Purification of laccase I from *Armillaria mellea*

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(Received 18 December 1991; accepted 28 February 1992)

Laccase activity accumulated during rhizomorph development in malt extract cultures of *Armillaria mellea*. The activity was readily separated into two bands by PAGE under non-denaturing conditions. The less rapidly migrating activity (on PAGE) was purified by ammonium sulphate precipitation, anion-exchange chromatography and gel filtration. The purified enzyme (laccase I) showed a main polypeptide of M_r 59000. Activity of the purified enzyme was greatest with 2,6-dimethoxyphenol (DMOP) as substrate. Syringaldazine, *p*-phenylenediamine and other typical laccase substrates were readily oxidized. No oxidation of tyrosine was detected. The K_m for DMOP was 0.178 mM and for *p*-phenylenediamine was 1.69 mM. The pH optimum for *p*-phenylenediamine oxidation was 3.5. Electrophoretically purified main polypeptide of laccase I was used to raise a specific antibody. Immunoblot analysis showed that whilst the antibody bound strongly to laccase I, no binding to laccase II was detectable. Antibody raised against pure laccase from *Agaricus bisporus* reacted with laccase I from *Armillaria mellea* but not with laccase II. The isoelectric pH was 4.1 for laccase I and 3.3 for laccase II.

Introduction

Secretion of extracellular laccase (polyphenoloxidase, EC 1.10.3.2) is a common property amongst basidiomycete fungi. In organisms such as *Sporotrichum pulverulentum* and *Phlebia radiata* a function related to lignin degradation has been proposed (Ander & Eriksson, 1976; Kantelinen *et al.*, 1989). A similar function may apply to the constitutively produced laccase of *Agaricus bisporus* (Wood, 1985). In contrast, other basidiomycetes such as *Lentinus edodes* and *Schizophyllum commune* secrete laccase in association with fruit body development (Leatham & Stahmann, 1981; De Vries *et al.*, 1986). *Armillaria mellea* secretes laccase activity in association with the differentiation of vegetative mycelium to form rhizomorphs (Worrell *et al.*, 1986). Although not all *Armillaria* spp. produce rhizomorphs, root infection by these strand-like structures formed of numerous hyphae is a principal means of vegetative spread of the fungus in forest stands (Wargo, 1990). In this paper some properties of laccase secreted

by *A. mellea* during rhizomorph formation in liquid culture are reported.

Methods

Organism. *Armillaria mellea* 8R was obtained from J. Rishbeth (University of Cambridge, UK). It was originally isolated from dead *Rosa* spp. in January 1983 at Cambridge. The culture was maintained on 2% (w/v) malt extract 1% (w/v) agar slopes grown at 25 °C and stored at 4 °C.

Growth medium. Experimental cultures were grown in 3% (v/v) malt extract syrup (Boots plc, England) adjusted to pH 5.0, except where stated otherwise, and autoclaved at 118 °C for 10 min. It was determined experimentally that 3% (v/v) malt extract syrup was equivalent to 2.2% (w/v) dry matter of malt extract.

Culture conditions. For purification of laccase enzymes 100 ml medium in 1 litre Roux bottles was inoculated with six 6 mm plugs cut from the growing margin of an *A. mellea* colony growing on malt extract agar. Generally 20–24 bottles were used. The bottles were sealed with vaccine stoppers or rubber bungs and incubated laid flat at 25 °C in the dark. Incubation was static but the bottle contents were mixed gently once per day. Where other bottles and medium volumes were used, details are given in figure legends. Culture filtrate was recovered by filtration first through cheese-cloth and subsequently through sintered glass filters (porosity no. 2).

Laccase assay. Laccase activity was determined quantitatively by polarographic measurement of oxygen consumption (Wood, 1980).

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Abbreviation: DMOP, 2,6-dimethoxyphenol.

Assay mixtures contained (in 1 ml): 27 μ mol citric acid/disodium hydrogen phosphate buffer, pH 4.0, 15 μ mol *p*-phenylenediamine and culture filtrate or enzyme fraction added as up to 30% of the total volume. The reaction was initiated by addition of the *p*-phenylenediamine and oxygen consumption was followed at 25 °C in a stirred reaction vessel with a Clark-type oxygen electrode in its base (Rank Brothers, Cambridge). Enzyme activity was expressed as μ mol oxygen consumed min^{-1} .

The position of laccase activity on gels after non-denaturing electrophoresis or isoelectric focussing was revealed by incubation with 10 mM-guaiacol (Wood, 1980) or 0.05% syringaldazine, from which laccase gives an intensely purple-coloured product (Harkin *et al.*, 1974).

Electrophoresis. SDS-PAGE was performed as described by Laemmli (1970). Non-denaturing electrophoresis was performed under the same conditions except that β -mercaptoethanol and SDS were omitted.

Isoelectric focussing (IEF). The Phast Gel system (Pharmacia) was used with gels that generated a gradient from pH 3 to pH 9.

Protein detection and quantification. After electrophoresis and IEF, protein on gels was stained with Coomassie brilliant blue or with silver using a Bio-Rad kit according to the manufacturer's instructions. Protein concentration was determined using the method of Hess *et al.* (1978) with bovine serum albumin as standard. During protein purification, protein concentration was monitored as A_{280} after the first anion-exchange chromatography step.

Purification procedures. All steps were carried out at 0–4 °C. Culture filtrate (2.21 litres) was concentrated fivefold in an Amicon 8400 cell using PM10 membranes (M_r cut-off 10000). The concentrated filtrate was made 90% saturated in ammonium sulphate. The precipitate was recovered by centrifugation and resuspended in 300 ml 60% saturated ammonium sulphate in 0.1 M-potassium phosphate, pH 7.0. After stirring for 1 h the undissolved precipitate was recovered by centrifugation (retaining 98% of the laccase activity of the first precipitate) and dissolved in 440 ml 0.01 M-potassium phosphate, pH 7.0 (hereafter, buffer A). This solution was dialysed for 18 h against buffer A prior to loading onto a DEAE-Sephadex A-50 column (Pharmacia, 2.6 $\times$ 40 cm), previously equilibrated with buffer A. The column was washed with 5 vols buffer A and eluted with a 600 ml gradient of 0.2–0.6 M-NaCl in buffer A. Fractions containing laccase activity were pooled and concentrated to 3–3 ml by ultrafiltration over a PM10 membrane. The concentrated sample was applied to a Sephacryl S-300 Superfine column (Pharmacia, 2.6 $\times$ 60 cm) at a flow rate of 20 ml h^{-1} and eluted with buffer A at 40 ml h^{-1} . Active fractions from gel filtration were pooled and loaded onto a second DEAE-Sephadex A-50 column which was equilibrated, washed and eluted as above. Laccase I activity was eluted in two or three 6 ml fractions which were pooled, dialysed against buffer A and concentrated 5 to 10-fold by ultrafiltration prior to storage at –70 °C as 150 μ l aliquots. Laccase II activity was retained as the most active fractions from a less well-defined peak and treated similarly.

Anti-laccase I antibody. Laccase I purified as described above was subjected to SDS-PAGE. The main polypeptide band was eluted from the gel in the presence of 0.1% SDS. This preparation (Fig. 5) was used as an immunogen. A rabbit was initially injected subcutaneously with 200 μ g laccase protein in complete Freund's adjuvant. Subsequently 100 μ g laccase protein in incomplete Freund's adjuvant was injected at 2–3 week intervals for 12 weeks. Antibody titre was monitored by ELISA assays (data not shown). IgG was purified from antiserum as described by Krugger & Hammond (1988).

Immunoblot analysis. The IgG fraction described above was used at 1 in 100 dilution. Anti-*Agaricus bisporus* laccase IgG fraction (prepared by C. R. Perry and C. F. Thurston) was used at 1 in 800 dilution. The method was as described previously (Pacy & Thurston, 1987). Pure

laccase from *Agaricus bisporus* was a gift from Dr D. A. Wood, Horticulture Research International, Littlehampton, UK.

Results

Accumulation of laccase activity in the medium of A. mellea cultures

Appearance of laccase activity in the medium of *A. mellea* cultures correlates with synthesis of rhizomorphs (Worrell *et al.*, 1986). In a series of preliminary experiments, it was established that higher levels of laccase activity were obtained in malt extract medium than in the defined medium employed by Worrell *et al.* (1986) and that shallow layers of liquid medium that allowed the relatively rapid development of aerial mycelium gave higher levels of laccase activity (and rhizomorph dry weight). Although the dry weight of rhizomorph tissue was marginally greater in medium adjusted initially to pH 7.0, more laccase activity was found in cultures with medium adjusted to pH 5.0 (Fig. 1).

Production of extracellular laccase was affected by the aeration of the cultures. When the culture bottles were sealed (rubber bungs or vaccine stoppers) after inoculation, the amount of laccase activity that accumulated was greater than when the bottles were closed with foam plugs (Fig. 2). Sealed flasks also resulted in the maintenance of high laccase activity for longer periods; with foam bungs the activity declined rapidly after reaching a maximum about 2 weeks after inoculation. Non-denaturing PAGE showed that two laccase activities were present in the medium (see Fig. 4a, track 1) and the relative amount of the two bands (stained for enzyme activity) was similar at all stages of culture on the basis of visual inspection.

Purification of laccase enzyme protein

Table 1 shows data for a typical purification. Culture medium was concentrated about fivefold by ultrafiltration prior to ammonium sulphate precipitation. The dialysed ammonium sulphate precipitate gave a dark brown solution. The majority of the pigmented material adsorbed to the DEAE-Sephadex column and could not be displaced by increasing the ionic strength (up to 1.0 M). Laccase activity was eluted as a single peak which was again concentrated by ultrafiltration. It should be noted that only 57% of the activity applied to the DEAE-Sephadex column was recovered. This step of the purification was necessary, however, to remove pigmented polyphenolic material. Gel filtration gave a single peak of eluted laccase activity, removing about half the

Fig. 1. Effect of culture medium pH on laccase synthesis and rhizomorph development. Cultures (20 ml) in 100 ml flat bottles were set up with malt extract medium adjusted to pH 5 (a) or pH 7 (b). Laccase activity (●; upper panels) was assayed (at pH 4) as described in Methods. The lower panels show undifferentiated mycelium (■) and rhizomorph (▲) dry weight. Mycelium and rhizomorph tissue were dissected apart prior to drying. All values are means of four determinations. Vertical lines show the range where this exceeds the size of the data symbols.

Fig. 2. Effect of aeration on laccase synthesis. Cultures (100 ml) in 1 litre Roux bottles were incubated either sealed with vaccine stoppers (○), or closed only with porous foam plugs (●), allowing exchange of atmosphere between the inside and outside of the bottles during incubation. The data are means of triplicate assays on samples withdrawn via a syringe needle, thus not requiring the sealed bottles to be opened.

Fig. 3. Elution of laccase activity (●) and protein from the second DEAE-Sephadex column step of laccase purification. Fraction volume was 6 ml. See Table 1 and Methods for further details.

contaminating protein present at this stage. Finally a second salt-gradient elution from a DEAE-Sephadex column gave two active fractions (Fig. 3). The first, eluting at 0.3 M-NaCl, constituted nearly a third of the

Fig. 4. (a) Separation of laccase I from laccase II. Non-denaturing 10% PAGE was followed by staining for laccase activity with guaiacol as substrate. Tracks: 1, concentrated culture filtrate (13 μg protein); 2, ammonium sulphate precipitate (15 μg); 3, first DEAE-Sephadex column eluate (15 μg); 4, Sephacryl gel filtration eluate (8 μg); 5, first peak (laccase I) from the second DEAE-Sephadex column steps (2 μg); 6, second peak (laccase II) from the second DEAE-Sephadex column (2 μg). (b) SDS-PAGE (12% gel) analysis of purified laccase I. Track 1, M_r markers: phosphorylase *b* (97000), bovine serum albumin (66000), ovalbumin (43000), carbonic anhydrase (31000), soybean trypsin inhibitor (21000) and hen egg lysozyme (14000). Tracks 2, 3 and 5, purified laccase I (10, 20 and 80 μg protein, respectively). Track 4, electro-eluted protein from a non-denaturing PAGE gel loaded with purified laccase I, from the region of the gel showing laccase activity. Protein stained with Coomassie brilliant blue.

Table 1. *Purification of laccase from A. mellea culture filtrate*

Purification step	Volume (ml)	Total protein (mg)	Total enzyme activity ($\mu\text{mol min}^{-1}$)	Specific activity [$\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$]	Purification (-fold)	Yield (%)
Culture filtrate	2120	190	148	0.78	1	100
Concentrated filtrate	440	118	140	1.18	1.51	94
Ammonium sulphate (60% satd)	307	31	102	3.31	4.25	68
DEAE-Sephadex column I	3.3	8.25	57.2	6.93	8.89	38
Sephacryl S-300	90	4.50	56.7	12.6	16.1	38
DEAE-Sephadex column II						
First peak	3.6	1.08	43.6	40.4	51.7*	29*
Second peak	1.2	0.36	12.1	33.7	43.1*	8*

* The calculation of these values does not correct for the presence of two separable activities. For instance, if the culture filtrate contained equal amounts of first- and second-peak activities, the true purification and yield values would be double those shown.

Fig. 5. Gel-purified laccase I main polypeptide. Purified laccase I was separated by SDS-PAGE (10% gel) and the main band cut out and electro-eluted, after location by brief staining with Coomassie brilliant blue. Tracks 1 and 4, M_r markers as described in the legend to Fig. 4(b). Tracks 2 and 3, 10 μ g and 20 μ g, respectively, of gel-purified laccase I main polypeptide. Proteins were silver stained.

Fig. 6. Non-denaturing PAGE of purified laccase I. Tracks 1 and 2 show purified laccase I from two separate purification experiments, stained for laccase activity with guaiacol as substrate (both tracks loaded with approx. 20 μ g protein). Tracks 3 and 4 show samples from the same preparations stained for protein with Coomassie brilliant blue (both tracks loaded with approx. 80 μ g protein).

original activity and is referred to subsequently as laccase I. The second peak, eluting at about 0.38 M-NaCl, was always only a small fraction (<10%) of the initial activity and is referred to as laccase II. The two activities present in culture filtrate were therefore separated (Fig. 4).

Laccase I gave a single predominant band on SDS-PAGE with an apparent M_r of 59000 (Figs 4b and 5). When purified laccase I was analysed by non-denaturing PAGE, the protein resolved as up to four bands close together. All bands that stained for protein also stained for laccase activity (Fig. 6). It is probable that most if not all of the minor protein bands seen in SDS-PAGE analysis of laccase I (Fig. 4b) are fragments of the full-length polypeptide as they reacted with antibodies raised against the gel-purified full-length polypeptide.

Properties of laccase I from *A. mellea*

Purified laccase I showed optimum activity at pH 3.5 and only about 10% of this activity at pH 6.5 (Fig. 7).

Fig. 7. Effect of assay pH on laccase activity. Laccase activity was assayed in phosphate/citrate buffers adjusted to pH values between 2.5 and 6.5, testing concentrated *A. mellea* culture filtrate (O) and purified laccase I (●).

The broader pH optimum of laccase activity in unfractionated culture filtrate may indicate a higher pH optimum for laccase II. Laccase I was active against a range of typical laccase substrates (Table 2), with DMOP showing the greatest rate of oxidation. Tyrosine was not oxidized. The enzyme exhibited normal substrate

Fig. 8. Immunoblot of pure laccase from *Agaricus bisporus* (8 µg, track 1), pure laccase I from *Armillaria mellea* (2 µg, track 2) and partially purified laccase II from *A. mellea* (4 µg, track 3). After electrophoresis under non-denaturing conditions proteins were transferred to nitrocellulose paper and reacted with anti-*A. mellea* laccase I IgG (a) or anti-*Ag. bisporus* laccase IgG (b).

Table 2. Substrate specificity of purified laccase I from *A. mellea*

Enzyme activity was measured as the rate of oxygen consumption. Data are mean values from quadruplicate assays.

Substrate (all at 15 mM)	Relative activity
<i>p</i> -Phenylenediamine	1.0
Syringaldazine	0.82
Guaiacol	0.95
<i>N,N</i> -Dimethyl-1,4-phenylenediamine	1.19
Catechol	1.18
2,6-Dimethoxyphenol (DMOP)	1.59
Tyrosine	0

saturation kinetics when tested with a range of concentrations of *p*-phenylenediamine and DMOP. The K_m for *p*-phenylenediamine was 1.69 mM and for DMOP was 0.178 mM. Purified laccase I retained full activity during storage at -70°C for six months.

Comparison of laccase I and laccase II from *A. mellea*

The purification procedure described above yielded a fraction containing laccase II, but the amount of this

material was insufficient for complete characterization. Not all purification runs gave a laccase II fraction that was free of laccase I activity. Where complete separation was achieved the laccase II fraction contained insufficient protein for the extent of its purity to be clearly demonstrated. Two experiments were performed that clearly show laccase I and laccase II to be distinct.

First, the two enzymes had clearly different properties during IEF. When culture filtrate was analysed by IEF two bands were revealed by activity staining for laccase, at pH 3.3 and pH 4.1 (results not shown). Pure laccase I focused at pH 4.1 and the purified laccase II fraction showed a strong band at pH 3.3 (a weak band at pH 4.1 probably indicated residual contaminating laccase I in this sample).

Secondly, the response of the two enzymes to antibodies was different. Anti-laccase I antiserum detected laccase I in concentrated culture filtrate and gave a strong band with pure laccase I in immunoblot analysis, but laccase II was not detected (Fig. 8a). Immunoblot analysis was also performed using antibodies raised against purified laccase from *Agaricus bisporus* (Fig. 8b). Although anti-*Ag. bisporus* antibodies reacted weakly with *A. mellea* laccase I, no binding to laccase II was detected.

Discussion

The properties so far demonstrated for *A. mellea* laccase I (i.e. size, pH optimum, substrate specificity and pI) are not untypical of extracellular fungal laccases. A polypeptide of M_r about 60000 has been verified by DNA sequencing for laccases from *Neurospora crassa* (German *et al.*, 1988), *Aspergillus nidulans* (Aramayo & Timberlake, 1990), *Coriolus hirsutus* (Kojima *et al.*, 1990) and *Phlebia radiata* (Saloheimo *et al.*, 1991). In common with these and all other fungal laccases that have been studied, it is to be expected that the *A. mellea* laccases are copper-containing glycoproteins. Direct confirmation of a carbohydrate component of the *A. mellea* laccases has yet to be sought. However, anti-laccase I IgG has been used to immunoprecipitate protein from cell-free translation of *A. mellea* poly(A)-containing RNA in the rabbit reticulocyte lysate system, yielding a single band of M_r 52000 (data not shown). This is consistent with the mature M_r 59000 polypeptide containing approximately 15% carbohydrate as found, for instance, in *Ag. bisporus* laccase (Wood, 1980). Indeed, variability of glycosylation is a likely contributor to the microheterogeneity shown by laccase I on non-denaturing PAGE analysis (Fig. 6).

The evidence for production of two distinct laccases by *A. mellea* is clear. Laccase I and laccase II were separated

by ion-exchange chromatography, had different pI values, and antibodies to laccase I did not recognize antigenic determinants on laccase II. Further, antibodies raised against *Ag. bisporus* laccase recognized laccase I, but not laccase II. The properties of laccase II require further investigation. In this study the yield of laccase II was never sufficient to allow characterization. As crude culture filtrates contained essentially similar amounts of laccase I and laccase II activity (Fig. 4a) it is apparent that the majority of laccase II activity was lost during the purification procedure we have described. From Table 1 it can be seen that the largest single loss of activity occurred at the first DEAE-Sephadex column step. It is conceivable that the majority of laccase II protein was adsorbed to the pigmented polyphenolic material that stuck to this column. There is of course the alternative that most of the laccase II was irreversibly inactivated during this step of the purification.

The correlation between laccase synthesis and rhizomorph development in *A. mellea* is intriguing. It is clear that the initiation of both processes requires the development of aerial mycelium, is favoured by high oxygen tension and is not affected by CO₂ tension (Worrell, *et al.*, 1986). Under continuing high oxygen tension, however, laccase activity rapidly declines after reaching a maximum (Fig. 2) and the cultures develop extensive dark pigmentation, presumably as a result of laccase action (not shown; cf. Worrell *et al.*, 1986). If oxygen availability is limited (by sealing culture vessels after inoculation), not only is more laccase recovered (Fig. 2), but the cultures remain substantially unpigmented and rhizomorph synthesis continues for longer (results not shown). The part that laccase might play in rhizomorph development remains unclear. As can be seen from Fig. 1, cultures at pH 7 make marginally more rhizomorphs than cultures at pH 5, but develop only about two-thirds the amount of laccase activity. If the enzymes' response to pH (Fig. 7) is taken into account, the amount of laccase action in pH 7 cultures is likely to be significantly less than in pH 5 cultures. Thus, if laccase is required for rhizomorph development as others have proposed (Worrell *et al.*, 1986), the amount produced in cultures at pH 5 must be in great excess of this requirement. Furthermore the functions of two quite distinct laccases are at present inexplicable.

We thank Dr J. Rishbeth for generously providing *Armillaria* strains and for information about their origin. Advice from Professor D. A. Wood on numerous points concerning fungal laccases and the gift of

pure laccase from *Agaricus bisporus* are also most gratefully acknowledged.

References

- ANDER, P. & ERIKSSON, K.-E. (1976). The importance of phenol oxidase activity in lignin degradation by the white rot fungus *Sporotrichum pulverulentum*. *Archives of Microbiology* **109**, 1-8.
- ARAMAYO, R. & TIMBERLAKE, W. E. (1990). Sequence and molecular structure of the *Aspergillus nidulans* yA (laccase 1) gene. *Nucleic Acids Research* **18**, 3415.
- DE VRIES, O. M. H., KOOISTRA, W. H. C. F. & WESSELS, G. H. (1986). Formation of an extracellular laccase by *Schizophyllum commune* dikaryon. *Journal of General Microbiology* **132**, 2817-2826.
- GERMAN, U. A., MULLER, G., HUNZIKER, P. E. & LERCH, K. (1988). Characterisation of two allelic forms of *Neurospora crassa* laccase. Amino- and carboxyl-terminal processing of a precursor. *Journal of Biological Chemistry* **263**, 885-896.
- HARKIN, J. M., LARSEN, M. J. & OBST, J. R. (1974). Use of syringaldazine for detection of laccase in sporophores of wood rotting fungi. *Mycologia* **66**, 469-476.
- HESS, H. H., LEES, M. B. & DERR, J. E. (1978). A linear Lowry-Folin assay for both water soluble and SDS-solubilized proteins. *Analytical Biochemistry* **85**, 295-300.
- KANTELINEN, A., HATAKKA, A. & VIKARI, L. (1989). Production of lignin peroxidase and laccase by *Phlebia radiata*. *Applied Microbiology and Biotechnology* **31**, 234-239.
- KOJIMA, Y., TSUKUDA, Y., KAWAI, Y., TSUKAMOTO, A., SUGIURA J., SAKAIMO, M. & KITA, Y. (1990). Cloning, sequence analysis, and expression of lignolytic phenoloxidase genes of the white-rot basidiomycete *Coriolus hirsutus*. *Journal of Biological Chemistry* **265**, 15224-15230.
- KRUGGER, N. J. & HAMMOND, J. B. W. (1988). Purification of immunoglobulins using protein A-sepharose. In *Methods in Molecular Biology*, vol. 3, *New Protein Techniques*, pp. 363-371. Edited by J. M. Walker. Clifton, NJ: Humana Press.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680-685.
- LEATHAM, G. F. & STAHMANN, M. A. (1981). Studies on the laccase of *Lentinus edodes*: specificity, localization and association with the development of fruiting bodies. *Journal of General Microbiology* **125**, 147-157.
- PACY, J. & THURSTON, C. F. (1987). Distribution of isocitrate lyase in induced and de-adapting cells of the green alga *Chlorella fusca*. *Journal of General Microbiology* **133**, 3341-3346.
- SALOHEIMO, M., NIKU-PAAVOLA, M.-L. & KNOWLES, J. K. C. (1991). Isolation and structural analysis of the laccase gene from the lignin-degrading fungus *Phlebia radiata*. *Journal of General Microbiology* **137**, 1537-1544.
- WARGO, P. M. (1990). Spread of *Armillaria* by rhizomorphs in forest stands. In *Fourth International Mycological Congress Abstracts*, p. 92. Edited by A. Reisenger & A. Bresinsky. Regensburg: Botanical Institute.
- WOOD, D. A. (1980). Production, purification and properties of extracellular laccase of *Agaricus bisporus*. *Journal of General Microbiology* **117**, 327-338.
- WOOD, D. A. (1985). Production and roles of extracellular enzymes during morphogenesis of basidiomycete fungi. In *Development of Higher Fungi*, pp. 375-387. Edited by D. Moore, L. A. Casselton & J. C. Frankland. Cambridge: Cambridge University Press.
- WORRELL, J. J., CHET, I. & HÜTTERMANN, A. (1986). Association of rhizomorph formation with laccase activity in *Armillaria* spp. *Journal of General Microbiology* **132**, 2527-2533.