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Purification and characterization of laccase II of *Aspergillus nidulans*

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Abstract Sexual development in *Aspergillus nidulans* is a morphogenetic differentiation process triggered by internal and environmental signals. As a first step in analyzing the developmental pathway at the molecular level, laccase II (EC 1.10.3.2), which is specifically expressed in early stages of fruitbodies, was isolated. The enzyme was purified to apparent homogeneity from a mutant strain (SMS1) in which the sexual cycle dominates and the number of cleistothecia is increased tenfold. Laccase II was enriched 560-fold to a specific activity of 892 U (mg protein)$^{-1}$. The apparent molecular mass was determined to be 80 kDa under denaturing conditions and to be 100–120 kDa under native conditions. The internal peptide sequences gained from the protein will allow the isolation of the corresponding gene as a first step in determining the key regulators of sexual development.

Key words *Aspergillus nidulans* · Filamentous fungi · Sexual development · Cleistothecia · Laccase

Introduction

Aspergillus nidulans is a eukaryotic soil microorganism that is found in virtually all soil types where it lives as a saprophyte with a broad range of substrates (Christensen 1989). The original laboratory strain was isolated near Glasgow (for an overview see Pontecorvo et al. 1953). Because soil habitats are characterized by an inhomogeneous distribution of organic material, sessile organisms, such as filamentous fungi, have to be able to adapt to changing environmental conditions. *A. nidulans* is perfectly adapted to this situation by having different growth forms (Fig. 1 Adams et al. 1998; Clutterbuck and Timberlake 1992; Timberlake 1990).

Vegetative growth occurs by apical extension of the hyphal tips. This enables filamentous fungi to spread on solid substrates. In addition, *A. nidulans* undergoes an asexual developmental cycle, which is initiated in the laboratory after 18 h of hyphal growth (Axelrod et al. 1973). With the single-cell haploid spores (conidia) produced in this pathway *A. nidulans* is able to spread across large distances by water or wind transportation. In addition to the vegetative reproductive cycle, *A. nidulans* is capable of developing sexually derived fruitbodies, called cleistothecia, which harbor meiotically produced ascospores. A cross between different strains is not necessary for the initiation of the pathway (Käfer 1977; Pontecorvo et al. 1953). The development of ripe fruitbodies requires 10–14 days (Zonneveld 1974, 1975, 1976, 1977). The resulting ascospores are very durable and survive in dry soil for many decades.

Both asexual and sexual developmental pathways are fascinating model systems to study morphogenetic changes in lower eukaryotes. *A. nidulans* is especially attractive because it is amenable to genetic, biochemical, cell biological, and molecular biological techniques (Timberlake and Marshall 1989). The asexual developmental pathway has been studied for many years by analysis of mutants and the corresponding genes (Clutterbuck 1969; for reviews see Adams et al. 1998; Timberlake 1990; Ward 1991). A cascade of transcriptional activators that trigger expression of downstream structural genes have been discovered (Timberlake 1993). In contrast, knowledge of the molecular biology underlying sexual development is very limited. Generation of mutants is rather difficult because of the fast hyphal growth and the slow process of fruitbody development. In addition, it was found that many genes affect the sexual cycle when mutated although their primary function is not in sexual development (Adams et al. 1998; Clutterbuck 1969). Therefore, a different approach was taken in this work with the purification of a phenol oxidase, laccase II. This enzyme is differentially

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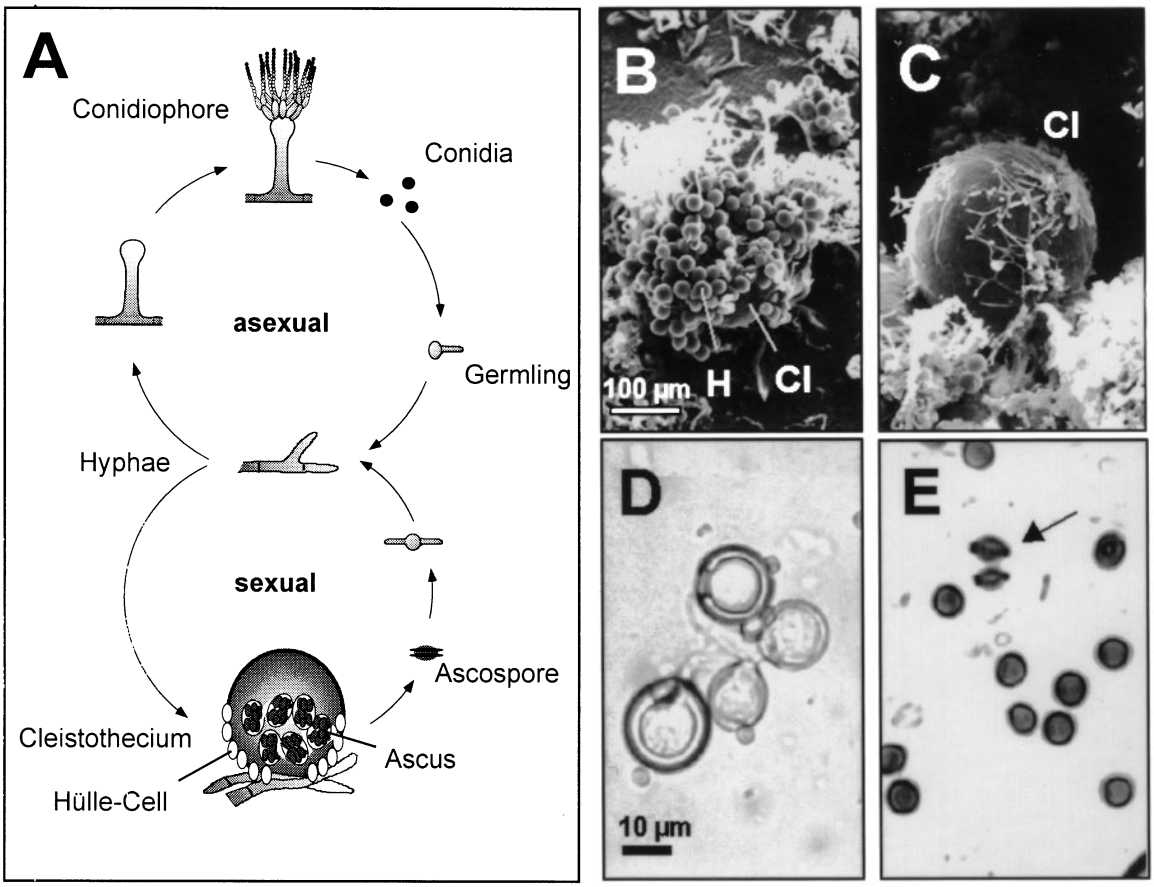

Fig. 1 A–E Life cycle of *A. nidulans*. **A** After a period of vegetative hyphal growth cells enter the asexual developmental pathway or the sexual developmental pathway. The intitiation of the pathways is triggered by internal and environmental signals. **B** SEM of young cleistothecia *(Cl)* which are embedded into thick-walled Hülle cells *(H)*. **C** Mature cleistothecia *(Cl)* with a black shell. **D** Phase-contrast view of yellow, thick-walled, Hülle cells. **E** Phase-contrast view of red-pigmented ascospores. At ascospores viewed from the side, two rings are visible *(arrow)*. The scanning electron microscopic pictures in **B** and **C** were taken by M. Krüger (Marburg). The *bar* represents 100 µm in **B** and **C** and 10 µm in **D** and **E**

expressed during fruitbody development (Hermann et al. 1983). *A. nidulans* contains at least two laccases, I and II. Laccase I (gene *yA*), defined by mutagenesis, is specifically expressed late during asexual development and is required for the conversion from a yellow precursor to a green pigment (Aramayo and Timberlake 1990; Aramayo and Timberlake 1993). Corresponding mutant colonies are yellow. The *yA* gene has been shown to be highly regulated by the transcriptional activator *abacus* (Andrianopoulos and Timberlake 1994). In contrast, laccase II has been found to be expressed in primordia and Hülle cells during sexual development. Yellow pigment formation and hyphal fusion during cleistothecial wall formation have been discussed as functions of the enzyme. The protein had been enriched to 20% of the soluble protein and histologically localized within the fruitbody tissues (Hermann et al. 1983).

In the work described here, laccase II was purified to apparent homogeneity using an *A. nidulans* mutant strain in which cleistothecial density was increased tenfold. The substrate spectrum, inhibitors, and internal peptide sequences of the enzyme were determined.

Materials and methods

Aspergillus nidulans strains and growth conditions

Supplemented minimal and complete media for *A. nidulans* were prepared as described and standard strain construction procedures were used (Käfer 1977). The strain with the dense cleistotheciation mutation *(dcl)* was identified following mutagenesis with diethylsulfate (Krüger and Fischer 1996). This strain was crossed with RMSO11 and SMS1 *(yA2, veA1, dcl)* was selected from the progeny. For the preparation of laccase II, SMS1 was grown for 3 days on miracloth membranes spread on complete media agar surfaces. Mycelia from 0.7 m^2 of agar were used for one purification.

Preparation of crude cell extract

Mycelia (60 g wet weight) were suspended in 120 ml buffer (10 mM Tris-HCl pH 7.5 supplemented with the proteinase inhibitors pefabloc (2.5 mM), leupeptin (10 µg/ml), and pepstatin (10 µg/ml) (Biomol) and homogenized (Ultra-Turrax T25) at 13,500 rpm. Cells were ruptured by two passages through a French pressure cell at 140 MPa. After one centrifugation at 16,000 × *g*, resuspension of the pellet, and a second centrifugation, the two supernatants were combined and centrifuged at 67,000 × *g* for 1 h. The supernatant was filtered (Filtropur S, 0.45 µm pores) and the filtrate was designated as crude cell extract.

Determination of the extinction coefficient of DMP (*N*,*N*-dimethyl-*p*-phenylendiamine) and enzyme assay

DMP (0–1 mM) was completely oxidized by peroxidase (1.3 U) in the presence of 0.03% H_2O_2 in laccase buffer (37 mM, citric acid monohydrate; 126 mM Na_2HPO_4 pH 6) and the extinction was measured at 550 nm. The molar extinction coefficient was determined to 1.8 mM^{-1} cm^{-1}. Laccase activity was determined in 900 µl laccase buffer, 50 µl DMP (86 mM stock solution), and 50 µl enzyme solution. Extinction was followed at 550 nm at 25 °C.

Enzyme purification

All purification steps were carried out at 4 °C. The FPLC system and columns were from Pharmacia (Freiburg, Germany). The first step was chromatography on a DEAE-Sephacel column, equilibrated with 10 mM Tris-HCl buffer (pH 7.5) using a gradient from 100 to 250 mM NaCl. Laccase II eluted at 200 mM NaCl. Laccase II was further purified by $(NH_4)_2SO_4$ precipitation. At 40% saturation laccase II remained in solution and was separated from the precipitated proteins by centrifugation. The supernatant was applied to a Phenyl-Superose column equilibrated with 10 mM Tris-HCl buffer pH 7.5 and 2 M $(NH_4)_2SO_4$. Laccase II eluted between 720 and 610 mM NH_4SO_4. The buffer was changed to 50 mM MOPS pH 6, followed by chromatography on Mono Q at pH 6. The activity was recovered at 180–240 mM NaCl and then applied to a Mono S column equilibrated with 10 mM acetate buffer pH 5. Laccase II was found in the fractions with 160–200 mM NaCl. Finally, chromatography on Mono Q at pH 5 led to the purified protein.

Protein electrophoresis and blotting

Laccase II was blotted after SDS-PAGE (10% acrylamide) on a polyvinylindendifluoride membrane (PVDF; Millipore) with the trans-blot SD semi-dry electrophoretic transfer cell (BioRad, Munich, Germany) as described in the protocol of the manufacturer. Because the N-terminus of laccase II was blocked, peptides were generated and analyzed (WITA, Teltow, Germany). For detection of laccase II activity in native gels (4%–15% acrylamide, BioRad, Munich, Germany) the gels were incubated with 0.5 mM DMP in laccase buffer.

Results

Generation of a dense cleistotheciation mutation

The asexual and sexual developmental pathways are induced in surface cultures of *A. nidulans*. While asexual structures are produced within 24 h, sexual fruitbodies are developed after 2 weeks and are embedded in a lawn of conidiophores (Axelrod et al. 1973). The number of cleistothecia is rather low and laccase II purification from this material is difficult. In addition, wild-type *A. nidulans* strains produce large amounts of laccase I in conidiophores, and laccase I cannot be distinguished from laccase II by activity measurements. Therefore, we isolated a mutant strain in which cleistothecial development was preco-

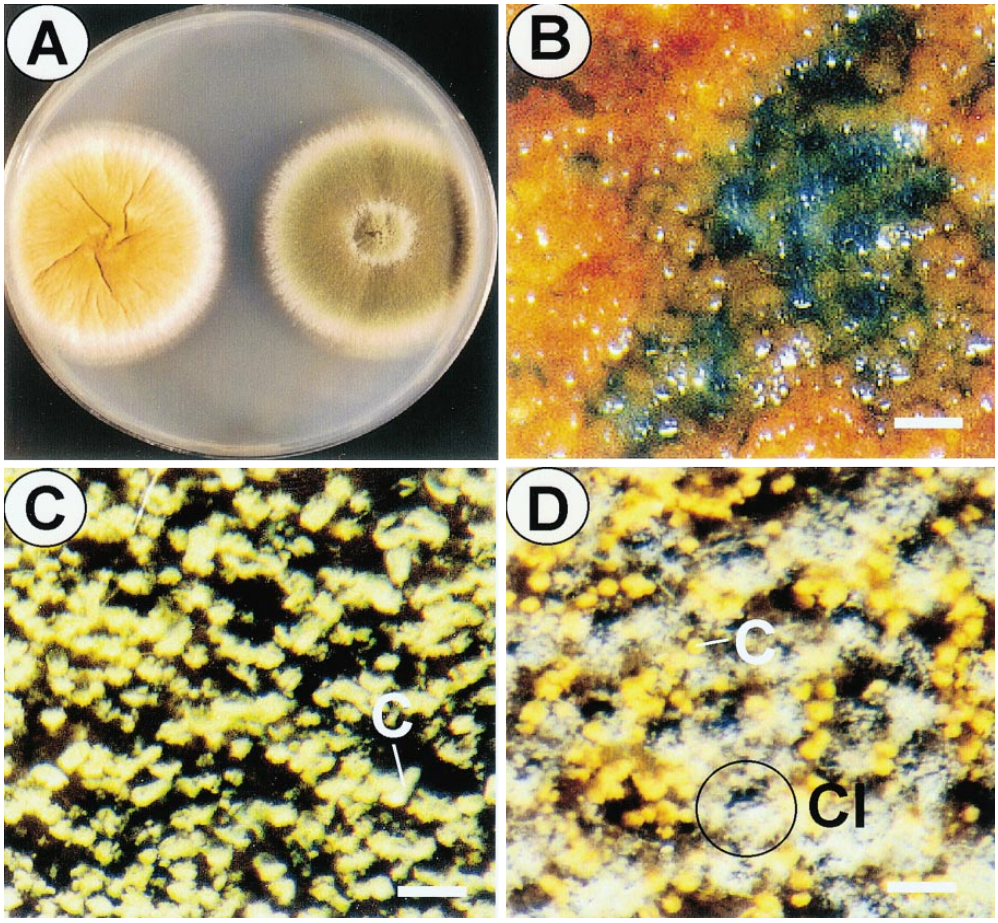

Fig. 2 A–D Characterization of the dense cleistotheciation strain SMS1 in comparison with the wild-type strain FGSC26. **A** Colonies of SMS1 *(left)* and FGSC26 *(right)* after 3 days of growth at 37 °C on a complete medium agar plate. **B** Laccase staining in the colony of SMS1. The histological staining was performed as described Hermann et al. (1983). **C, D** Microscopic view of a region of an FGSC26 (**C**) and an SMS1 (**D**) colony. *C* conidiophore, *Cl* cleistothecium in Hülle cells, *bar* 200 µm

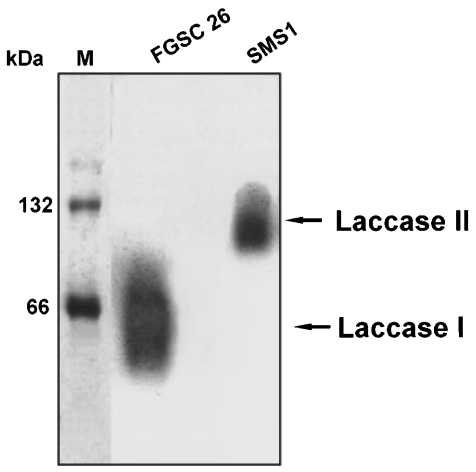

Fig. 3 Detection of laccase activity in extracts of FGSC26 and SMS1. Conidia of FGSC26 were washed with protein extraction buffer and loaded onto a native polyacrylamide gel. Laccase I is located outside the conidia and is detectable with this procedure. Laccase II is not detectable because of the time point and also because of the isolation method applied. For laccase II detection, protein extract of SMS1 was prepared and activity visualized as described in the Materials and methods section. BSA (66 kDa as monomer and 132 kDa as dimer) was used as molecular mass markers *(M)*. For a time course of laccase I and laccase II synthesis during development in wild-type strains, see Hermann et al. (1983)

cious and the number of cleistothecia ten times higher than in wild-type strains. A similar phenotype has already been described in the literature and the gene is named *dcl-1* (dense cleistotheciation; Zonneveld 1974). Whether the *dcl* mutation of our strain is allelic to the one isolated by Zonneveld was not tested. The *dcl* mutation was introduced into a strain with yellow conidiospores in which laccase I was inactive. From this cross, a strain with the *dcl* and the *yA2* mutation (SMS1) was selected and used for all further experiments (Fig. 2). This strain initiated cleistothecia formation after 2 days of cultivation, while the wild type was still completely asexual at this time. The number of conidiophores was also dramatically reduced. Analysis of protein extracts in native gels revealed the presence of laccase II only (Fig. 3).

Purification of laccase II

For the determination of laccase II activity, the artificial substrate DMP (*N,N*-dimethyl-*p*-phenylendiamine) was used in a photometric assay. Crude cell extracts of SMS1 were prepared after growth on complete media for 3 days. The hyphal mat was scraped off the agar surface (approximately 0.7 m^2) and suspended in protein extraction buffer supplemented with proteinase inhibitors. Cells were ruptured with two passages through a French pressure cell. Laccase II was purified according to the procedure out-

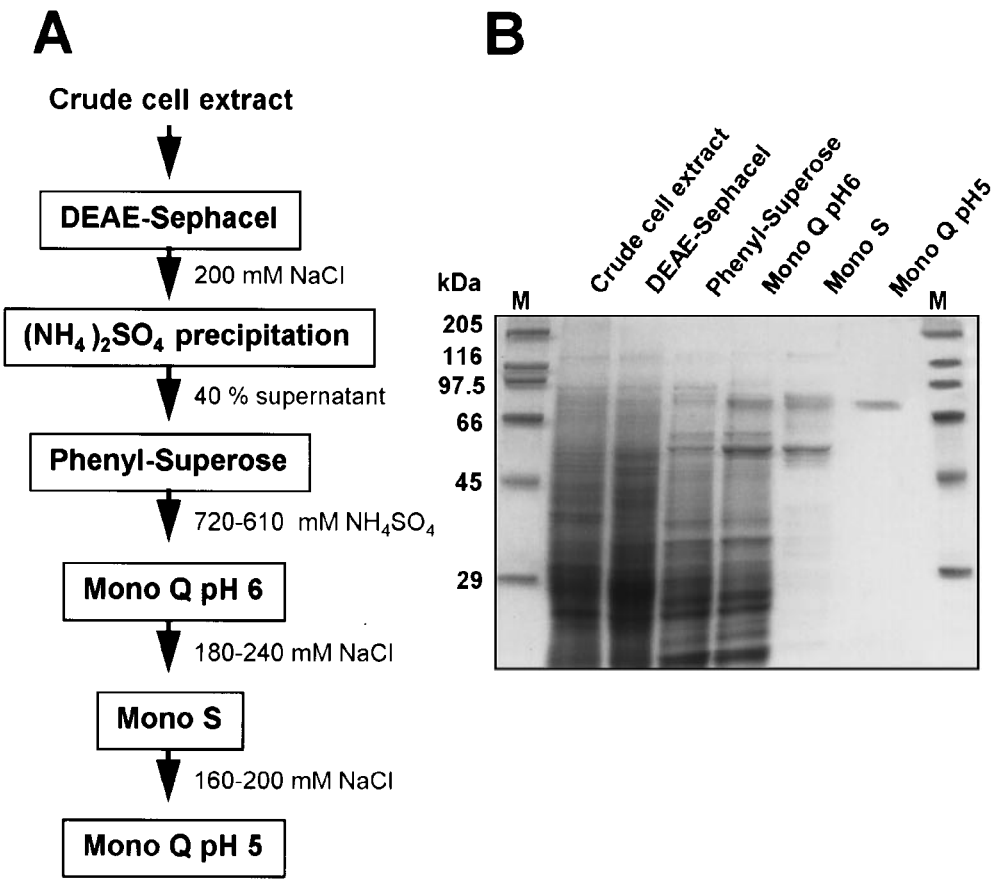

Fig. 4 A, B Purification of laccase II. **A** Schematic diagram of the purification procedure. **B** SDS-PAGE of the fractions obtained. Protein (5 µg) from the last purification fraction was loaded

Table 1 Purification of laccase II. Activity was measured at 33 °C with DMP as electron acceptor. 1 U = 1 µmol DMP oxidized per min

Fraction	Activity (U)	Protein (mg)	Yield (%)	Specific activity (U/mg)	Purification (-fold enrichment)
Crude cell extract	1170	731.0	100	1.6	1.0
DEAE-Sephacel	882	226.0	75	3.9	2.4
Phenyl-Superose	611	31.0	52	19.7	12.3
Mono Q, pH 6	281	2.4	24	117.1	73.2
Mono S	162	0.4	14	405.0	253.1
Mono Q, pH 5	107	0.12	9	891.7	557.3

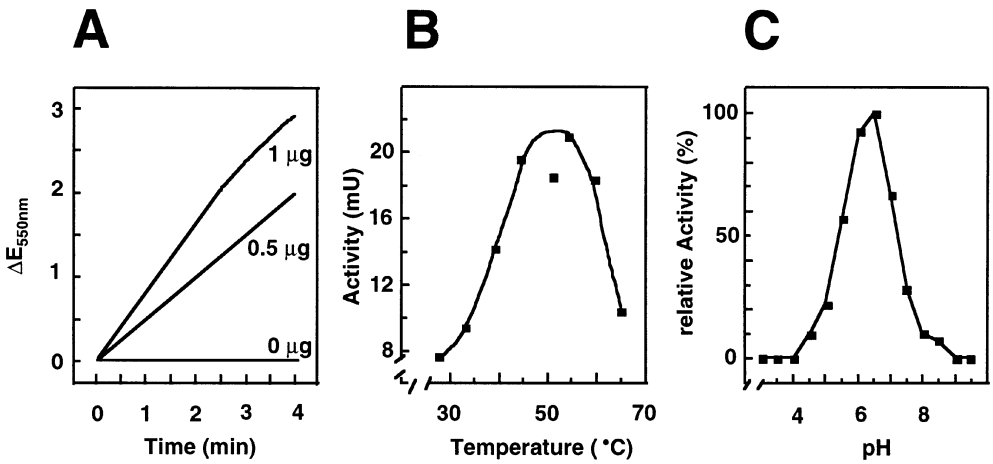

Fig. 5 Dependence of the DMP oxidation activity of laccase II on (**A**) protein concentration, (**B**) temperature, and (**C**) pH

lined in Fig. 4 with a yield of 9%, a specific acitvity of 890 U (mg protein)$^{-1}$, and a 560-fold enrichment (Table 1). Only one DMP-oxidizing activity was detected in all chromatographic steps. The protein fractions obtained during the purification procedure were analyzed on a denaturing SDS-polyacrylamide gel (Fig. 4). The apparent molecular mass was determined to be 80 kDa.

Catalytic properties of laccase II

DMP is oxidized by a variety of different enzymes, one class of which are laccases. Therefore, the purified protein was further characterized. The dependency of laccase II activity on the DMP concentration revealed an apparent K_M for DMP of 1.3 mM. This is in the same range as the K_M values determined for other laccases, such as laccase I of *A. nidulans* (3.9 mM; Clutterbuck, 1972). Catalysis of oxidation of the substrate by the laccase II protein fraction depended on the amount of protein added to the assay. The temperature optimum was determined to be 55 °C. Oxidation of DMP was dependent on the pH of the assay and was optimal at pH 6.5 (Fig. 5). The bell-shaped pH dependency is typical for the oxidation of phenolic compounds (Xu 1997). In addition to DMP, other potential laccase substrates were tested for activity. Whereas *p*-phenylendiamin, *p*-aminophenol, and 4-amino-2,6-dibromophenol were oxidized, as previously reported for a laccase-II-enriched protein fraction (Hermann et al. 1983), guiaicol, syringaldazin, 2,2′-azino-bis-(3-ethylbenzthiazolin-6-sulfonate), or potassium-ferricyanide could not serve as electron donors. To characterize the enzyme further, several potential inhibitors for laccases were tested (Table 2). The strong inhibition of the enzyme by cyanide and azide suggests the presence of a metal in the catalytic center. Even more specific is the inhibition by fluoride; this inhibition is typical for laccases and has been explained by the interaction of fluoride with the Cu center (Xu 1996).

Table 2 Inhibition of laccase II activity

Inhibitor	Concentration (mM)	Inhibition (%)
EDTA	1	31
	5	32
	10	31
NH_2OH	1	9
	10	37
KCN	0.05	31
	0.1	31
	0.2	40
	0.5	69
	1	78
	2	94
NaN_3	0.05	17
	0.1	34
	0.2	52
	0.5	68
	1	72
	2	85
NaF	1	9
	5	42
	10	51

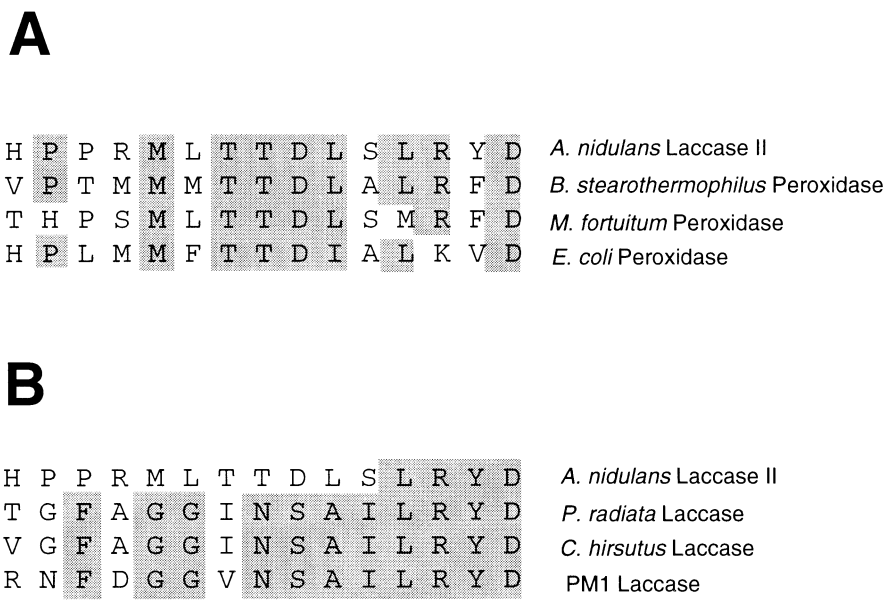

Fig. 6 Alignment of one internal peptide with known enzyme sequences. Amino acids found in three or more sequences are shaded

Because peroxidases also catalyze the oxidation of DMP, we wanted to exclude the possibility that the observed activity in the purified enzyme preparation was due to these enzymes. Peroxidases require H_2O_2 as an electron acceptor, whereas laccases use O_2. Therefore, DMP oxidation was measured in the presence (0.03%) and absence (addition of catalase to remove traces of peroxide) of H_2O_2. No change in DMP oxidation activity was found. Thus, the enzyme preparation contained only laccase II.

Determination of internal peptide sequences

The purified laccase protein was loaded onto an SDS-polyacrylamide gel, the band was cut out from the gel, and the eluted protein was used for sequencing. The N-terminus of the protein was blocked. Therefore peptides were generated and used for N-terminal sequencing. Three sequences were obtained: (1) HPP(C)RMLTTDLSLRYD, (2) AILNSGISHTDL, and (3) GVEPFIPDPFDPSIK. The first sequence has similarity to bacterial peroxidases and to fungal laccases (Fig. 6).

Discussion

Laccase II and other laccases

As a first step to a molecular analysis of the sexual cycle, we purified laccase II from primordia of *A. nidulans* cleistothecia. Several lines of evidence show that the purified enzyme is laccase II: (1) one DMP (*N*,*N*-dimethyl-*p*-phenylendiamine) oxidation activity was detected in native gels and one activity peak was detected throughout all purification steps, (2) the substrate spectrum is identical to that determined earlier with a partially purified enzyme (Hermann et al. 1983), (3) the inhibitor study indicates the presence of a Cu center, (4) H_2O_2 had no effect on the DMP oxidation activity, (5) the molecular mass of 80 kDa is similar to that of other laccases (Thurston 1994), and (6) one of the internal peptide sequences showed similarity to laccases. Although many similarities of laccase II to other laccases were found, the sequence similarity is apparently not very high. Otherwise one would have expected to find all three peptides in other laccase sequences. The same holds true for laccase I of *A. nidulans* which also has low sequence similarity to other fungal laccases. Because no sequence similarity was found between the identified peptide sequences of laccase II and laccase I, it also can be concluded that laccase I and laccase II are only distantly related enzymes. This could reflect the different substrates of the two proteins. While the substrate of laccase I is quite well defined as a polyketide derivative, the substrate of laccase II is unknown (Aramayo and Timberlake 1990; Mayorga and Timberlake 1992). It has been speculated that laccase II is required for pigment synthesis in primordia and thus is not essential for fruitbody development. A dominant mutation, *yB*, was identified which abolished pigment conversion in conidiospores and also pigmentation in primordia (Hermann et al. 1983). Thus, *yB* could be a regulator of both laccases. In addition, it was also suggested that laccase II could be involved in hyphal fusion, which is required for the shell formation of the cleistothecia. This is supported by the fact that *yB* mutants are acleistothecial (Kurtz and Champe 1981). However, the effect could also be secondary if *yB* also regulates other genes which are essential for fruitbody formation. Deletion of the corresponding gene should allow a definitive determination of the molecular function of laccase II during sexual development.

Many other laccases have been cloned by PCR approaches based on conserved regions within the enzymes. However, laccase I of *A. nidulans* shares only 20%–30% amino acid sequence identity with laccases of *Neurospora crassa* (accession number M18333), *Podospora anserina* (accession number Y08827), and *Cryphonectria parasit-*

ica (accession number Q03966). Therefore, we decided to initiate the approach described in this work to isolate the laccase II gene of *A. nidulans*. During preparation of this paper, a fragment of a cDNA which was detected in an *A. nidulans* cDNA sequencing project at the University of Oklahoma (http://www.genome.ou.edu/asper.html) was found to have significant sequence similarity to laccase I. The sequences, however, were only 40% identical in 106 amino acids of the published sequence with longer stretches of identity in the Cu-binding domains. If the isolated partial cDNA sequence is part of the laccase II cDNA, it is obvious that the two laccases of *A. nidulans* are quite different from each other and differ also from all other fungal laccases.

Laccase II is a good candidate to study sexual development in *A. nidulans*. Laccase II expression occurs very early during sexual development (Hermann et al. 1983). Because transcriptional regulation is the dominant regulatory principle in fungi, laccase II is probably a target gene of transcriptional regulators that are involved in the initiation of this process. Therefore, promoter analysis and definition of *cis*-acting regulatory boxes should lead to the identification of early regulators of sexual development. Since laccase II is expressed at a high level, promoter analyses should be straightforward. The experiments performed with laccase I are examples of how successful this approach could be. The laccase I gene was identified by mutagenesis, and it was found that one of the key regulators of asexual development, *abaA*, activates transcription of this gene (Andrianopoulos and Timberlake 1994; Aramayo and Timberlake 1993).

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References

Adams TH, Wieser JK, Yu JH (1998) Asexual sporulation in *Aspergillus nidulans*. Microbiol Mol Biol Rev 62:35–54

Andrianopoulos A, Timberlake WE (1994) The *Aspergillus nidulans abaA* gene encodes a transcriptional activator that acts as a genetic switch to control development. Mol Cell Biol 14: 2503–2515

Aramayo R, Timberlake WE (1990) Sequence and molecular structure of the *Aspergillus nidulans yA* (laccase I) gene. Nucleic Acids Res 18:3415

Aramayo R, Timberlake WE (1993) The *Aspergillus nidulans yA* gene is regulated by *abaA*. EMBO J 12:2039–2048

Axelrod DE, Grealt M, Pastushok M (1973) Gene control of developmental competence in *Aspergillus nidulans*. Dev Biol 34: 9–15

Christensen M (1989) A view of fungal ecology. Mycologia 81: 1–19

Clutterbuck AJ (1969) A mutational analysis of conidial development in *Aspergillus nidulans*. Genetics 63:317–327

Clutterbuck AJ (1972) Absence of laccase from yellow-spored mutants of *Aspergillus nidulans*. J Gen Microbiol 70:423–435

Clutterbuck AJ, Timberlake WE (1992) Genetic regulation of sporulation in the fungus *Aspergillus nidulans*. In: Russo EA, Brody S, Cove D, Ottolenghi S (eds) Development: the molecular genetic approach. V. Springer, Berlin Heidelberg New York, pp 103–120

Hermann TE, Kurtz MB, Champe SP (1983) Laccase localized in Hulle cells and cleistothecial primordia of *Aspergillus nidulans*. J Bacteriol 154:955–964

Käfer E (1977) Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. Adv Genet 19:33–131

Krüger M, Fischer R (1996) Isolation of two *apsA* suppressor strains in *Aspergillus nidulans*. Genetics 144:533–540

Kurtz MB, Champe SP (1981) Dominant spore color mutants of *Aspergillus nidulans* defective in germination and sexual development. J Bacteriol 148:629–638

Mayorga ME, Timberlake WE (1992) The developmentally regulated *Aspergillus nidulans wA* gene encodes a polypeptide homologous to polyketide and fatty acid synthases. Mol Gen Genet 235:205–212

Pontecorvo G, Roper JA, Hemmons LM, MacDonald KD, Bufton AWJ (1953) The genetics of *Aspergillus nidulans*. Adv Genet 5:141–238

Thurston CF (1994) The structure and function of fungal laccases. Microbiology 140:19–26

Timberlake WE (1990) Molecular genetics of *Aspergillus* development. Ann Rev Genetics 24:5–36

Timberlake WE (1993) Translational triggering and feedback fixation in the control of fungal development. Plant Cell 5:1453–1460

Timberlake WE, Marshall MA (1989) Genetic engineering of filamentous fungi. Science 244:1313–1317

Ward M (1991) *Aspergillus nidulans* and other filamentous fungi as genetic systems. In: Modern microbial genetics. Wiley-Liss, London, pp 455–496

Xu F (1996) Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. Biochemistry 35:7608–7614

Xu F (1997) Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases. J Biol Chem 272: 924–928

Zonneveld BJM (1974) α-1,3 glucan synthesis is correlated with α-1,3 glucanase synthesis, conidiation and fructification in morphogenetic mutants of *Aspergillus nidulans*. J Gen Microbiol 81:445–451

Zonneveld BJM (1975) Sexual differentiation in *Aspergillus nidulans*. The requirement for manganese and the correlation between phosphoglucomutase and the synthesis of reserve material. Arch Microbiol 105:105–108

Zonneveld BJM (1976) The effect of glucose and manganese on adenosine-3′,5′-monophosphate levels during growth and differentiation of *Aspergillus nidulans*. Arch Microbiol 108:41–44

Zonneveld BJM (1977) Biochemistry and ultrastructure of sexual development in Aspergillus. In: Smith JE, Pateman JA (eds) Genetics and physiology of *Aspergillus*. Academic Press, London, pp 58–80