). The mixture was stirred for 20 min and centrifuged (3000 g, 10 min). The supernatant was free of laccase activity and contained 38% of the protein originally present in the dialysate. The DEAE-Sephacel was washed in the centrifuge with 60 ml 0.01 M-KPB (pH 6.0), packed into a column (diam. 1 cm), and washed with 20 ml 0.01 M-KPB followed by 50 ml 0.1 M-KPB, which eluted another 31% of the protein and an orange pigment but only negligible laccase activity. The column was then eluted with a linear gradient of 0.1 to 0.5 M-KBP (pH 6.0) in a total volume of 100 ml at a flow rate of 20 ml h⁻¹. Fractions (2 ml) were collected and monitored for A_{280} , conductivity and laccase activity. Fractions containing laccase were pooled, concentrated by dehydration with sucrose in dialysis tubing and stored at -20 °C.

Analytical gel electrophoresis. Samples containing 3–7 μ g protein, unless indicated otherwise, were run in 10% (w/v) polyacrylamide gels with or without 0.1% (w/v) SDS using the discontinuous Tris/glycine buffer system of Laemmli (1970). Gels were stained for laccase activity by incubating at 24 °C in 0.1 M-KBP (pH 6.0) containing substrate as indicated for 1–2 h. Proteins were stained with Coomassie Brilliant Blue.

Preparation of antibodies. The laccase fraction (fraction II) obtained after DEAE-Sephacel chromatography was further purified by electrophoresis through a preparative polyacrylamide gel (7.5%, w/v; thickness 3 mm) using the buffer system of Laemmli (1970) but without SDS. The position of the laccase bands was located on strips from the gel by staining with 0.01% (w/v) diaminobenzidine (DAB) in 0.1 M-KPB (pH 6.0). The area containing the major laccase band was then cut from the gel, ground in a mortar with liquid nitrogen and taken up in 3 ml 0.1 M-KPB (pH 7.4)–0.9% (w/v) NaCl. A rabbit was immunized by subcutaneous injections repeated every 10–14 d with 0.5 ml of the gel homogenate containing 5 μ g protein. Before injection 0.5 ml of incomplete Freund's adjuvant was added to the homogenate. Blood samples (10 ml) were taken every 10–14 d. The serum used in this work was obtained 2 weeks after the fourth injection.

Immunoblotting. Proteins were separated by SDS-PAGE according to Laemmli (1970) using 10% (w/v) polyacrylamide gels (thickness 1 mm). The separated proteins were electrophoretically transferred to nitrocellulose (Millipore HA, 0.45 μ m) according to Towbin *et al.* (1979) except that methanol was omitted from the electrode solution and the transfer was done at 0.4 A for 2 h. For direct visualization of proteins the nitrocellulose was stained for 30 min in 0.1% (w/v) amidoblack 10 B in 25% (v/v) 2-propanol–10% (v/v) acetic acid

in water and destained in the same solvent. For immunological detection of laccase unstained blots were soaked in 0.1% (w/v) BSA–0.05% (v/v) Triton X-100 in saline [9% (w/v) NaCl, 10 mM-KPB (pH 7.4)] for 1–2 h, followed by incubation in 1% (w/v) BSA in saline for 3–5 h to saturate non-specific protein binding sites. The blots were then incubated overnight at 20 °C in a 1/400 dilution of antiserum in 0.1% (w/v) BSA in saline under constant agitation. After washing with 0.1% (v/v) Triton X-100 in saline, the blots were incubated with goat-anti-rabbit IgG (Calbiochem) at 0.2 U ml⁻¹ in 0.1% (w/v) BSA in saline for 1 h, washed as before, and incubated with peroxidase-anti-peroxidase complex (Miles 61242-Z, dilution 1/250) in 0.1% (w/v) BSA in saline for 1 h. After washing as before, the bound peroxidase complex was detected with 4-chloro-1-naphthol and hydrogen peroxide as described by Hawkes *et al.* (1982).

Immunoprecipitation. Laccase was immunoprecipitated from solution using a modification of the procedure described by Vos-Scheperkeuter & Witholt (1984). Laccase purified by DEAE-Sephacel chromatography (1 µl containing 0.5 µg laccase protein) was diluted in 20 µl of a solution containing 0.9% (v/v) Triton X-100, 0.1% (w/v) SDS and 0.1% (w/v) BSA in 50 mM-Tris/HCl (pH 8.0), followed by addition of 4 µl antiserum or pre-immune serum. After 30 min incubation on a rotator (30 r.p.m.) at 20 °C, 20 µl 10% (w/v) fixed and washed *Staphylococcus aureus* cells were added and incubation was continued for 15 min. The suspension was centrifuged in an Eppendorf microfuge for 5 min. The pellet was washed twice with 0.1 M-KPB (pH 6.0) and resuspended in 1 ml of the same buffer. The suspension and samples of the first supernatant were then assayed for laccase activity using ADBP/DMA.

RESULTS

Genetic and environmental regulation of extracellular laccase

Surface cultures were grown from spread mycelial macerates on agar at 24 or 30 °C in continuous darkness or light. After 72 h at 24 °C in the light the dikaryon had produced numerous fruit-body primordia (about 50 cm⁻²) and few aerial hyphae. At 30 °C in the light the dikaryon developed very few fruit bodies but much more aerial mycelium. In darkness at 24 and 30 °C the dikaryon formed no fruit bodies at all and developed a very copious aerial mycelium. The two component monokaryons remained vegetative under all conditions.

The media were analysed for the presence of laccase activity (Table 1). Only the medium of the dikaryon grown at 30 °C in continuous darkness contained considerable laccase activity. These results show that both the dikaryotic condition and the synergistic action of high temperature and darkness are required for high extracellular laccase production.

The effect of putative inducers of laccase activity (cf. Reinhammar, 1984) on the dikaryon grown in the light at 24 °C was tested. The mycelium was grown on liquid minimal medium with one of the following additions: xylinine, toluidine, guaiacol, gallic acid, ferulic acid, *p*-coumaric acid (all at 0.5 mM), yeast extract (0.2%, w/v), malt extract (0.3%, w/v) or peptone (0.3%, w/v). After cultivation for 2 d the media were tested for laccase activity using ADBP/DMA. Only in the media supplemented with guaiacol or peptone was laccase activity detected (about 10 units ml⁻¹), which was low when compared to the laccase activity obtained for the dikaryon grown in the dark at 30 °C without inducer (Table 1).

Purification of laccase by DEAE-Sephacel chromatography

The purification scheme is outlined in Table 2. The laccase activity was eluted as a non-symmetrical peak by gradually increasing the phosphate buffer concentration as depicted in Fig. 1. The laccase fractions were recovered in two pools (fractions I and II) which were concentrated with sucrose. Both fractions were pale-yellow. Fraction II contained about 7% (1.9 mg) of the protein added to the DEAE-Sephacel and about 74% of the eluted laccase activity. As can be seen from Table 2 there was an appreciable loss of activity during concentration and particularly during DEAE-Sephacel fractionation. This accounts for the rather low increase in specific activity of the enzyme after purification.

The pooled fractions obtained after DEAE-Sephacel chromatography were analysed by polyacrylamide gel (10%) electrophoresis using the Laemmli buffer system in the absence and presence of 0.1% (w/v) SDS. Because in this and all further analyses the laccases in fractions I and II behaved similarly, results are only given for fraction II which contained most (74%) of the laccase activity. The presence of three bands of laccase activity which appeared irrespective of

Table 1. *Laccase activity present in the medium of surface cultures of monokaryons and dikaryon of S. commune grown for 72 h under various conditions*

Laccase activity was determined with ADBP/DMA as described in Methods.

Growth conditions*	Laccase activity (units ml ⁻¹)†		
	Monokaryons		Dikaryon 4-39/4-40
	4-39	4-40	
24 °C, light	0	0	0
24 °C, darkness	0	0	5
30 °C, light	0	0	20
30 °C, darkness	11	4	1429

* Mycelia were grown from spread macerates on plates containing minimal medium and 0.7% (w/v) agar.

† The mycelial mats were removed and the agar medium from five plates was pooled and frozen. After thawing the agar was removed by centrifugation; for enzyme assays the medium was concentrated 10 times by sucrose-dehydration.

Table 2. *Purification of extracellular laccase from S. commune*

Laccase activity was determined with ADBP/DMA as described in Methods.

Fraction	Volume (ml)	10 ⁻³ × Activity (units ml ⁻¹)	10 ⁻³ × Total activity (units)	Total protein* (mg)	10 ⁻³ × Specific activity (units mg ⁻¹)	Yield (%)
Medium	5400	1.1	5760	—†	—	100
Ultrafiltration	28	14.7	4243	35.3	120	74
Dialysed sucrose- concentrate	95	41.2	3912	26.8	146	68
DEAE-Sephacel						
Conc. fraction I	0.6	546	328	0.76	432	6
Conc. fraction II	1.4	1080	1512	1.90	796	26

* Determined with the Bradford reagent.

† The low concentration present prevented accurate determination of protein.

the substrate used [*o*-dianisidine, DAB, tetramethylbenzidine, ADBP (0.01%, w/v) or L-DOPA (0.1%, w/v)] is shown in Fig. 2(a, c). A prolonged incubation (15 h; required only for L-DOPA) did not reveal additional bands with any of the substrates. The presence of SDS in the gels increased the mobility of the three bands and enhanced the intensity of the staining reaction. To estimate the relative activities of the three laccases, SDS-polyacrylamide gels (10%) were run with serial dilutions of the laccase preparations and stained with diaminobenzidine and ADBP/DMA. By visual comparison of the intensities of the bands it was estimated that the major band contained 98% of the total activity. Coomassie Brilliant Blue staining (Fig. 2b, d) and densitometric scanning revealed that 40% of the protein loaded on the gels was present at this location.

The major laccase band (running at a position equivalent to that of a denatured protein of M_r 36×10^3 , Fig. 2d) was cut from the gel and boiled for 5 min in SDS-sample buffer. The sample (including the gel material) was then transferred into a well of another SDS-polyacrylamide gel. Electrophoresis followed by staining for protein resulted in the appearance of two proteins of M_r 64×10^3 and 62×10^3 (Fig. 2f). These protein bands were also visible when the whole of fraction II was boiled in SDS-sample buffer and applied to the gel (Fig. 2e).

Fig. 1. DEAE-Sephacel purification of extracellular laccase from *S. commune* dikaryon grown in surface culture at 30 °C in darkness. The column (4 × 1 cm) was washed with 0.1 M-potassium phosphate buffer (pH 6.0) (50 ml), after which a linear gradient of 0.1–0.5 M-potassium phosphate buffer (pH 6.0) (100 ml total) was started (arrowed). The flow rate was 20 ml h⁻¹; 2 ml fractions were collected. Fractions with laccase activity were pooled as indicated (fraction I and II). ○, Laccase activity; ●, A_{280} ; □, buffer concentration.

Fig. 2. PAGE of laccase in fraction II in the absence (A) and presence (B) of 0.1% (w/v) SDS. (a), (c), Laccase activity as revealed by DAB; (b), (d), corresponding protein patterns; (e), fraction II boiled in SDS-sample buffer and stained for protein; (f), the major laccase band excised from (b), boiled in SDS-sample buffer and stained for protein. Bands with laccase activity are arrowed.

Properties of the major laccase

Fraction II was used to determine some properties of the major laccase.

pH activity curves for the substrates ADBP and *o*-dianisidine revealed optimal activity between pH 5.4 and 6.0 while above pH 7.0 almost no activity was observed. The enzyme produced coloured products within minutes from syringaldazine (0.05 mM), ADBP (0.375 mM), *p*-phenylenediamine (0.09 mM), guaiacol (5 mM), 4-chloro-1-naphthol (1.1 mM), and from the diaminoaromatics *o*-dianisidine (0.08 mM), DAB (0.4 mM) and tetramethylbenzidine (0.4 mM). L-DOPA (5 mM) was oxidized at a low rate. Tyrosine and *p*-cresol were not oxidized. Typical K_m values found were 0.030 mM (ADBP), 0.026 mM (tetramethylbenzidine) and 0.033 mM (syringaldazine). Addition of catalase (0.01 mg ml⁻¹, 240 U ml⁻¹) had no effect, excluding a role of H₂O₂ in the oxidations; H₂O₂ above 150 mM completely inhibited the activity of laccase with all substrates. Cinnamic acid (0.34 mM) had no effect on the laccase activity. The activity was, however, completely inhibited by sodium azide (1 mM), potassium cyanide (1 mM), diethyldithiocarbamate (2 mM) and the anionic detergent cetyltrimethylammonium bromide (20 mM). On the other hand, addition of SDS (0.5%, w/v) seemingly enhanced the reaction rate as measured with ADBP/DMA at 690 nm. However, the reaction products were blue in the absence of SDS ($\lambda_{\max}$ 690 nm) and blue-green in the presence of SDS ($\lambda_{\max}$ 710 nm). Addition of SDS during the course of the reaction caused an immediate shift in the absorbance maximum and an increase in A_{690} to the level of the sample with SDS, after which the increase of the absorbance continued as in the sample with SDS. This suggests that SDS neither stimulates nor inhibits the enzyme but only interacts with the product of the ADBP/DMA reaction.

The purified enzyme was unstable. On storage at -20 °C a purified laccase preparation lost 80% of its activity in one year.

Detection of laccase with antiserum

The major laccase band cut from a gel not containing SDS (Fig. 2*a, b*) was used to raise antibodies. In contrast to the pre-immune serum the antiserum quantitatively immunoprecipitated laccase from solutions. The immunoprecipitate retained laccase activity.

To examine the effectiveness and specificity of the antiserum in immunoblotting, fraction II before and after boiling in SDS-sample buffer was fractionated by SDS-PAGE. The gel was blotted onto nitrocellulose and the blot was subjected to the immunodetection procedure using the antiserum at a dilution of 1/150. As shown in Fig. 3, in the unboiled sample of fraction II one strong band was present at the position of the active laccase (corresponding to the position of a denatured protein of M_r 36×10^3), while a weaker band was visible at the position of the denatured inactive form (M_r 64×10^3). With the boiled sample of fraction II two strong bands were visible, corresponding to the denatured forms of laccase (M_r 64×10^3 and 62×10^3). No other bands could be seen.

With a 1/400 dilution of the antiserum 0.02 µg of laccase protein could be reliably detected using 15 min development of the peroxidase-anti-peroxidase reaction. Above 10 µg of laccase protein the capacity of the nitrocellulose was exceeded. To estimate the amount of laccase protein in samples, the signals obtained by immunodetection were visually compared with those obtained with known amounts of laccase. Laccase detection on the basis of activity was about five times more sensitive than immunodetection.

Formation and inactivation of extracellular laccase

During growth of the dikaryon in surface cultures in the dark at 30 °C extracellular laccase activity increased until glucose was depleted and then declined steadily to zero activity in 120 h-old-cultures (Fig. 4).

When the medium was separated from the mycelium at the time of peak activity of laccase, then sterilized by filtration and further incubated, the laccase activity disappeared at the same rate as in the culture with mycelium. Mixing equal amounts of media collected from 44 h-old- and 120 h-old-cultures gave no evidence for the presence of an inhibitor in the 120 h medium. Electrophoresis and immunoblotting of an unboiled concentrate of the medium of a 120 h-old-culture, which had no laccase activity, showed the presence of the three forms of laccase protein

Fig. 3. Immunoblots of extracellular laccase in fraction II (1.4 μg protein) (a, b) and in medium (35 μg protein) from a 120 h-old-culture (c, d) after SDS-PAGE. (a), (c), unboiled; (b), (d), boiled in SDS-sample buffer.

Fig. 4. Extracellular laccase activity (○), immunodetectable laccase protein (●) and glucose consumption (□) during growth of the dikaryon 4-39/4-40 of *S. commune* in surface culture at 30 °C in the dark.

(Fig. 3c), which were converted into the $62 \times 10^3 M_r$ protein by boiling the concentrate in SDS (Fig. 3d). The amount of laccase protein as estimated by the binding of antibodies appeared to be 0.20 μg laccase protein ml^{-1} , close to the amount of laccase protein found in the medium at peak laccase activity (Fig. 4). Thus the disappearance of laccase activity in older cultures was not due to loss of the enzyme protein, but to some kind of inactivation.

This raised the question as to whether the low activities recorded when the dikaryon was grown in the light at 30 °C, or at 24 °C in either the dark or in the light (Table 1) could be explained by normal formation of laccase but increased inactivation. However, using immunodetection laccase protein was found only in the medium of the dikaryon grown at 30 °C in darkness. Similarly no laccase protein was detected in medium of the monokaryons grown under the various conditions.

Fig. 5. Immunodetection of intracellular laccase. (a), Coomassie-Blue-stained, denatured mycelial proteins (200 μ g) of the dikaryon grown for 44 h at 30 °C in darkness, after SDS-PAGE; (b), immunoblot of an unstained duplicate of (a); (c), immunoblot of denatured purified laccase (fraction II) (1.0 μ g total protein) for comparison. The positions of immuno-reacting bands are arrowed.

Intracellular laccase

Mycelial extracts were prepared from the laccase-excreting dikaryon grown for 44 h at 30 °C in the dark. An extract made in the presence of SDS but in the absence of β -mercaptoethanol was examined for laccase activity using DAB after SDS-PAGE. The profile after staining with DAB showed the presence of five laccase bands at positions corresponding to denatured protein standards of M_r 36, 38, 42, 45 and 48×10^3 ; the band with the lowest mobility was the most intensely stained. Coomassie Blue did not reveal protein bands corresponding with the laccases. After blotting no immunostaining was observed. An intracellular extract boiled in the presence of SDS and β -mercaptoethanol was also examined for laccase protein after SDS-PAGE and blotting onto nitrocellulose. The protein patterns on the gel and on the nitrocellulose blot were identical, indicating that no differential blotting had occurred. As shown in Fig. 5a a major protein band was present at the position of extracellular laccase (62×10^3 ; Fig. 5c) but the intracellular band did not react with the antiserum. Instead the immunoblot showed the presence of one reacting band at the position M_r 72×10^3 (Fig. 5b). Both the M_r 72×10^3 and 62×10^3 bands were present after mixing the extract with denatured extracellular laccase, excluding the possibility that a decrease in mobility occurred during electrophoresis of the complex extract. The absence of any other bands after immunoblotting of mycelial proteins demonstrated that the antiserum was highly specific.

DISCUSSION

With the mycelia of *S. commune* used in this study, two co-isogenic monokaryons and the derived dikaryon, no laccase was detected in the medium when growth occurred at 24 °C in the light.

The dikaryon formed numerous fruit-bodies under these conditions and our results are thus at variance with the findings of Leonard and co-workers (Leonard, 1971; Leonard & Phillips, 1973; Phillips & Leonard, 1976; Leslie & Leonard, 1979), who found a correlation between fruit-body formation in *S. commune* and the presence of extracellular laccase. The reasons for this discrepancy are not clear but may be related to differences in strains and methods of cultivation. Significantly, we did find laccase activity in the dikaryon during fruiting but only localized in the young fruit bodies (Wessels *et al.*, 1985). A functional significance of laccase for the process of fruiting can thus still be entertained.

Growth at 30 °C in darkness prevented fruit body formation and induced the dikaryon, but not the progenitor monokaryons, to excrete laccase into the medium in amounts up to 3% of the extracellular proteins. The competence of the dikaryon to produce laccase activity, in contrast to the component monokaryons, was also noted by Leonard & Phillips (1973). Purification of the enzyme and determination of some of its properties revealed the presence of a typical fungal laccase (*p*-diphenol: oxygen oxidoreductase, EC 1.10.3.2; Walker & McCallion, 1980).

Under non-denaturing conditions electrophoresis showed one major and two minor forms of the enzyme, the major form accounting for 98% of the laccase protein and activity. Under denaturing conditions this major form was resolved into two proteins of M_r 64×10^3 and 62×10^3 . The M_r 64×10^3 form was convertible into the M_r 62×10^3 form but the mechanism by which this occurs is not clear. Sometimes only the latter form was observed. These M_r values are in the range recorded for various other fungal laccases (cf. Bollag & Leonowicz, 1984). The purified enzyme solution was pale-yellow but the laccase concentration (0.5 mg ml^{-1}) might have been too low to reveal the blue colour of copper proteins. A yellow laccase has been reported for *Agaricus bisporus* (Wood, 1980a).

The major form of the laccase of *S. commune*, isolated from a non-denaturing gel, was used to raise antibodies. The antiserum obtained was highly specific. It quantitatively precipitated the laccase activity from the medium and apparently reacted with none of the *S. commune* proteins except laccase as shown on Western blots. On electrophoresis of intracellular extracts under non-denaturing conditions five bands with laccase activity were detected but the low concentration of protein probably precluded reaction with the antiserum. However, on denaturation a single antiserum-reactive protein of M_r 72×10^3 was visible, possibly representing the precursor for the extracellular laccase.

The antiserum did not detect laccase protein in the media of the dikaryon grown at 30 °C in the light, or at 24 °C either in the dark or in the light (i.e. in the presence of fruit-body formation), nor in the media of the monokaryons grown under any condition tested. This indicates that at 30 °C in the dark the presence of different alleles of the incompatibility genes in the dikaryon is required for the formation of extracellular laccase. In addition, these results indicate that our failure to detect extracellular laccase in the medium during fruiting (Leonard & Phillips, 1973; Phillips & Leonard, 1976) was not due to inactivation of the enzyme.

The laccase activity in the medium of the dikaryon grown at 30 °C in the dark disappeared after glucose exhaustion. Using the antiserum as a probe for laccase on Western blots it was shown that the loss of activity was not due to loss of the laccase protein. Mixing experiments did not indicate the production of inhibitors by the mycelium during ageing. In *S. commune* a decline in extracellular laccase has been observed after the formation of mature fruit bodies (Leonard & Phillips, 1973; Phillips & Leonard, 1976), whereas such a decline was observed at the start of fruiting in *A. bisporus* (Turner, 1974; Wood & Goodenough, 1977; Wood, 1980b). On the basis of mixing experiments Phillips & Leonard (1976) concluded that in *S. commune* the decrease in activity was due to the production of diffusible inhibitors by the fruiting mycelium. However, similar experiments in *A. bisporus* (Wood, 1980b, 1985) did not indicate the production of such inhibitors. In the latter case, it was also shown that the loss of activity was not due to the loss of the laccase protein. Whatever the nature of the inactivation mechanism, the major difference with the present study is that here the decline in laccase activity occurred in the absence of fruit-body formation, and even in the absence of the mycelium.

A major result of the present study is that it has provided us with a specific antiserum against laccase of *S. commune*. Preliminary results showed that the antiserum also reacts specifically with a protein of M_r 64×10^3 synthesized *in vitro* on RNA extracted from a dikaryon grown at

30 °C in the dark. The antiserum could therefore be suitable for the isolation of the coding sequence for this laccase.

Note added in proof. After purification over DEAE-Sephacel and PAGE in the absence of SDS the enzyme solution with an OD₂₈₀ of 0.7 was colourless. An atomic absorption spectrophotometric determination of the copper and manganese content, of this solution revealed the presence of 0.015 mM-Cu and 0.001 mM-Mn. This indicates a copper content of two atoms per polypeptide chain assuming an M_r of 62×10^3 and a specific OD₂₈₀ of 1.5.

We thank Mr W. Schuurmans at the Institute for Soil Fertility (Haren) for performing this analysis.

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