Production, Purification and Properties of Extracellular Laccase of *Agaricus bisporus*

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Extracellular laccase (EC 1.14.18.1) of the cultivated mushroom *Agaricus bisporus* was produced constitutively in defined or complex media. No enzyme induction was found after treatment with cycloheximide or with other potential inducers such as toluidine or xylidine. The enzyme was purified to homogeneity by ammonium sulphate precipitation, ion-exchange chromatography, gel filtration and affinity chromatography. It eluted as a single peak from ion-exchange, gel filtration and affinity columns and sedimented as a single band on centrifugation. It showed four enzymically active bands on electrophoresis and a diffuse band on isoelectric focusing. Its molecular weight was estimated to be about 100000 and the enzyme contained 15% carbohydrate and two atoms of copper per molecule. The substrate specificity was similar to that of other fungal laccases. Antiserum prepared against the purified enzyme gave one major precipitin band.

INTRODUCTION

Production of extracellular laccase (EC 1.14.18.1) is a common feature of many higher basidiomycete fungi, particularly those associated with wood decay or the terminal stages of decomposition of leaf litter (Kent Kirk, 1971; Higuchi, 1971; Kent Kirk *et al.*, 1977). Ascomycete fungi can also produce extracellular laccase (Walker, 1968; Froehner & Eriksson, 1974*a*). The production, regulation and properties of fungal laccases have been examined in a number of species. In cultures of *Polyporus versicolor* extracellular laccase can be induced above basal levels by treatment of cultures with the aromatic amines toluidine or xylidine (Fahraeus *et al.*, 1958). Similar inducing effects are found with the extracellular laccase of *Neurospora crassa* and in this organism increased laccase production is also induced by treatment with cycloheximide or actinomycin D (Froehner & Eriksson, 1974*b*). The laccase of *Aspergillus nidulans* appears during conidiation and is responsible for a step in the synthesis of conidium pigment (Clutterbuck, 1972). In the basidiomycete *Schizophyllum commune* laccase activity is positively correlated with the ability of monokaryons to fruit (Leonard, 1971) and with the stage of fruiting development in dikaryotic cultures (Leonard & Philips, 1973).

The purified fungal laccases are normally blue, copper-containing, proteins capable of oxidizing *o*- and *p*-phenols and aromatic amines and differ from tyrosinases on the basis of their substrate specificity (Hewitt & Smith, 1975). Differences have been reported in many of the properties of the various fungal laccases (Mosbach, 1963; Fahraeus & Reinhammar, 1967; Cheung & Marshall, 1969; Molitoris, 1976; Froehner & Eriksson, 1974*a*, *b*; Bocks, 1967; Walker, 1968; Matsubara & Iwasaki, 1972; Blaich & Esser, 1975; Leonowicz & Trojanowski, 1975; Dubernet *et al.*, 1977).

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During growth of *Agaricus bisporus* on composted straw, extracellular laccase activity increases and then declines rapidly at the time of fruiting (Wood & Goodenough, 1977). This present investigation had two aims: firstly, to obtain further knowledge of the properties of the laccase from *A. bisporus* and to compare them with those of other fungal laccases in respect of the production, inducibility and physico-chemical properties of the enzyme; secondly, to obtain sufficient pure enzyme to produce enzyme antibody for use in studies of the loss of enzyme activity during fruiting (Wood, 1980).

METHODS

Organism. *Agaricus bisporus* D621 was used. This is a heterokaryotic fertile strain of direct commercial origin. Cultures of the organism have been deposited in the Glasshouse Crops Research Institute collection of basidiomycete fungi. This collection is maintained in liquid nitrogen storage in the Plant Breeding Department of the Institute.

Media and culture. Mycelium was grown in a 2% (w/v) malt extract medium as previously described but with the omission of agar (Wood, 1976). For small-scale cultures, two agar cubes (2 × 2 × 2 mm) cut from colonized malt extract agar plates were inoculated into 50 or 100 ml sterilized malt extract medium in a 250 ml conical flask. The cultures were grown at 25 °C either static or shaken at 200 rev. min^{-1} on a Gallenkamp orbital shaker. Culture filtrates and mycelium were harvested either by filtration through glass-fibre discs (9·0 cm diam., Whatman GF/C) or by centrifugation at 10000 ***g*** for 15 min. Duplicate or triplicate cultures were removed at suitable intervals to follow growth and enzyme production. Mycelial dry weights were estimated after oven drying at 95 °C for 24 h.

Similar small-scale cultures were also set up with basal medium (Treschow, 1944) supplemented with various sole carbon sources (1%, w/v), L-glutamic acid [0·07% (w/v); neutralized with KOH] as nitrogen source, and thiamin (0·2 mg l^{-1}) and biotin (0·02 mg l^{-1}) to satisfy vitamin requirements. These cultures were grown for 6 weeks, then the mycelium was removed, its dry weight was determined, and the laccase activity of the culture filtrate was measured.

For enzyme purification, large-scale cultures were grown. These consisted of 10 l malt extract medium in a 15 l glass vessel. The sterile medium was inoculated with 400 ml of a suspension of 4-week-old small-scale cultures which were homogenized in a sterile Waring blendor prior to inoculation of the bulk medium. The bulk culture was aerated with sterile air that had been passed through a Whatman Gamma filter and distributed in the medium through a glass sparger. Foam control was maintained by adding 2 or 3 drops of sterile antifoam (Polyglycol P2000; Dow Chemical Co., Michigan, U.S.A.) when required. The culture was grown for 4 weeks and then the mycelium was removed by filtration through muslin sheet and the filtrate was clarified by centrifuging at 10000 ***g*** for 15 min. The supernatant containing the crude laccase activity was stored in 2 l batches at −20 °C. Frozen supernatants remained fully active for at least 2 years.

Enzyme localization. Samples of mycelium (3 g fresh weight) suspended in 0·1 M-Tris/HCl buffer pH 7·5 were homogenized using a Silverson blendor. Approximately 70% of the hyphae were ruptured. The resulting homogenate was centrifuged at 10000 ***g*** for 20 min and the pellet and supernatant were separated. The supernatant was centrifuged again at 100000 ***g*** for 60 min and the pellet and supernatant were separated. Each fraction and the original culture filtrate were assayed for laccase and tyrosinase activity.

Enzyme assays. Laccase was assayed polarographically as previously described (Wood & Goodenough, 1977). Laccase activity was qualitatively determined in column fractions by means of a rapid spot test. Small volumes of column fractions (20 to 50 μl) were mixed with 200 μl 0·1 M-sodium acetate/acetic acid buffer pH 5·6 containing *N,N*-dimethyl-*p*-phenylenediamine (1 mg ml^{-1}) in the wells of a spotting tile. A red quinone product appeared in wells containing significant laccase activity within 5 min. Alkaline phosphatase activity was assayed as previously described using *p*-nitrophenylphosphate as substrate (Wood & Goodenough, 1977). Tyrosinase was assayed polarographically using an assay similar to that for laccase but substituting 3,4-dihydroxyphenylalanine as substrate. One unit of laccase or tyrosinase is defined as that amount of enzyme catalysing the consumption of 1 μmol O_2 min^{-1}.

Enzyme purification. Samples (3 l) of the bulk culture filtrates were reduced to approximately one-tenth volume by ultrafiltration in an Amicon model 202 ultrafiltration cell using a PM10 membrane (molecular weight cut-off, 10000). All of the enzyme activity was retained in the concentrate. Subsequent operations were then carried out at 5 °C. Laccase was precipitated by ammonium sulphate between 50 and 90% saturation and the precipitate was collected by centrifugation (20000 ***g*** for 30 min). The precipitate was dissolved in approximately 40 ml 0·01 M-phosphate buffer pH 7·0 and dialysed for 24 h against two changes of similar buffer. Batches (10 ml) of the dialysed extract, a dark brown liquid, were applied to the top of a column (1·5 × 30 cm) of DE52 ion-exchange cellulose (Whatman) previously equilibrated with 0·01 M-

Table 1. *Purification of extracellular laccase from Agaricus bisporus*

Fraction	Volume (ml)	Protein (mg ml⁻¹)	Activity (units ml⁻¹)	Specific activity (units mg⁻¹)	Yield (%)	Purification factor
Culture supernatant	3000	1·88	0·43	0·229	100	—
Ultrafiltration	350	5·20	3·28	0·630	88	2·7
Ammonium sulphate precipitation	55	15·00	19·8	1·320	84	5·8
DEAE-cellulose chromatography	6·0	18·00	129·7	7·200	60	31·4
Sephadex G-100 gel filtration	6·8	6·56	80·3	13·250	42	57·8
Con A–Sepharose affinity chromatography	5·0	7·24	99·2	13·700	38	59·8

phosphate buffer pH 7·0. Several column volumes of similar buffer were then passed through but, though a brown-pigmented material eluted, no enzyme activity was eluted. The column was then developed with a linear gradient of 0 to 0·5 M-NaCl in 0·01 M-phosphate buffer pH 7·0 in a total volume of 500 ml at a flow rate of 20 ml h^{-1}. The column eluate was monitored for absorbance at 280 nm and 2 ml fractions were collected. When the enzyme activity had been located it was assayed quantitatively and the appropriate fractions (numbers 80 to 116) were then pooled and concentrated to a small volume (5 to 6 ml) by ultrafiltration. Samples of the concentrate (2 ml) were mixed with 0·5 ml glycerol and layered on to the top of a column (1·5 × 90 cm) of Sephadex G-100 previously equilibrated with 0·01 M-phosphate buffer pH 7·0. The column was eluted with similar buffer and fractions were collected and assayed as described above. Suitable fractions (numbers 24 to 34) were pooled, concentrated and applied to the top of a column (0·9 × 30 cm) of Concanavalin A–Sepharose previously equilibrated in 0·01 M-phosphate buffer pH 7·0. The enzyme was adsorbed on to the column and 6 to 7 column volumes of buffer were passed through without elution of the enzyme. It was desorbed by elution with 100 ml 10% (w/v) α-methyl-D-mannopyranoside dissolved in 0·01 M-phosphate buffer pH 7·0. Enzyme elution was monitored and suitable fractions (numbers 58 to 100) were dialysed against two changes of 0·01 M-phosphate buffer for 24 h. The dialysed enzyme was concentrated by ultrafiltration and stored frozen at −20 °C in batches of 0·5 ml until required. Purified frozen enzyme retained complete activity for at least one year. The purification scheme is outlined in Table 1.

Disc electrophoresis. Polyacrylamide gels (5 or 7·5%, w/v) at pH 8·9 were prepared by the method of Davis (1964) but with the omission of both spacer and small pore gel layers. Samples (10 to 150 μl) containing enzyme (10 to 200 μg protein) were mixed with 25% (v/v) glycerol containing 0·03% (w/v) bromophenol blue and then loaded on the gel surface and electrophoresed at 4 mA per tube until the tracker dye was 0·5 cm from the end of the tube. Gels were stained for protein using Coomassie blue by the method of Chrambach *et al.* (1967). Enzyme activity was located by immersing the gels in solutions (1 mg ml^{-1}) of *p*-phenylenediamine, *N*,*N*-dimethyl-*p*-phenylenediamine hydrochloride or guiacol in 0·05 M-sodium acetate/acetic acid buffer pH 5·6 or 3·5. The activity was visualized as coloured bands which were photographed for permanent record. Polyacrylamide gels at pH 4·5 were prepared by the method of Gabriel (1971) and protein was stained and enzyme activity was localized as described above.

The molecular weights of the different electrophoretic forms of the native enzyme were estimated by a polyacrylamide gel method (Barton, 1972) using gel concentrations of 5, 7, 10 and 11·25% (w/v). The mobilities of the different forms were compared with those of proteins of known molecular weight. The following proteins, with their molecular weights as given by Sober (1970), were used for calibration: pepsin (35000), ovalbumin (46000), haemoglobin (64000) and calf intestinal alkaline phosphatase (100000). Sodium dodecyl sulphate (SDS) gel electrophoresis was carried out on denatured enzyme by the method of Dunker & Rueckert (1969) using 10% (w/v) gels. A series of 11 marker proteins over the molecular weight range 12400 to 67000 (Brunt, 1977) were run in duplicate and the molecular weights of the laccase polypeptides were estimated from the plot of logarithm of molecular weight versus mobility.

Isoelectric focusing. Samples of crude and purified enzyme were examined by isoelectric focusing in polyacrylamide gels as described by Wrigley (1968). The carrier ampholine was broad range pH 3·5 to 10·0 (LKB). Samples of the enzyme (10 μg protein) were polymerized *in situ* throughout the gel column. Cytochrome *c* and haemoglobin, proteins of known isoelectric point, were similarly treated, 10 μg of each being incorporated into separate gels. Current was applied at 1 mA per tube, and when the marker proteins had formed sharp bands, focusing was terminated and the laccase-containing gels were stained for activity. The slope of the pH gradient was estimated by measuring the pH value of eluates of 1 cm long slices of three

blank gels focused simultaneously with the laccase preparation. Isoelectric points were then obtained graphically from the plot of gel length versus pH.

Sedimentation coefficient and molecular weight. This was determined by analytical ultracentrifugation of samples of purified enzyme (5 mg protein ml^{-1}) using a synthetic boundary cell method. The sedimentation coefficient was obtained by the graphical method of Markham (1960). Sedimentation coefficient and molecular weight were also estimated by use of sucrose density gradient analysis (Martin & Ames, 1961). Samples of enzyme (0·1 ml, containing 50 μg protein) were layered on to 13 ml gradients of 5 to 20% (w/v) sucrose and centrifuged at 110000 ***g*** for 36 h. Haemoglobin was used as an internal marker. The molecular weight of the enzyme was also estimated from its elution volume through calibrated columns of Sephadex G-100 or G-200. The following marker proteins, with their molecular weights as given by Sober (1970), were used for calibration: cytochrome *c* (12400), myoglobin (17200), trypsin (23500), ovalbumin (46000), bovine serum albumin (67000) and calf intestinal alkaline phosphatase (100000). The elution volumes of all these, except alkaline phosphatase, were obtained from the absorption of eluted peaks at 280 nm. Alkaline phosphatase was assayed enzymically.

Carbohydrate determination. The amount of carbohydrate bound to the purified enzyme was determined by an orcinol method (Froehner & Eriksson, 1974*a*) using glucose as standard.

Copper determination. Samples of purified enzyme (3·0 ml, 0·6 mg protein ml^{-1}) were mixed with 3·0 ml 12 M-HCl and incubated at 100 °C for 24 h. The HCl was then removed by evaporation and the copper was determined by a colorimetric method based on that of Andrus (1955) as described by Graves & Sutcliffe (1974). Duplicates of 3·0 ml of the buffer used for the enzyme solution were also analysed and the values were subtracted from those obtained with enzyme solution to give the true copper content.

Amino acid analysis. Samples of enzyme (1 mg) were lyophilized in glass ampoules and then resuspended in 0·9 ml 6 M-HCl. Oxygen was removed by repeated evacuation and flushing with oxygen-free nitrogen. The ampoule was sealed and the protein was hydrolysed by incubation for 21 h at 100 °C. Part of the hydrolysed sample was analysed on a Locarte analyser using norleucine as internal standard. Values were corrected for degradation of serine, glycine, methionine and cysteine.

Antibody production and serology. The purified enzyme from the Concanavalin A–Sepharose stage was used as antigen in an immunization procedure. Enzyme (1·5 ml, 0·6 mg protein ml^{-1}) was mixed with an equal volume of complete Freund's adjuvant (Difco) by vigorous mixing with a syringe until an emulsion formed. Samples of the emulsion (0·25 ml) were injected intradermally into several sites on the depilated flanks of two rabbits. The protein/adjuvant mixture was stored at −20 °C and the injection was repeated via the same route after 2 weeks. A test bleed was taken after a further week but the antibody titre was low, so a further injection was given 1 week later. The antibody-containing serum was obtained 1 week after the final injection.

Precipitin reactions were examined by the double diffusion technique on glass slides (Ouchterlony, 1949). After precipitin band formation the agar was washed with several changes of saline, followed by several distilled water washes and finally dried on the slide by incubation for 12 h at 50 °C. The precipitin bands were stained by immersion in 0·1% (w/v) Amido black in 7% (w/v) acetic acid, and then destained in 7% (w/v) acetic acid and photographed.

For immunoelectrophoresis, 100 μg enzyme protein was electrophoresed in a pH 8·9 polyacrylamide gel and then the gel was laid in molten immunodiffusion agar maintained at 44 °C in a 9 cm diam. Petri dish. A trough to hold 0·3 ml antiserum was made by placing a small plastic former in the dish and removing it when the agar had solidified. The dish was placed in a moist chamber and a precipitin arc formed overnight.

Protein determination. Protein was determined by the Lowry method, with bovine albumin, fraction V (Sigma) as standard.

RESULTS

Enzyme localization, production and purification

Assays of the culture filtrate and centrifuged homogenates of the mycelium showed that extracellular laccase accounted for nearly 90% of the total activity. The bulk of the remaining intracellular activity was also found in the soluble fraction (Table 2). No extracellular tyrosinase was found and the intracellular tyrosinase activity was distributed between the particulate and soluble fractions.

A range of sole carbon source media were examined to determine if extracellular enzyme production was inducible or constitutive. The fungus produced extracellular laccase on all the carbon sources examined (Table 3) with approximately a twofold variation in the yield of enzyme per unit weight of mycelium. In contrast, in malt extract medium the enzyme

Table 2. *Localization of laccase and tyrosinase of Agaricus bisporus*

Substrates were *p*-phenylenediamine (10 mM) for laccase and 3,4-dihydroxyphenylalanine (2·5 mM) for tyrosinase.

Fraction	Activity (% of total) Laccase	Tyrosinase
Culture supernatant	88·5	0
1st particulate (10000 *g* pellet)	3·5	71·8
1st soluble (10000 *g* supernatant)	8·0	28·2
2nd particulate (100000 *g* pellet)	1·5	25·9
2nd soluble (100000 *g* supernatant)	6·5	2·3

Table 3. *Production of extracellular laccase by cultures of Agaricus bisporus on defined or complex media*

Carbon source (1%, w/v)	Extracellular laccase [units (g mycelium)$^{-1}$]	Carbon source (1%, w/v)	Extracellular laccase [units (g mycelium)$^{-1}$]
Glucose	29	Maltose	28
Mannitol	26	Sucrose	20
Fructose	25	Ribose	29
Trehalose	21	Rhamnose	11
Xylose	19	Malt extract (2%, w/v)	102
Cellobiose	26		

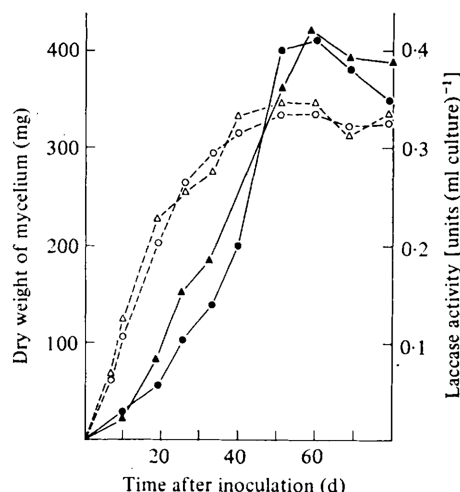

Fig. 1. Mycelial growth and laccase production of *A. bisporus* in static cultures (○, △) or shaken cultures (●, ▲) of malt extract medium at 25 °C: ○, ●, dry weight of mycelium; △, ▲, laccase activity.

yields were three- to fourfold greater than the best yields from the single carbon source media (Table 3) and for this reason malt extract medium was used for bulk enzyme production. The maximum yield of enzyme was found at the end of the exponential growth phase of the fungus (Fig. 1) when grown in shaken or static cultures. Enzyme production paralleled fungal growth in both types of culture but enzyme yield and fungal biomass were slightly greater in shaken cultures (Fig. 1).

Attempts were made to determine the effect on enzyme yield of compounds employed for

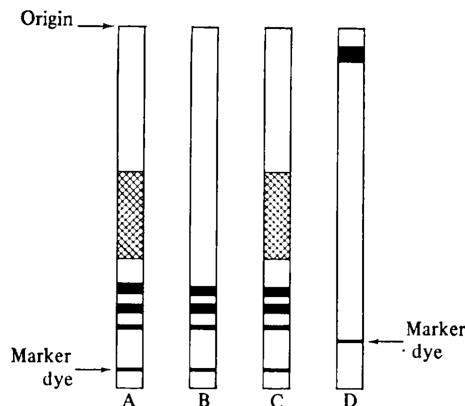

Fig. 2. Disc electrophoresis of purified laccase of *A. bisporus* on 5% acrylamide gels. For running conditions and staining methods, see Methods. Gels A, B and C were at pH 8·9: A, stained with *p*-phenylenediamine; B, stained with guiacol; C, stained with *N*,*N*-dimethyl-*p*-phenylenediamine. Gel D was at pH 4·5: stained with *p*-phenylenediamine. Cross-hatching represents diffuse staining of enzyme activity.

laccase induction in other fungi. Xylidine and toluidine were tested at final concentrations of 1·0 and 0·1 mM using 2 and 4 week-old cultures, grown in both static and shaken conditions and using both malt extract and glucose minimal medium. No increase in enzyme yield above that in control cultures was found, and at the higher concentrations tested both compounds were inhibitory to growth and enzyme production. Cycloheximide tested in a similar manner at a final concentration of 1·0 and 0·1 mM was also inhibitory to both growth and enzyme production.

Five purification steps were required to obtain a preparation free of both pigment and other contaminating proteins (Table 1). Several criteria were used to establish that the final preparation was highly pure. No non-enzymic bands of protein were detected on staining replicate polyacrylamide gels separately for enzyme and protein. The purified enzyme eluted as a single peak of activity and ultraviolet absorbance from columns of Sephadex G-100 and G-200. The enzyme protein gave only a single peak on centrifugation in the analytical ultracentrifuge.

Physical properties of laccase

Sedimentation of the enzyme in the analytical ultracentrifuge showed that a single protein was present with an $s_{20,w}$ value of 5·5. In sucrose density gradients the enzyme centrifuged as a single band with an $s_{20,w}$ value of 5·35. A molecular weight of 102000 was calculated using the method of Martin & Ames (1961). The molecular weight estimated by measurement of elution volume from gel filtration columns was 100000. The gel electrophoretic method used gave estimates for the molecular weights of the enzymically staining bands found on pH 8·9 polyacrylamide gels as 62000, 66000, 67500, and 76000 to 117000 for the broad band (Fig. 2).

Both crude and purified enzyme showed the same pattern of several distinct bands of activity after electrophoresis on polyacrylamide gels at pH 8·9, but both only gave a single enzymically active band at pH 4·5 (Fig. 2). The least mobile diffuse band showed little or no activity with the substrate guiacol (Fig. 2). Isoelectric focusing in polyacrylamide gels of crude or purified enzyme showed that enzyme activity focused over a broad pH range from 3·4 to 4·0 whereas the marker proteins focused as one or more sharp bands in comparable gels. Denatured purified enzyme electrophoresed on SDS–polyacrylamide gels showed that the enzyme protein comprised several polypeptides with molecular weights of 56000, 53100,

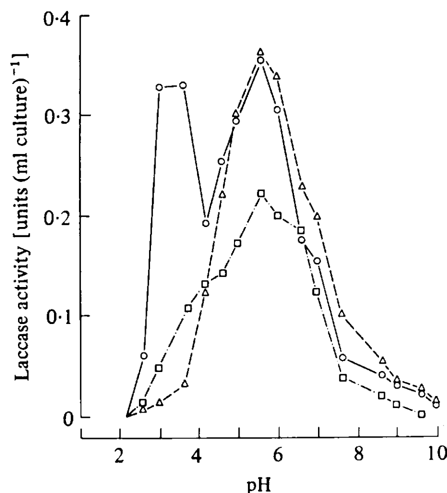

Fig. 3. pH–activity profiles for *A. bisporus* laccase with three different substrates: ○, *p*-phenylenediamine; □, guiacol; △, *N*,*N*-dimethyl-*p*-phenylenediamine. Buffers: pH 2·2 to 3·0, 0·1 M-glycine/HCl; pH 3·8 to 5·6, 0·1 M-sodium acetate/acetic acid; pH 6·0 to 8·0, 0·1 M-sodium phosphate; pH 8·6 to 10·0, 0·1 M-glycine/NaOH.

Table 4. *Substrate specificity of laccase from Agaricus bisporus*

The rate of oxidation of each substrate is expressed relative to the rate of *p*-phenylenediamine oxidation (100%).

Substrate (1 mM)	Relative activity (%)	Substrate (1 mM)	Relative activity (%)
Quinone	0	1-Naphthol	60
Anthraquinone	0	*m*-Phenylenediamine	0
N,*N*-Dimethyl-*p*-phenylenediamine	94	Resorcinol	0
Guiacol	72	Phloroglucinol	8
Catechol	26	Orcinol	0
DL-3,4-Dihydroxyphenylalanine	0	Tyrosine	0
Gallic acid	14	Ascorbic acid	16
Pyrogallol	62	Ferrocyanide	104

51900, 45400, 31900 and 21400, with that of 53100 molecular weight being the most prominent.

The absorption spectrum of the purified enzyme had a peak at 280 nm and a 280/260 ratio of 1·003. Concentrated enzyme samples (> 10 mg ml^{-1}) were pale yellow.

Kinetic properties of laccase

pH-activity curves for the three substrates *p*-phenylenediamine, guiacol and *N*,*N*-dimethyl-*p*-phenylenediamine are shown in Fig. 3. The curve for *p*-phenylenediamine showed two distinct peaks, one at pH 3·6 and one at 5·6, but the other two substrates gave only a single peak with optimal activity at pH 5·6 (Fig. 3).

The Michaelis constant was estimated from a Lineweaver–Burk plot to be 0·1 mM for *p*-phenylenediamine. A series of *o*-, *m*- and *p*-phenols and aromatic amines were examined as possible enzyme substrates. The enzyme oxidized both *o*- and *p*-phenols and aromatic amines and, at a low rate, the *m*-phenol phloroglucinol (Table 4). No activity was found with diphenols such as 3,4-dihydroxyphenylalanine and tyrosine. This pattern of substrate specificity corresponded to that of a polyphenoloxidase of the laccase type.

Table 5. *Effects of various potential inhibitors on the activity of laccase from Agaricus bisporus*

Laccase activity was assayed with *p*-phenylenediamine (5 mM) as substrate.

Potential inhibitor	Inhibition (%)	Potential inhibitor	Inhibition (%)
Sodium azide (1 mM)	100	8-Hydroxyquinoline (1 mM)	0
Sodium azide (0·1 mM)	90	Cetyltrimethylammonium bromide (1 mM)	80
Potassium cyanide (1 mM)	100	Cetyltrimethylammonium bromide (0·1 mM)	5
Potassium cyanide (0·1 mM)	86	Cetylpyridinium bromide (1 mM)	87
EDTA (1 mM)	26	Cetylpyridinium bromide (0·1 mM)	5
EDTA (0·1 mM)	5		
Diethyldithiocarbamate (1 mM)	0		
Cysteine (1 mM)	0		

Table 6. *Amino acid composition of laccase from Agaricus bisporus*

Amino acid	Amount [g (100 g protein)$^{-1}$]	Residues per 85000 mol. wt* as nearest integer	Amino acid	Amount [g (100 g protein)$^{-1}$]	Residues per 85000 mol. wt* as nearest integer
Aspartic acid, Asparagine	11·53	74	Valine	6·59	48
			Methionine	0·49	3
Threonine	6·45	46	Isoleucine	9·80	63
Serine	8·68	70	Leucine	7·77	50
Glutamic acid, Glutamine	7·17	41	Tyrosine	1·96	9
			Phenylalanine	3·93	20
Proline	7·62	56	Lysine	1·89	11
Glycine	12·52	141	Histidine	2·17	12
Alanine	7·50	71	Arginine	3·51	17
Cysteine	0·39	3			

* Assuming a molecular weight for laccase of 100000 and a carbohydrate content of 15%, the protein molecular weight is about 85000.

Several other inhibitors of enzyme activities, either metal-binding agents or detergents, were tested for their action on the purified enzyme (Table 5). The enzyme was totally inhibited by sodium azide and potassium cyanide and partly inhibited by EDTA, but no inhibition was found with diethyldithiocarbamate or cysteine.

Enzyme activity was also inhibited by the detergents cetyltrimethylammonium bromide and cetylpyridinium bromide.

Other properties

The enzyme was thermostable with a half-life of 10 min at 70 °C, 40 min at 60 °C and 3 h at 50 °C. It remained completely stable at 40 °C and below for at least 24 h. Samples of crude and purified enzyme remained completely active on incubation with solutions of trypsin or *Streptomyces* protease (10 mg ml^{-1}, final concentration) for 24 h at 25 °C.

The purified enzyme contained 1·17 μg copper per mg enzyme, which corresponded to 1·8 atoms of copper per molecule assuming a molecular weight of 100000. Analysis for carbohydrate showed the purified enzyme to be a glycoprotein containing 14·6% carbohydrate estimated as orcinol-positive material. Amino acid analysis of the purified enzyme showed no unusual values for individual amino acid residues (Table 6).

Tests with crude or purified enzyme by the Ouchterlony double diffusion technique showed that a single major precipitin band was formed against enzyme antiserum and at high antigen loadings a further faintly distinguishable precipitin band was present. Extracts

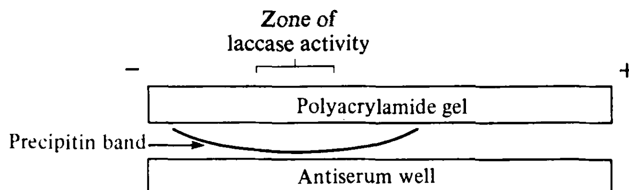

Fig. 4. Immunoelectrophoresis of laccase of *A. bisporus*. Enzyme was electrophoresed in polyacrylamide gel (10 %) and then tested against antibody. Note the single continuous arc formed.

of mycelium gave a single precipitin band continuous with the extracellular enzyme precipitin line. Enzyme samples analysed by immunoelectrophoresis showed that a single precipitin arc was formed (Fig. 4).

DISCUSSION

Most of the laccase produced by the mycelium of *A. bisporus* appeared in the culture medium. Since no tyrosinase, an intracellular enzyme, appeared in this fraction it can be concluded that laccase is a true excreted enzyme and is not found in the medium as a result of cellular autolysis. Laccase was also present in the intracellular extracts at much lower levels and in soluble form. This type of distribution has been observed for other fungal laccases (Froehner & Eriksson, 1974*a*). Laccase excretion paralleled growth in both static and shaken cultures, enzyme yields being similar in both conditions. Other workers have found much lower laccase production in shaken fungal cultures (Bocks, 1967).

Laccase was produced on all the media examined but at threefold higher concentrations in malt extract medium. The latter type of medium also gives increased yields of laccase in cultures of *Polyporus versicolor* (Fahraeus *et al.*, 1958). The identity of the inducing compounds which stimulate increased laccase production in malt extract medium remains undetermined. A more exhaustive study employing a large range of aromatic compounds might reveal whether the laccase of *A. bisporus* can be induced above the levels reported here.

The specific activity of the purified enzyme is close to that reported for *Neurospora* laccase (Froehner & Eriksson, 1974*a*). The final enzyme preparation was pale yellow in contrast to the blue of the other fungal laccases (Malmstrom *et al.*, 1975; Hewitt & Smith, 1975). Nevertheless, analysis for copper showed the presence of approximately 2 copper atoms per molecule of enzyme. The difference in colour of this laccase presumably indicates that the copper atoms exist in a state different from those of other previously characterized laccases (Malmstrom *et al.*, 1975).

The value for the specific activity allows a calculation of the amount of excreted enzyme produced and shows that this accounts for 0·7 % of the fungal biomass or 2·1 % of the fungal protein assuming a protein content of 33 % of dry weight (Maw & Flegg, 1975). Thus, laccase is a major product of protein synthesis and this probably reflects its importance in the nutrition of the organism. It has recently been demonstrated that laccase activity is an obligatory requirement for the degradation and subsequent metabolism of lignin by basidiomycete fungi (Ander & Eriksson, 1976). It is surprising, therefore, that such high enzyme levels are produced on simple defined media where the enzyme has no obvious nutritional function. The constitutive synthesis of enzyme parallels the finding of lack of inducibility in the presence of aromatic and other compounds. This is in contrast to *Neurospora* laccase, where non-induced cultures have very low levels of enzyme (Froehner & Eriksson, 1974*a*) but can be induced to high levels, but it is similar to the findings that in cultures of *Polyporus versicolor* laccase is produced without a requirement for inducers (Fahraeus *et al.*, 1958).

Most of the criteria used showed that the final preparation of enzyme was highly purified.

The binding of the enzyme to Concanavalin A–Sepharose and its subsequent desorption by solutions of α-methyl mannoside was evidence that the enzyme is a glycoprotein, and this was confirmed by analysis for the presence of carbohydrate showing that the enzyme contains approximately 15% of orcinol-positive material. Many excreted proteins from fungi and other organisms have been shown to be glycoproteins. Whether the carbohydrate moiety has any role in enzymic activity or structural integrity of the enzyme remains to be determined. The molecular weight and sedimentation coefficient of the purified enzyme differed from those reported for laccases from other fungi, e.g. *Polyporus versicolor* (molecular weight 64000; Malmstrom *et al.*, 1975), *Russula delica* (molecular weight 63000; Matsubara & Iwasaki, 1972), *Podospora anserina* (forms I, II and III, molecular weights 340000, 78000 and 82000, respectively, sedimentation coefficients 14·47, 5·37 and 5·26; Esser & Minuth, 1970; Molitoris, 1976), *Botrytis cinerea* (molecular weight 64800; sedimentation coefficient 3·2; Mayer *et al.*, 1977). The closest molecular weight was that of the laccase from *Aspergillus nidulans*, 117000 (Clutterbuck, 1972). It would be of interest to determine if there are any detectable antigenic relationships among this apparently diverse group of enzymes, using the antiserum prepared during this study. Use of antiserum prepared against the enzyme 3-carboxymuconate cyclase showed close intergeneric relationships between *Penicillium* and *Aspergillus* but no cross-reaction was observed with extracts from a basidiomycete (*Schizophyllum commune*) or prokaryotes (*Nocardia* sp. and *Pseudomonas* sp.) (Cook & Cain, 1977).

Although the *A. bisporus* laccase behaved as a single molecular species on chromatographic and centrifugation analyses, it exhibited multiple enzymically active forms on gel electrophoresis at pH 8·9. The apparent molecular weight of these forms differed but this behaviour may be due to minor differences in charge on fractions of the population of purified enzyme molecules. Multiple electrophoretic forms of other fungal polyphenoloxidases have been shown to be due to small differences in the length and properties of the glycan chains on sub-fractions of the enzyme population (Esser & Minuth, 1971). Similar examples of fungal laccases behaving as single proteins in some separation systems but as multiple forms in others have been reported for *Neurospora crassa* (Froehner & Eriksson, 1974*b*), *Trametes (Polyporus) versicolor* (Jonsson *et al.*, 1968; Cheung & Marshall, 1969) and *Russula pseudodelica* (Matsubara & Iwasaki, 1972). The broad band of activity found in isoelectric focusing gel columns may also indicate charge heterogeneity of the enzyme population. The enzyme protein was shown to be composed of several polypeptides when analysed on SDS–polyacrylamide gels, but the structure has not been further investigated. The laccase of *Podospora anserina* is also composed of several subunits (Molitoris, 1976). It is possible that the multiplicity of bands found with the *A. bisporus* laccase may also be due to differences in the length of associated glycan chains, and the subunit structure may well be of a simple dimeric form as suggested recently for the tyrosinase of *A. bisporus* (Strothkamp *et al.*, 1976).

The substrate specificity of the enzyme corresponded with that of a laccase type of polyphenoloxidase. The pH optima for the substrate *p*-phenylenediamine was unusual in showing a bimodal distribution, but whether the more acid activity has any physiological significance is not known. The substrates colonized by the organism in nature, such as composted leaf litter, are likely to be at near neutral pH values.

Although evidence was obtained to show that the enzyme was a copper-containing protein, it was not inhibited by diethyldithiocarbamate or 8-hydroxyquinoline. Possibly the copper is bound in a form which is not accessible to these inhibitors. The enzyme was completely inhibited by azide and cyanide which are classical inhibitors of metal-containing oxidases. Inhibition by the quaternary ammonium detergents, such as cetylpyridinium bromide, has been observed for the laccase of the ascomycete *Glomerella cingulata* (Walker, 1968). The enzyme was of similar thermostability to other fungal laccases and tyrosinases (Bull & Carter, 1973), a feature which is characteristic of this group of enzymes. The enzyme

protein was also resistant to certain proteases. This is obviously a suitable property for an extracellular enzyme, especially as cultures of *A. bisporus* in composted straw or conventional media produce proteases that are active over a broad pH range (Wood & Goodenough, 1977; Wood, 1980). The amino acid content of the purified *A. bisporus* enzyme was similar to the published data for the *Polyporus* enzyme (Malmstrom *et al.*, 1975).

The antiserum produced when the purified enzyme was used as antigen showed one major and one minor precipitin band in double diffusion tests. Extracts of intracellular protein gave a precipitin band continuous with the extracellular enzyme. The cross-reacting intracellular band is presumably the intracellular laccase which must have similar antigenic properties to the extracellular enzyme. Since the antigen used was pure with respect to several criteria, the minor band is almost certainly due to enzyme–antibody precipitin formation and may be a result of cross-reaction with a minor species of laccase not separable by chromatographic methods or of cross-reaction with one or more of the different electrophoretic forms. That these forms showed one continuous arc in immunoelectrophoretic analysis is further evidence that antibody to a single antigen was prepared. This antiserum was of sufficient titre and specificity to allow its use in studies of the loss of laccase activity during fruiting (Wood, 1980).

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