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Heterologous expression of laccase cDNA from *Ceriporiopsis subvermispora* yields copper-activated apoprotein and complex isoform patterns

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Analysis of genomic clones encoding a putative laccase in homokaryon strains of *Ceriporiopsis subvermispora* led to the identification of an allelic variant of the previously described *lcs-1* gene. A cDNA clone corresponding to this gene was expressed in *Aspergillus nidulans* and in *Aspergillus niger*. Enzyme assays and Western blots showed that both hosts secreted active laccase. Relative to the isozymic forms of the native *C. subvermispora* enzyme, the *A. niger*-produced laccase had a higher molecular mass and gave a single band on IEF gels. In contrast, *A. nidulans* transformants secreted several isoforms remarkably similar to those of the native system. Considered together with previously reported Southern blots and protein sequencing, expression in *A. nidulans* supports the view that *C. subvermispora* has a single laccase gene and that multiple isoforms result from post-translational processes. In addition, several lines of evidence strongly suggest that under copper limitation, *A. nidulans* secretes apoprotein which can be reconstituted by a short incubation with Cu(I) and to a lesser extent with Cu(II).

INTRODUCTION

Ceriporiopsis subvermispora is one of the most widely used filamentous fungi in laboratory studies of lignin biodegradation. It secretes manganese peroxidase (MnP), a haem protein that catalyses the H_2O_2-dependent oxidation of Mn(II) to Mn(III) (Glenn & Gold, 1985; Paszczynski *et al.*, 1985), and laccase (EC 1.10.3.2), a member of the multi-copper oxidase family of proteins, which includes ascorbate oxidase and ceruloplasmin (Mayer, 1987; Reinhammer, 1984). Laccases catalyse one-electron oxidation of a variety of phenolic compounds, with the concomitant four-electron reduction of O_2 to H_2O. They are involved in lignin biogenesis in higher plants, and lignin depolymerization by fungi (Eggert *et al.*, 1997; Kirk & Farrell, 1987). Laccases also participate in a broad range of cellular processes such as sporulation (Leatham & Stahman, 1981), fruit body formation and plant pathogenesis (Choi *et al.*, 1992; Geiger *et al.*, 1986; Marbach *et al.*, 1985).

The active site of laccases possesses four copper ions, which can be classified according to their spectrophotometric properties. Type I copper (blue copper) exhibits an intense absorption at about 600 nm, owing to the charge transfer between Cu(II) and a cysteine residue. Type II copper shows a very weak absorption and functions as a one-electron acceptor. Type III copper contains two copper centres absorbing at 330 nm and functions as a two-electron acceptor (Jonsson *et al.*, 1995; Reinhammer, 1984).

Several laccase isoforms can be identified in *C. subvermispora* cultures depending on the composition of the medium (Salas *et al.*, 1995). Low-stringency Southern blots (Karahanian *et al.*, 1998) and N-terminal sequences of laccases isoenzymes (Salas *et al.*, 1995) suggest a single laccase gene, and its expression is strongly regulated by copper at the transcriptional level (Karahanian *et al.*, 1998). The phenomenon of isoenzyme multiplicity is commonly observed among ligninolytic fungi, although its physiological significance is not known.

To further biochemical analysis of the *C. subvermispora* laccase we describe here the cloning and expression of Lcs-1 cDNA in *Aspergillus nidulans* and *Aspergillus niger*. The *A. nidulans* system produced multiple isoforms in a pattern that was similar to the native *C. subvermispora* enzyme, and copper was found to play a post-transcriptional role in laccase expression.

Abbreviations: ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate); MnP, manganese peroxidase.

The GenBank accession numbers for the sequences reported in this paper are AY219235 and AY219236.

METHODS

Strains. *Ceriporiopsis subvermispora* strain FP-105752 was obtained from the Center for Mycology Research, Forest Products Laboratory, Madison, WI, USA. *Aspergillus nidulans* A722 (*pyrG89*, *pabaA1*; *fwA1*; *uaY9*) and *Aspergillus niger* A969 (*cspA1*; *fwnA1*; *pyrG5*; *metB10*) were obtained from the Fungal Genetic Stock Center (FGSC, Kansas City, KS, USA).

Cloning and sequencing. RT-PCR was used to isolate a full-length cDNA as described by Karahanian *et al.* (1998). Design of primers was based on the genomic sequence of *lcs-1* (GenBank accession number AF053472). The upstream primer was located at position −30 from the ATG, whereas the downstream primer was at position +7 with respect to the stop codon. The PCR product, designated Lcs-1, was directly cloned in pBluescript KSII+ as described by Lobos *et al.* (1998). To obtain genomic clones, *lcs-1* was amplified from *C. subvermispora* homokaryons 3 and 8 (Tello *et al.*, 2001). Nucleotide sequence was determined with the ABI-Prism Dye terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems) with an ABI373 DNA sequencer. Sequence editing and analysis were done with DNAstar software.

***Aspergillus* expression.** The expression vector pexLcs-1 was constructed by placing the Lcs-1 cDNA coding region, including the sequence for its signal sequence, under the control of the TAKA amylase promoter (Andersen *et al.*, 1992) and the *Aspergillus awamori* glucoamylase terminator (Kersten *et al.*, 1995) by the PCR overlap extension technique (Horton *et al.*, 1989) using proofreading polymerase *Pfu* (Stratagene). The coding region and junctions of the expression cassette were sequenced. The selection marker, ppyrG, was obtained from the Fungal Genetic Stock Center.

A. nidulans A722 and *A. niger* A969 cotransformations were as described by Larrondo *et al.* (2001), except that *A. niger* protoplasts were prepared from germinated conidia after an overnight incubation at 300 r.p.m. at 30 °C. Protoplasts were cotransformed with 5 μg ppyrG and 5 μg pexLcs-1. Selection was based on complementation of uridine auxotrophy by the selectable marker *pyrG*. Transformants carrying the genetic construction pexLcs-1 were confirmed by PCR.

One hundred millilitres of *Aspergillus* minimal medium (AMM) (Cullen *et al.*, 1987) (5 % maltose) or YEM (0·5 % yeast extract, 5 % maltose) was inoculated with 1×10^7 spores and incubated for 3 days at 30 °C in an orbital shaker (250 r.p.m.). In the case of *A. niger* A969, the medium was also supplemented with 1 mM methionine. Mycelium was harvested by filtration through Miracloth, snap frozen in liquid nitrogen and stored at −70 °C. For routine cultivations, the medium was supplemented with $CuSO_4$ up to a final concentration of 100 μM. In copper-deficient cultures, only traces of copper were present (1–3 μM).

We screened rLcs-1 production by plate assay. Isolated transformants were inoculated and selected in agar-minimal medium plates containing maltose (5 %) and 1 mM 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS; Sigma). Characteristic ABTS oxidation could be observed after 2–3 days. Transformants were also evaluated for rLcs-1 production in liquid media by spectrophotometric measurements of ABTS oxidation

Enzyme purification and characterization. One litre of extracellular fluid from *A. nidulans* Lcs-1 transformant (AnidL-6) culture was directly fractionated by chromatography on Q-Sepharose. One litre of supernatant from *A. niger* Lcs-1 transformant (AnigL-2) was concentrated 10-fold by filtration in a 185 ml Amicon cell possessing a 10 kDa cut-off membrane, and then dialysed twice against 500 ml 25 mM sodium acetate pH 4·5 and fractionated by chromatography on Q-Sepharose (Lobos *et al.*, 1998). Enzyme activity was measured at 30 °C in a Shimadzu 160 UV-visible recording spectrophotometer. Reaction mixtures (1 ml) contained 50 mM glycine, pH 3·0, and 4·33 mM ABTS as substrate. K_m values for ABTS were determined with 0·01 U laccase, using Eadie–Hofstee plots. For endoglycosidase treatment, 5 μg enzyme was treated as described by Larrondo *et al.* (2001).

Zymograms were as previously reported (Larrondo *et al.*, 2001). Briefly, samples were applied with nonreducing denaturing loading buffer without boiling and subjected to SDS-PAGE at 4 °C. Gels were then fixed in a solution containing 10 % acetic acid and 40 % methanol for 10 min followed by incubation in 50 mM glycine buffer, pH 3·0, and 5 mM ABTS for 10 min, or until activity could be detected.

Reconstitution assays. Q-Sepharose-purified laccase (0·5 μg) from low-copper-containing media was incubated at 25 °C with various concentrations of copper as indicated in the corresponding figure legend. Thereafter, samples were withdrawn at the indicated times to measure laccase activity.

Other methods. Analytical IEF and protein concentration measurements were as described by Larrondo *et al.* (2001); RNA extraction and Northern blot hybridization were as described by Karahanian *et al.* (1998). The DNA probe corresponding to the entire coding region of Lcs-1 was labelled with [α-^{32}P]dCTP by nick translation (Gibco-BRL).

RESULTS

Characterization of a new *lcs-1* allele

In further attempts to isolate and characterize laccase genes from *C. subvermispora*, we have cloned laccase-like amplicons from homokaryotic strains. Each homokaryon harboured a highly conserved, but distinct sequence. Presumed to be allelic variants, the sequences are >99 % identical with all but one mismatch occurring in non-coding regions. The single coding region mismatch occurred in the third position of an alanine codon, (GCT to GCC), located in exon X, and did not affect translation. Designated *lcs-1A* and *lcs-1B* (accession number AY219235), the former corresponds to the previously deposited *lcs-1* sequence AF053472 (Karahanian *et al.*, 1998). (Minor sequencing errors in *lcs-1A* have been recently corrected.)

Cloning of *lcs-1* cDNA

A unique and abundant product was obtained when RT-PCR was conducted with RNA extracted from dikaryotic strain FP105752 of *C. subvermispora* grown in liquid culture containing 100 μM $CuSO_4$. The cDNA was cloned and sequenced, and based on the nucleotide difference mentioned above, it corresponded to *lcs-1B* (accession number AY219236).

Production of rLcs-1 in *Aspergillus* spp.

Transformants confirmed by PCR were screened for extracellular laccase activity when grown in minimal medium. Similar levels of enzyme activity were obtained for both *Aspergillus* hosts. No major variations in enzyme activity were detected when AMM or YEM media (both supplemented to 100 μM $CuSO_4$ final) were used. Laccase activity

appeared on day 2, reaching a peak of 0·23 U ml^{-1} on day 4 and then detected without major variation up to day 5 or 6. For purification purposes, cultures were routinely collected on day 3. The levels of rLcs-1 produced were about 1·5 mg per litre of culture.

Enzyme purification. Q-Sepharose purification of laccase from *A. nidulans*-Lcs-1 cultures yielded one major protein (Anid-rLcs-1) with the same molecular mass and immunoreactivity as the native *C. subvermispora* enzyme (Fig. 1A). Zymograms showed the presence of only one major band, with a higher electrophoretic mobility than the enzyme under reducing conditions (Fig. 1B). The enzyme purified from *A. niger*-Lcs-1 cultures (Anig-rLcs-1) also reacted with antibodies against *C. subvermispora* laccase although its molecular mass was higher than expected. It was also detected as a single band in zymograms, with a lower electrophoretic mobility than Anid-rLcs-1 (Figs 1A and 1B, respectively).

Isoelectric focusing. IEF of Anid-rLcs-1 gave a pattern resembling the native *Ceriporiopsis* laccases. In contrast, Anig-rLcs-1 laccase yielded a single IEF band, which was similar in pI to the most basic isoform observed for *C. subvermipora* laccases (Fig. 2).

Glycosylation. Treatment of Anid-rLcs-1 with endoglycosidase H revealed *N*-glycosylation of about 25 % of the original molecular mass, a composition similar to the native *C. subvermispora* laccase. A comparable degree of glycosylation was observed with Anig-rLcs-1, but its molecular mass after the treatment remained higher than that of native *C. subvermispora* laccase (Fig. 3) (Salas *et al.*, 1995).

K_m for ABTS. The K_m values determined for the oxidation of ABTS were 0·042 mM for Anid-rLcs-1 and 0·038 mM for Anig-rLcs-1, which is in accordance with the value of 0·03 mM reported for this enzyme (Fukushima & Kirk, 1995).

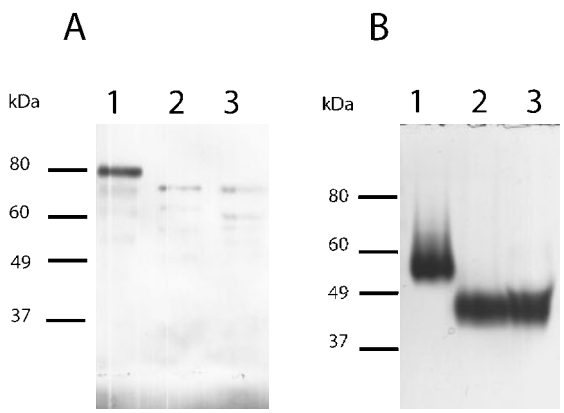

Fig. 1. PAGE of native and recombinant laccases. (A) SDS-PAGE and Western blot of Anig-rLcs-1 (lane 1), Anid-rLcs-1 (lane 2) and native *C. subvermispora* laccase pool (lane 3). (B) Zymogram of 0·02 U Anig-rLcs-1 (lane 1), Anid-rLcs-1 (lane 2) and native *C. subvermispora* laccase pool (lane 3). Activity was developed with ABTS.

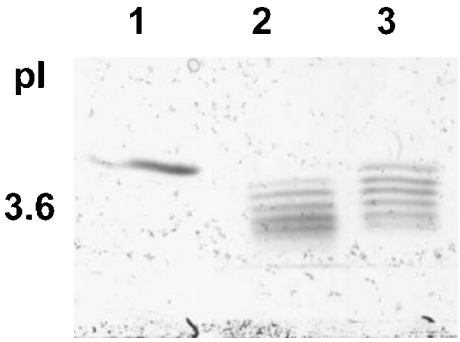

Fig. 2. IEF of recombinant and native laccase: 0·04 U of Anig-Lcs-1 (lane 1), Anid-Lcs-1 (lane 2) and *C. subvermispora* laccase (lane 3) was loaded and after running the gel, activity was developed with 4-chloronaphthol.

Effect of copper on rLcs-1 production

Copper dramatically affected laccase titres in YEM cultures of *A. nidulans* Lcs-1. YEM cultures supplemented with 100 μM $CuSO_4$ yielded laccase levels up to 200 U l^{-1}, as compared to less than 20 U l^{-1} obtained in unsupplemented growth medium. When normalized by biomass, this represents a sixfold stimulation by copper (Fig. 4A). A similar result was observed when *A. niger* transformants were analysed (data not shown). This effect was not exerted at the transcriptional or the translational levels, as determined by Northern (Fig. 4C) and Western (Fig. 4B) blots, respectively. This suggests that apoprotein is efficiently produced and secreted at low copper levels. The effect of

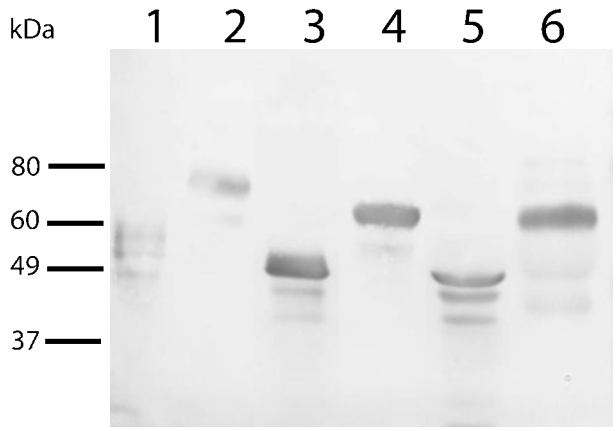

Fig. 3. Endoglycosidase H treatment of laccases. Western blot of endoglycosidase H treated (End+) and untreated (End−) laccases. Anig-rLcs-1 End+ (lane 1) and End− (lane 2); Anid-rLcs-1 End+ (lane 3) and End− (lane 4); *C. subvermispora* Lcs1 End+ (lane 5) and End− (lane 6).

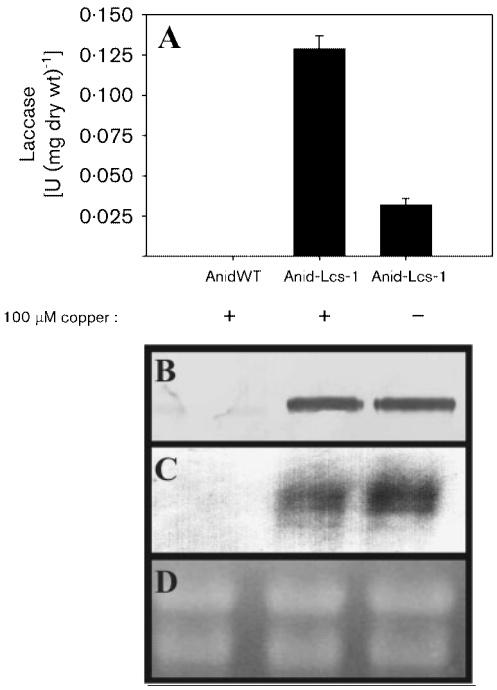

Fig. 4. Effect of copper supplementation (100 μM) on rLcs-1 expression in *A. nidulans*. (A) Specific laccase activity from 3-day-old YEM cultures. (B) Western blot analysis of samples from each culture, containing 2·5 μg protein. (C) Northern blot analysis of *lcs-1*. (D) Loading control: ethidium-bromide-stained formaldehyde gel shows approximately equal amount of rRNA.

copper was also assessed in AMM, with and without added copper. In the absence of externally added copper (copper levels of 1–3 μM), three and five times less activity was observed in *A. nidulans* and *A. niger* transformants, respectively.

Partial reconstitution of laccase activity. Copper could be incorporated into the apoprotein, giving rise to active enzyme. When supernatant or Q-Sepharose supernatant pools of Anid-Lcs1 cultures grown at low copper levels were incubated with different copper concentrations, laccase activity could be recovered in a concentration- and time-dependent manner. Concentrations up to 5 mM $CuSO_4$ were tested. At this copper level, a fourfold increase in activity was achieved (Fig. 5). A larger effect was observed when copper was present as Cu(I) due to incubation of Cu(II) with ascorbate. Maximal reconstitution levels were achieved after 20 min incubation of apoprotein in a solution containing 5 mM $CuSO_4$ and 1 mM ascorbate. Under these conditions, a ninefold increase in activity was reached, which constitutes about 75 % of the maximal activity present in the Q-Sepharose pool obtained from *A. nidulans*-Lcs-1 grown in YEM medium in the presence of 100 μM $CuSO_4$ (data not shown). Incubation of the latter enzyme preparation with copper and ascorbate resulted in only a slight increase of

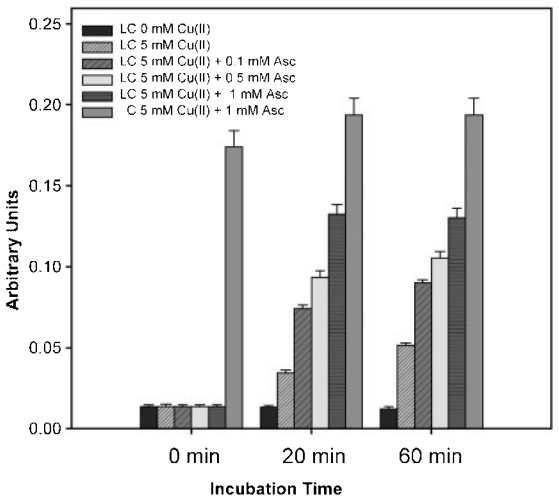

Fig. 5. Reconstitution of laccase activity with copper. Q-Sepharose-purified laccase (0·5 μg) from low-copper medium (LC) and copper-supplemented medium (C) was incubated with various amounts of copper, with or without ascorbate (Asc), and samples were taken at the indicated times to measure laccase activity.

activity (15 %), which is consistent with the loss of copper during the purification procedure.

DISCUSSION

Laccase isoenzyme multiplicity is a phenomenon observed in several fungi, and in the case of *Pleurotus sajor-caju* (Soden & Dobson, 2001), the basidiomycete CECT 20197 (Mansur *et al.*, 1997), *Pleurotus ostreatus* (Palmieri *et al.*, 2000), *Trametes villosa* (Yaver & Golightly, 1996), *Agaricus bisporus* (Perry *et al.*, 1993) and *Rhizoctonia solani* (Wahleithmer *et al.*, 1995), families of structurally related genes have been described. In the case of *C. subvermispora*, over four isoforms are observed, with different substrate specificity, but only one gene, *lcs-1*, has been identified (Karahanian *et al.*, 1998; Salas *et al.*, 1995). The search for a new laccase gene in *C. subvermispora* led to the identification of the allelic variant *lcs-1B*. This allele cannot contribute to the isoform multiplicity observed in this fungus, because the two alleles are predicted to encode identical proteins. Taken together with previously reported low-stringency Southern blots and N-terminal protein sequencing, the results support the existence of a single laccase gene in *C. subvermispora*.

We were able to successfully express Lcs-1 cDNA in *A. nidulans* and *A. niger*. To our knowledge, this is the first report of expression of a white-rot fungus laccase in *A. nidulans* and the second instance of using *A. niger* (Record *et al.*, 2002) as a host. Enzyme titres were substantially lower than those reported for other laccases in

Aspergillus oryzae systems (Wahleithmer *et al.*, 1995; Yaver *et al.*, 1996, 1999; Record *et al.*, 2002; Berka *et al.*, 1997).

The molecular mass obtained for Anid-rLcs-1 was similar to that of the native enzyme, whereas Anig-rLcs-1 had a higher molecular mass. After treatment with endoglycosidase H, the molecular mass of Anig-rLcs-1 remained higher than that of the native enzyme, which suggests that modifications other than *N*-glycosylation may alter molecular mass. This was also associated with an altered pI, although the K_m for ABTS remained unchanged.

The IEF pattern of Anid-rLcs-1 was strikingly similar to that obtained with laccase activity from *C. subvermispora*. The phenomenon of isoform multiplicity was previously observed with recombinant Cs-MnP1, as well as recombinant *Phanerochaete chrysosporium* MnP1, both expressed in *A. nidulans* (Larrondo *et al.*, 2001). We have also observed multiple isoforms for *P. chrysosporium* multicopper oxidase when expressed in *Aspergillus* (L. F. Larrondo and others, unpublished). Thus, our experimental data clearly show that multiple isoforms can arise from a single cDNA, which is consistent with the presence of a single laccase gene in *C. subvermispora*. The precise nature of isoform modifications *in A. nidulans* and *C. subvermispora* remains unknown. Possibilities of post-translational modification include glycosylation and phosphorylation. Notably, IEF of *A. niger*-produced laccase revealed a single isoform, indicating that post-translational modifications differ among species of *Aspergillus*. Further work is required to understand the biochemical basis of this divergence. Prior to this work, there had been a single report of laccase expression in *A. niger*. In that study, the recombinant protein had the same characteristics as the native enzyme (Record *et al.*, 2002).

It has been reported that the expression of the *Trichoderma reesei* β-mannanase gene in yeast gives rise to two proteins with different pIs, which match the pIs described for the same activity in *T. reesei* (Stalbrand *et al.*, 1995). A detailed study of *T. reesei* cellobiohydrolase I (CBHI) secretion revealed several pI forms during processing in the secretion pathway (Pakula *et al.*, 2000). Both *N*- and *O*-glycans might be responsible for the final IEF pattern, but at least in the case of CBHI, it seems plausible that isoform multiplicity is due to phosphorylation of *O*-glycans. In addition, sulfatation of the glycopeptide region, as described in CBHI produced by the *T. reesei* strain ALKO2788 (Harrison *et al.*, 1998), can not be excluded.

Previous investigations have stressed the importance of copper in the regulation of laccase at the transcriptional level (Karahanian *et al.*, 1998; Soden & Dobson, 2001; Palmieri *et al.*, 2000; Galhaup *et al.*, 2002). We previously demonstrated that under low-copper conditions, *lcs-1* is not efficiently transcribed in *C. subvermispora* (Karahanian *et al.*, 1998). Here, we assessed the influence of copper on the laccase titres in a foreign host. Copper limitation decreased production of fully active laccase, but transcript levels remained constant. At low concentrations of this metal, laccase activity was barely detectable. Interestingly, when AMM medium was used, the effect of copper limitation was not as striking as in YEM medium. Biomass and the total extracellular protein produced in YEM cultures were not drastically affected by supplementation with 100 μM $CuSO_4$. We corroborated this observation by partially recovering activity from supernatants by incubation with copper concentrations in the millimolar range. This implies that the apoprotein lacks some or all of the structural copper atoms necessary for its activity.

There has been some interest in the study of laccase copper centres, by depletion and reconstitution after addition of this metal (Hanna *et al.*, 1988). Usually, this has been achieved by extensive dialysis against cyanide ion. In one case, apoprotein was produced under low copper levels (Bligny *et al.*, 1986), but the activity could not be reconstituted by incubation with copper. On the other hand, reconstitution of laccase from the Chinese lacquer tree requires the addition of Cu(I) and not Cu(II) (Hauenstein & McMillin, 1978). Omura (1961) also reported that Cu(I), but not Cu(II), could be used to reconstitute the apoprotein, results that were confirmed by Ando (1970). In the case of *C. subvermispora* laccase produced by *A. nidulans*, spectroscopic studies may help to clarify whether all three types of copper or only a single one are affected in cultures with limiting concentrations of this metal.

The expression systems reported here should be useful for an array of biochemical investigations, including studies of post-transcriptional modification. The results also highlight the importance of adequate copper levels in the production of fully active laccase.

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