Molecular Characterization of Laccase Genes from the Basidiomycete *Coprinus cinereus* and Heterologous Expression of the Laccase Lcc1

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A laccase from *Coprinus cinereus* is active at alkaline pH, an essential property for some potential applications. We cloned and sequenced three laccase genes (lcc1, lcc2, and lcc3) from the ink cap basidiomycete *C. cinereus*. The lcc1 gene contained 7 introns, while both lcc2 and lcc3 contained 13 introns. The predicted mature proteins (Lcc1 to Lcc3) are 58 to 80% identical at the amino acid level. The predicted Lcc1 contains a 23-amino-acid C-terminal extension rich in arginine and lysine, suggesting that C-terminal processing may occur during its biosynthesis. We expressed the Lcc1 protein in *Aspergillus oryzae* and purified it. The Lcc1 protein as expressed in *A. oryzae* has an apparent molecular mass of 66 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and absorption maxima at 278 and 614 nm. Based on the N-terminal protein sequence of the laccase, a 4-residue propeptide was processed during the maturation of the enzyme. The dioxygen specificity of the laccase showed an apparent *Km* of 21 ± 2 μM and a catalytic constant of 200 ± 10 min−1 for O2 with 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) as the reducing substrate at pH 5.5. Lcc1 from *A. oryzae* may be useful in industrial applications. This is the first report of a basidiomycete laccase whose biosynthesis involves both N-terminal and C-terminal processing.

Laccases are multicopper enzymes (EC 1.10.3.2) that catalyze the oxidation of a variety of phenolic compounds and are widely distributed among plants (37) and fungi (6). In plants, laccase is involved in lignification (37, 39). In fungi, laccases may be involved in many cellular processes, including delignification (17, 30), sporulation (33), pigment production (2, 12, 14), and dye precursors (14), enzymatic conversion of chemical intermediates (1), and production of chemicals from lignin. Most fungi that produce laccases do so at levels that are far too low to be an economical source. Laccase genes have been cloned from *Neurospora crassa* (20), *Cryphonectria parasitica* (10), *Aspergillus nidulans* (2), *Agaricus bisporus* (41), *Coriolus versicolor* (31), *Phlebia radiata* (44), *Coriolus versicolor* (25), *Trametes versicolor* (24, 27, 28, 40), *Trametes villosa* (54, 55), *Rhizoctonia solani* (52), *Myceliophthora thermophila* (5), the linoxylytic basidiomycetes *PM1* (13) and CECT 20197 (34), *Podospora anserina* (18), and *Pycnoporus cinnabarinus* (16).

The *C. hirsutus* laccase has been expressed in *Saccharomyces cerevisiae* (31), and the *P. radiata* laccase has been expressed in *Trichoderma reesei* (43). The yields obtained for these laccases were too low to be commercially feasible. Recently, the expression of the *T. versicolor* Lcc1 laccase in *Pichia pastoris* was reported (27). The *M. thermophila* laccase, three of the laccases from *R. solani*, and one of the laccases from *T. villosa* have been expressed in *Aspergillus oryzae* (5, 52, 55). The work on expression in *A. oryzae* resulted in commercialization of laccase for use by the textile industry in denim processing (29).

*Coprinus cinereus* is an ink cap basidiomycete that secretes a laccase (4) which has been purified from culture broth and characterized enzymologically (47), and its three-dimensional crystal structure has been determined (15). Our objectives in this study were (i) to clone the gene for the purified *C. cinereus* laccase, (ii) to express the laccase in *A. oryzae* to produce enough material for further characterization and applications testing, and (iii) to determine if there was more than one laccase gene that was expressed under the growth conditions under which the laccase had been previously purified. The work to clone and express this laccase was undertaken due to its neutral-to-alkaline pH optimum, which is required for some of the potential industrial applications of laccases (8, 14, 42).

Because it has been demonstrated that, unlike other basidiomycetes, *C. cinereus* contains only a single peroxidase gene (4), we were also interested in whether *C. cinereus* had a laccase gene family, as do many other basidiomycetes. We found that *C. cinereus* contains a family of at least three laccase genes and that the previously biochemically characterized *C. cinereus* laccase can be expressed in *A. oryzae*.

### MATERIALS AND METHODS

#### Strains

Plasmid and library construction was done with Escherichia coli Y1090(ZL) (Gibco BRL, Gaithersburg, Md.), *E. coli DH10B(ZL)* (Gibco BRL), and *E. coli DH5α* (Stratagene, La Jolla, Calif.). The fungal strains were *Coprinus cinereus* var. *microsorus* IFO 8371 (Institute for Fermentation, Osaka, Japan) and *A. oryzae* HowBo14 (5), a pyr6 amy4 amyB amyC mutant of IFO4177.

#### RNA isolation

*C. cinereus* A3387 was cultivated in FG4 medium (1.5% maltodextrin, 5% soy flour, 0.5% Bacto Peptone, 0.2% PLURONIC L61 [BASF, Mount Olive, N.J.]) at 20°C; the mycelium was harvested after 6 days of growth, frozen in liquid N2, and stored at −80°C. Total RNA was prepared from frozen, powdered mycelia of *C. cinereus* A3387 by extraction with guanidinium thiocyanate followed by ultracentrifugation through a 3.7 M CsCl cushion (9). Poly(A)+ RNA was isolated by oligo(dT)-cellulose affinity chromatography (3).

#### Construction of the cDNA library

Double-stranded cDNA was synthesized from 5 μg of *C. cinereus* poly(A)+ RNA as described previously (21, 45), except...
that an oligo(dt)-Nool anchor primer instead of an oligo(dt)_{12-18} primer was used in the first-strand reaction. After synthesis the cDNA was treated with mung bean nuclease, blunt ended with T4 DNA polymerase, and ligated to nonpalindromic HindIII linker and cloned into the HindIII site of the cloning vector. The adaptors cDNA was digested with Nool, size fractionated for 1.2- to 3.0-kb cDNAs by agarose gel electrophoresis, and ligated into BstXI/Nool-ligated pYES.2 vector (Invitrogen), and the ligation mixture was transformed into DH10B cells ( Gibco BRL). The library consisted of 10^9 independent clones.

Generation of a cDNA probe for C. cinereus laccase by PCR. One µg of plasmid DNA from a C. cinereus cDNA library pool was used as a template in a PCR reaction. The primers were contained in high-fidelity Taq polymerase (Perkin-Elmer, Foster City, Calif.). The primers used were as follows: sense, 5'-ATICAGTGCTTCGTCGTCGTA-3' and antisense, 5'-GGIACAGAA/GAAGAAG/GAAC/GTA/GTA/GATCTC-3'. 1 denotes inosine. Thirty cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min were performed. The amplified fragment was subcloned into pCR2.1 (Invitrogen) with the TA cloning kit and sequenced with universal forward and reverse primers (45).

Genomic DNA isolation. A culture of C. cinereus A3387 was grown in YEG medium (0.5% yeast extract, 2% dextrose) at room temperature (23°C to 25°C) for 200 rpm for 4 days. Mycelia were harvested through Miracloth (Calbioche, La Jolla, Calif.), washed twice with TE, and frozen in liquid nitrogen. DNA was isolated as previously described (52).

Preparation of C. cinereus genomic library. A genomic library of C. cinereus A3387 was constructed with a ZIpiLox kit (EcoRI arms) (Gibco BRL). A partial digestion of genomic DNA with Tsp509I (New England Biolabs, Beverly, Mass.) was done at 65°C, and samples were taken after 3, 5, 7, 8, and 9 min of digestion. Fragments were separated on 0.7% agarose gel, and DNA from fragments of 3 to 8 kb in size were recovered from the gel slices with a Qiagen (Qiagen, Chatsworth, Calif.). The size-fractionated DNA was ligated overnight at room temperature to ZIpiLox EcoRI arms following the protocols provided with the kit. The ligations were packaged into phage with a Gigapak Gold packaging kit (Strategene).

Probe preparation for library screening. A digoxigenin (DIG)-labeled probe for nonradioactive screening of the library was prepared by PCR with the C. cinereus lcc1 partial cDNA probe as a template. The primers used in the reaction were 5'-ACTCGGATGGTCTCCGTGGTC-3' and 5'-GGIACAGAA/GAAGAAG/GAAC/GTA/GTA/GATCTC-3'. 1 denotes inosine. Thirty cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min were performed. The amplified fragment was subcloned into pCR2.1 (Invitrogen) with the TA cloning kit and sequenced with universal forward and reverse primers (45).

Library screening. Appropriate dilutions of the ZIpiLox C. cinereus genomic library were plated with E. coli Y1090 cells on NZY plates (0.5% NaCl, 0.2% MgSO_4, 0.5% yeast extract, 1% N-Aminoc, A, pH 7.5) with 0.7% top agarose. The plates were incubated for 4 days at 30°C. The library pools were filtered, and each filtrate was divided into 4 fractions. For screening with 32P-labeled probes, filter lifts were prehybridized at 65°C in 0.1 M Na-phosphate (pH 7), 0.9 mS (buffer A). After the Q-Sepharose column was loaded and washed with buffer A, a linear gradient with buffer B (buffer A plus 2 M NaCl) was applied and the active fractions were eluted around 7% buffer B. These fractions were dialyzed in buffer A and loaded onto a Mono-Q 16/10 (40-mL) column preequilibrated with buffer A.

Protein purification. Miraclofil-filtered culture supernatant (pH 7.2; 15 ml) was centrifuged, and the supernatant was filtered through Whatman no. 2 paper and loaded onto a cation-exchange Q-Sepharose HP column (16-fold; 0.8 mS). The broth was frozen overnight at −20°C, thawed the next day, filtered again on Whatman no. 2 paper, and loaded onto a Q-Sepharose XK26 column (120 µL), preequilibrated with 10 mM Tris, pH 7.7, 0.9 mM (buffer A). After the Q-Sepharose column was loaded and washed with buffer A, a linear gradient with buffer B (buffer A plus 2 M NaCl) was applied and the active fractions were eluted around 7% buffer B. These fractions were dialyzed in buffer A and loaded onto a Mono-Q 16/10 (40-mL) column preequilibrated with buffer A.

RESULTS

Screening of laccase transformants. Primary transformants were screened on minimal medium plates containing 1% glucose and 1 mM 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). Transformants producing green zones on plates sprayed with ABTS were purified by subcloning into BstXI/EcoRI-linearized plasmids. The transformed strains were grown at 37°C in MY51 (per liter) 30 g of maltose, 2 g of MgSO_4, 10 g of KHPO_4, 2 g of K_2SO_4, 2 g of citric acid, 10 g of yeast extract, 0.5 ml of trace metals, 1 g of urea, 2 g of (NH_4)_2SO_4, pH 6.0).
previously purified protein and determined that this protein was encoded by $lcc1$.

**Isolation and characterization of genomic clones.** We probed the genomic library with a DIG-labeled fragment of $lcc1$ and identified nine clones, two of which appeared to be identical. Four of the eight clones carried two fragments unique to $lcc1$. The nucleotide sequence of one clone containing the complete Lcc1 open reading frame was determined on both strands. The deduced amino acid sequence of this clone matches the NH$_2$-terminal sequence of the purified protein, although the predicted signal peptide cleavage site (S1) is between A18 and Q19 while the NH$_2$-terminal sequence begins 4 residues downstream at S23. The open reading frame of the $lcc1$ gene is interrupted by seven introns ranging in size from 54 to 77 bp. The positions of introns 3, 4, and 5 were confirmed from the partial cDNA. The positions of the other four introns were identified based on the consensus sequences found at the 5' and 3' splice sites of fungal introns (22) and by homology of the deduced protein to other laccases. The deduced protein contains three potential N-linked glycosylation sites (AsnXThr/Ser), and the predicted size of the mature protein after removal of the signal peptide is 521 amino acids. Lcc1 has the highest percent identity (58%) to the laccase from the unidentified basidiomycete PM1. Alignments of Lcc1 with other laccases suggest that Lcc1 may have either a COOH-terminal extension or a COOH-terminal peptide that is removed by processing (Fig. 2).

Two $lcc3$ clones were identified in the genomic library. The $lcc3$ gene contains 13 introns and codes for a precursor protein of 517 amino acids. There is one potential N-glycosylation site, and mature Lcc3 is predicted to be 501 amino acids long.

None of the four positive $lcc2$ clones contained the complete open reading frame. The largest clone was missing the last 300 bp of the gene, but subsequent screening identified a clone containing this sequence. The $lcc2$ gene contains 13 introns and codes for a precursor protein of 517 amino acids. There is one potential N-glycosylation site, and the predicted mature protein is 499 amino acids.

Neither Lcc2 nor Lcc3 contains the 23-amino-acid COOH-terminal extension present in Lcc1 (Fig. 2). Lcc1 has 59 and 58% amino acid identity with Lcc2 and Lcc3, respectively. Lcc2 and Lcc3 are similar (80%) to one another. The percent amino
acid identity with other fungal laccases ranges from 63% for Lcc2 and the basidiomycete PM1 laccase (13) to 18% for Lcc3 and the A. nidulans laccase (2).

The positions of the 13 introns in lcc2 and lcc3 are strictly conserved. The introns actually interrupt the coding sequence at the same codons. No significant regions of similarity are found when the lcc1, lcc2, and lcc3 promoter regions are compared.

Heterologous expression of lcc1 in A. oryzae. We transformed A. oryzae HowB425 with the expression vector pDSY67, and more than 90% of the transformants were positive for laccase activity. Transformants cultured in shake flasks of MY51 at 34°C for 3 days produced from 8.0 to 135 mg of laccase per liter.

Purification and characterization of recombinant C. cinereus laccase. During purification the active fractions passed through the Mono-Q column and showed apparent homogeneity on SDS-polyacrylamide gel electrophoresis. An overall 64-fold purification and a percent recovery of 23 were achieved. Purification of recombinant Lcc1 had absorbance maxima at 278 and 614 nm. During purification the active fractions passed through the Mono-Q column and showed apparent homogeneity on SDS-polyacrylamide gel electrophoresis. An overall 64-fold purification and a percent recovery of 23 were achieved. Purification and characterization of recombinant C. cinereus laccase.

Comparison of the predicted signal sequence processing site with the NH2-terminal residues (Fig. 2) (15). Therefore, the extension of the NH2-terminal extension similar in length to the extensions of the P. radiata, M. thermophila, and N. crassa laccases (Fig. 2). This extension is rich in arginine and lysine, and in the crystal structure of the recombinant Lcc1 no electron density is observed for the last 13 predicted COOH-terminal residues (Fig. 2) (15). Therefore, the extension of Lcc1 may be cleaved during its synthesis or secretion in A. oryzae. It is not known if the COOH-terminal extension of P. radiata is removed during its processing.

Laccases from the ascomycetes Myceliophthora (5), Neurospora (20), and Podospora (18) are processed at both the NH2 and COOH termini. Processing at the NH2 terminus removes propeptides of about 20 amino acids, which are larger than the 4-residue propeptide removed from P. radiata Lcc1. The COOH-terminal processing site in ascomycetes (Asp-Ser-Gly-

**FIG. 2.** Partial alignment of the deduced amino acid sequence of the C. cinereus Lcc1 laccase and other known laccase amino acid sequences. The sequences were aligned with the Clustal algorithm (DNASTAR, Madison, Wis.). The numbers refer to the amino acid sequence region. The region of boldface is the peptide that may be removed during the biosynthesis of Lcc1. C. cin lcc1, lcc2, and lcc3, C. cinereus Lcc1, Lcc2, and Lcc3, respectively; T. vil lcc1 and lcc2, T. villosa Lcc1 (GenBank accession no. L49376) and Lcc2 (L49377), respectively; T. ver lcc1, T. versicolor Lcc1 (X84683); C. hirs and F. rad, C. hirsutus (M60560; J05562) and P. radiata (X52134), respectively; P. cin and PM1, P. cinnabarinus (AF025481) and the basidiomycete PM1 (Z12156) laccases, respectively; A. bispo, N. cra, and M. ther, A. bisporus Lcc1 (1L0664), N. crassa laccase (M18333; M18334), and M. thermophila Lcc1 (T10922), respectively; R. sol lcc1 and lcc4, R. solani Lcc1 (Z54275) and Lcc4 (Z54277), respectively.

C. cinereus, like several other basidiomycetes, contains a laccase gene family of at least three members. Laccase gene families have been reported in T. villosa (54, 55), R. solani (52), A. bisporus (41), the lignin-degrading basidiomycete CECT 20197 (34), and T. versicolor (40). The physiological importance of these gene families is unknown, but differential expression of the members of the families was observed in A. bisporus (48), T. villosa (55), R. solani (52), and the lignin-degrading basidiomycete CECT 20197 (35). In our study, no quantitative analysis of the expression of the three laccase genes was done to demonstrate differential expression. We do not know if the C. cinereus laccase genes are differentially expressed. We would like to systematically delete the genes in C. cinereus as a step towards understanding why this fungus contains multiple laccase genes.

Recently, laccases were purified from the Coprinaceae members Coprinus friesii, Panaeolus sphinctrinus, and Panaeolus papilionaceus (23). The NH2-terminal sequences of these laccases were identical and differ from the predicted NH2-terminal sequences of the three C. cinereus laccases. For example, Lcc2 differs from that of C. friesii at 6 of the 20 amino-terminal residues.

There are three potential N-linked glycosylation sites in the Lcl1 protein. The analysis of the crystal structure of the heterologously produced Lcl1 protein confirms that one of the three potential sites for N-linked glycosylation, N343, is glycosylated. The crystal structure also suggested O glycosylation. In addition to glycosylation, mature C. cinereus Lcc1 laccase requires at least three processing steps (signal peptide removal in the endoplasmic reticulum, propeptide cleavage, and removal of its COOH-terminal extension). Comparison of the NH2 terminus predicted after signal sequence cleavage to the NH2 terminus determined for both the native and recombinant proteins indicates that a 4-residue propeptide (QIVN-) is removed during the maturation of Lcc1. The fact that the NH2 terminus of the recombinant laccase is identical to that of the native protein demonstrates that A. oryzae contains the activity required for propeptide removal. Alignment of the deduced amino acid sequence of Lcc1 with those of other fungal laccases predicts a 23-amino-acid COOH-terminal extension similar in length to the extensions of the P. radiata, M. thermophila, and N. crassa laccases (Fig. 2). This extension is rich in arginine and lysine, and in the crystal structure of the recombinant Lcc1 no electron density is observed for the last 13 predicted COOH-terminal residues (Fig. 2) (15). Therefore, the extension of Lcc1 may be cleaved during its synthesis or secretion in A. oryzae. It is not known if the COOH-terminal extension of P. radiata is removed during its processing.

Laccases from the ascomycetes Myceliophthora (5), Neurospora (20), and Podospora (18) are processed at both the NH2 and COOH termini. Processing at the NH2 terminus removes propeptides of about 20 amino acids, which are larger than the 4-residue propeptide removed from C. cinereus Lcc1. The COOH-terminal processing site in ascomycetes (Asp-Ser-Gly-
are found in all of the introns; however, the coordination of the copper ions are strictly conserved (Fig. 3).

Among various laccases, Lcc1 has a “low redox potential (E°)” (53). Based on protein sequence, Lcc1 has only 24% identity to the low-E° M. thermophila laccase and 56, 56, and 33% homology to the high-E° T. villosoa Lcc1, T. versicolor laccase-1, and R. solani Lcc4, respectively. Thus, the microenvironment at the Cu sites in Lcc1 could be quite different from those in other laccases. However, the E° and kCAT observed for the other laccases, supporting the hypothesis that the ΔE° (or the thermodynamic driving force) dominates the rate-limiting step of the catalysis, the electron transfer from the substrate to the type 1 Cu in the O2-binding domain in this enzyme family.

The cloned lcc1, lcc2, and lcc3 genes have 7, 13, and 13 introns, respectively. The 3′ consensus splice sites (C/TAG) are found in all of the introns; however, the 5′ splice sites of many of the introns do not strictly match the consensus (GTANGT). The variant bases of the 5′ intron splice sites are at positions 3 and 6, with the most common being a C at position 6. These variant bases are similar to those in the introns of T. villosoa laccase genes (54, 55). The positions of the 13 introns in lcc2 and lcc3 are identical, and the predicted Lcc2 and Lcc3 proteins have the highest identity (80%). The Lcc2 and Lcc3 proteins were not isolated from the extracellular broth of C. cinereus or expressed in A. oryzae. The higher yield obtained in C. cinereus wild-type fermentations is probably due to the use of the highly expressed α-amylase promoter in the expression vector pDSY67. The expression of Lcc1 in A. oryzae should benefit from the years of experience with industrial scale-up, strain improvement and process development for other enzyme products produced in Aspergillus (11). Recombinant Lcc1 from A. oryzae will be used to test this enzyme for industrial applications.

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REFERENCES


