JOURNAL OF BACTERIOLOGY, Nov. 1989, p. 6288–6293
0021-9193/89/116288-06$02.00/0
Copyright © 1989, American Society for Microbiology
Vol. 171, No. 11

Isolation and Characterization of a Laccase-Derepressed Mutant of *Neurospora crassa*

HISASHI TAMARU AND HIROKAZU INOUE*

Laboratory of Genetics, Department of Regulation Biology, Faculty of Science, Saitama University, Urawa 338, Japan

Received 30 March 1989/Accepted 18 August 1989

Laccase from the ascomycete *Neurospora crassa* is an inducible secretory enzyme. Production of this enzyme is repressed in vegetative cultures but can be induced by treatment with low concentrations of cycloheximide. Isolation and characterization of a derepressed mutant, the *lah-1* mutant, that is capable of producing laccase in vegetative cultures without induction by cycloheximide are described. The *lah-1* mutation is mapped between *nit-2* and *leu-3* on linkage group I, and it behaved as a recessive mutation in a forced heterokaryon. No differences were detected biochemically or immunologically between the laccase protein produced by the *lah-1* mutant in the absence of cycloheximide and that induced with cycloheximide in the wild-type strain. This suggests that both laccases (66 kilodaltons) are products of the same structural gene. Relative amounts of laccase in the culture filtrate of the *lah-1* mutant were much higher than those induced with cycloheximide in the wild-type strain, demonstrating high efficiency of the *lah-1* mutant in production and secretion of laccase. The time course of laccase production by the *lah-1* mutant revealed that expression of 66-kilodalton laccase was repressed in conidia and derepressed during vegetative mycelial growth. This suggests that a multiple regulatory mechanism is involved in the production and/or maturation of *Neurospora* laccase. The *lah-1* mutant may be useful for identifying genes that regulate expression of the laccase gene in *N. crassa*.

Laccase is a ubiquitous enzyme in fungi (18). Its activity has been observed intra- and/or extracellularly at various stages of development. For example, in basidiomycetes, such as *Lentinus* sp. and *Coprinus* sp., laccase is produced actively in the developmental stage of primordia (12, 19). In *Aspergillus nidulans*, it is synthesized preferentially when vegetative hyphae begin to differentiate conidiophores, and it mediates formation of the green pigment in conidia (2, 11). In *Podospora anserina*, laccase is formed during cell lysis (1). These findings suggest that synthesis of this enzyme is developmentally regulated.

Induction of laccase by a variety of substances in various fungi independently of developmental regulation has been reported. Effects of inducers of laccase formation differ from fungus to fungus. In *Botrytis* sp., laccase is produced only in the presence of inducer, and the characteristics of laccase, such as its molecular weight, amino acid composition, electrophoretic mobility, and heat inactivation, vary depending on the nature of the inducer (16, 17). In *Neurospora crassa*, production of laccase is induced by addition of low concentrations of protein synthesis inhibitors, such as cycloheximide and puromycin, to the culture medium used for vegetative growth (4). The physical and chemical properties of *Neurospora* laccase excreted under such induction conditions have been characterized (5, 14). Furthermore, the laccase gene has been cloned and its nucleotide sequence has already been determined (6, 7), providing a basis for molecular analysis of this enzyme. However, only limited studies on how production of laccase is regulated in *N. crassa* during development or under induction conditions have been reported. To elucidate the mechanisms that regulate laccase production and secretion in *N. crassa*, we used genetic analysis. At least two alternative genetic approaches may allow identification of genes that affect laccase production: (i) isolation of mutants unable to produce laccase in the presence of cycloheximide and (ii) isolation of mutants able to produce laccase even in the absence of cycloheximide. In this report, we describe the isolation of a derepressed mutant, carrying a mutation designated *lah-1*, that produces and secretes excess laccase in vegetative cultures. We also report the genetic and physiological characterization of this mutant.

MATERIALS AND METHODS

Strains. The genotypes and sources of the strains used in this study are listed in Table 1. Wild-type strain C1-T10-37A was derived from a cross between OR8-1a and 74-OR31-16A. The *exo-1* exoamylase mutant, which produces β-amylase, α-amylase, glycoamylase, invertase, and trehalase abundantly (8), was used to isolate the *lah-1* mutant. Strains FGSC 982 (*nit-2*) FGSC 1216 (*leu-3 arg-1*) were used for mapping, and strains H8-17A and M80-C1-12A were used to make forced heterokaryons.

Genetic analysis. The genetic procedures used were those described by Davis and de Serres (3).

Isolation of mutants. Conidial suspensions (10^7/ml) of the *exo-1* mutant were prepared as described previously (9) and exposed to UV radiation at 550 J/m^2. This UV dose gave about 10% survival of conidia. The exposed conidia were plated on Vogel N agar medium (21) containing 0.5% xylose and 1% sorbose and incubated at 35°C. After 4 days, a solution containing *o*-tolidine was added to the petri dishes. Colonies that developed a blue halo of oxidized *o*-tolidine were isolated and subcultured. An isolate that showed the most intensive halo was backcrossed extensively to wild-type strain C1-T10-37A to exclude additional mutations, including the *exo-1* mutation.

Culture conditions. (i) Vegetative culture. Conidial suspensions were inoculated into 200 ml of Vogel minimal medium (21) containing 1.5% sucrose (final concentration, 10^6 conidia per ml) and incubated at 35°C in a 500-ml Erlenmeyer flask with gentle shaking in the dark.

(ii) Culture for laccase induction. For induction of laccase in the wild-type strain, cycloheximide was added to ensure a

* Corresponding author.

TABLE 1. *Neurospora* strains used

Strain or FGSC[a] no.	Genotype	Source (reference)
C1-T10-37A	*A*	Segregant from OR8-1a (FGSC 988) × 74-OR31-16A (FGSC 2583)
M80-C1-12A	*A nic-2*	Segregant from FGSC 81 × FGSC 142
H2-53a	*a lah-1*	Tamaru and Inoue (this paper)
H8-17A	*A lah-1 pan-2*	Tamaru and Inoue (this paper)
H12-1A	*A lah-1*	Tamaru and Inoue (this paper)
FGSC 982	*a nit-2*	Obtained from FGSC
FGSC 1216	*a leu-3 arg-1*	Obtained from FGSC
FGSC 2256	*a exo-1*	Obtained from FGSC

[a] FGSC, Fungal Genetic Stock Center, Department of Microbiology, University of Kansas Medical School, Kansas City.

final concentration of 2.8 μM at 24 h after the start of the vegetative culture, and incubation was continued under the same conditions for 48 h.

Assay for laccase activity. A standard assay for laccase activity was performed by measuring *N,N*-dimethyl-*p*-phenylenediamine oxidation spectrophotometrically as described by Clutterbuck (2). The reaction mixture (final volume, 2.2 ml) contained 5 mM *N,N*-dimethyl-*p*-phenylenediamine and the enzyme solution to be assayed in 0.1 M sodium phosphate buffer adjusted to pH 6.0. The initial rate of increase in A_{550} was measured with a Beckman DU-50 spectrophotometer using a Kinetic Soft-pak TM module. One unit of enzyme activity is defined as the amount capable of oxidizing 1 nmol of *N-N*-dimethyl-*p*-phenylenediamine per min at 25°C per 1 ml of reaction mixture. For quantitative determination of the substrate specificity and pH optimum of laccase, oxygen consumption in the reaction mixture was measured with an oxygen electrode as described by Froehner and Eriksson (5).

Purification of extracellular laccase in the *lah-1* mutant. The procedures used to purify laccase are a modification of those described by Lerch et al. (14) and Froehner and Eriksson (5). After the *lah-1* mutant had been cultured for 60 h, culture media were collected by filtration. Laccase protein in the filtrate was precipitated at 80% saturation with $(NH_4)_2SO_4$. The precipitate was dissolved in 80 ml of 0.05 M sodium phosphate buffer (pH 6.0). The sample was applied to a column of hydroxylapatite. The fractions containing laccase activity were pooled (fractions I and II; see Fig. 2) and concentrated to a 5.0-ml volume by ultrafiltration (Immersible CX-30TM; Millipore Corp.). The sample from fraction II was applied to a column of Sephadex G-100, and fractions containing laccase activity were pooled and concentrated to 6.0 ml as described above. The purified enzyme was stored frozen at −30°C.

Preparation of crude cell extracts. Mycelia were harvested, washed by filtration, and frozen at −30°C. The frozen mycelia (about 1 g) were ground with several pieces of dry ice in a mortar and pestled to a fine powder. The powder was suspended in 2 ml of 0.05 M sodium phosphate buffer (pH 6.8) containing 20 μg of phenylmethylsulfonyl fluoride per ml and mixed well. The homogenate was centrifuged at 10,000 × *g* for 20 min. The supernatant was collected and stored at −30°C. Crude cell extracts from ungerminated conidia were prepared in the same manner.

Antiserum generation. To generate anti-laccase polyclonal serum, two male White rabbits weighing 2 to 3 kg were used. Purified laccase (1 mg) in Freund complete adjuvant was injected subcutaneously. The immunoglobulin G fraction of antiserum raised against laccase was purified by the method described by Levy and Sober (15).

Immunoblot analysis. Sample proteins were fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose (0.45-μm pore size; Schleicher & Schuell, Inc.) electrophoretically, and immunologically detected as described by Towbin et al. (20). To detect the bound primary antibodies, the sheet was incubated at room temperature for 1 h with a 1:1,000 dilution of goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (A-6154 Sigma Chemical Co.) in phosphate-buffered saline plus 1% gelatin. They were visualized by incubation with 0.2 mg of *o*-tolidine per ml and 0.25 μl of H_2O_2 per ml.

Other procedures. SDS-PAGE was performed by the method of Laemmli (10). Samples were treated with 1% SDS and 2% mercaptoethanol and boiled at 100°C for 5 min. Electrophoresis was done in gels of 10% acrylamide containing 0.1% SDS. Coomassie brilliant blue R was used to stain proteins in the gel. For calibration of molecular weight in SDS-PAGE, Molecular Weight Standard-low (Bio-Rad Laboratories) was used. Protein concentration determination was performed with a Bio-Rad Protein Assay Kit.

RESULTS

Isolation of mutants. A screening procedure (13) used to isolate mutants affected in production of glucose-releasing exoenzymes, such as invertase, trehalase, and amylase, led us to isolate unexpectedly a derepressed mutant capable of producing laccase in vegetative cultures with no inducer. In this screening system, colonies producing those enzymes extracellularly develop a blue halo of oxidized *o*-tolidine around the colony, while colonies lacking the enzymes do not show the halo. During this screening, several colonies that unexpectedly developed a blue halo without glucose oxidase and peroxidase were found. We isolated colonies that possessed the ability to oxidize *o*-tolidine extracellularly without H_2O_2. An *lah-1* mutant which exhibited the most intensive halo was further characterized.

The h^+ phenotype and the *lah-1* mutation. To confirm whether the ability of the *lah-1* mutant to oxidize *o*-tolidine extracellularly, designated the h^+ phenotype, is caused by a single mutation, the *lah-1* mutant was backcrossed to the wild-type strain. Figure 1 shows the 4:4 segregation of the h^+ and h^- phenotypes in two tetrads. This result suggests that a single nuclear mutation, *lah-1*, confers the h^+ phenotype.

Mapping of the *lah-1* mutation. Backcrosses of the *lah-1* mutant to the wild-type strain revealed linkage of the *lah-1* mutation to the mating type locus. To map the *lah-1* locus, the *lah-1* mutant was crossed to strains carrying the *nit-2*, *leu-3*, and *arg-1* mutations, all linked to the mating locus. The result indicated that *lah-1* is located between *nit-2* and *leu-3* on linkage group I (Table 2). No mutations like *lah-1* have previously been reported. Thus, *lah-1* should be regarded as a new nuclear mutation.

Responsibility of laccase activity for the h^+ phenotype of the *lah-1* mutant. A primary problem in the biochemical characterization of the *lah-1* mutant was to identify the activity responsible for the h^+ phenotype. Peroxidase oxidizes *o*-tolidine only in the presence of H_2O_2 as an electron acceptor, but amounts of H_2O_2 sufficient for peroxidase action did not seem to be present in the culture medium in which the *lah-1* mutant expressed the h^+ phenotype. It was therefore inferred that an oxidase whose substrate specificity is similar

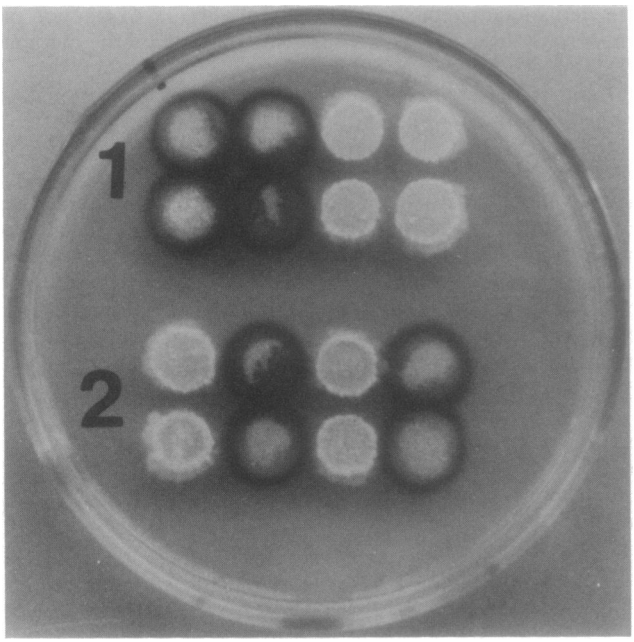

FIG. 1. The h^+ phenotype. Two tetrads (1 and 2) from a cross between the *lah-1* mutant (H2-53) and the wild-type strain were tested for the h^+ phenotype. Conidial suspensions of each progeny were spotted onto Vogel N agar medium containing 0.5% sucrose and 1% sorbose and incubated at 35°C for 2 days. The halo around the colonies is the result of treatment with 1 mg of *o*-tolidine per ml.

to that of peroxidase but whose requirement for the electron acceptor is not similar, might be responsible for the h^+ phenotype. Phenol oxidases were then regarded as candidates for this enzyme activity. Since these oxidases are known to require O_2 as an electron acceptor, O_2 consumption in the culture filtrate of the *lah-1* mutant was measured polarographically in the presence of *o*-tolidine with an oxygen electrode. O_2 consumption was observed only in the culture filtrate of the *lah-1* mutant and not in that of the wild-type strain. The pH optimum for O_2 consumption in this filtrate was estimated to be 6.0. Other substrates oxidized in the filtrate were guaiacol, 3-(3,4-dihydroxyphenyl)-L-alanine (DOPA), ascorbic acid, and homovanillic acid, but L-tyrosine was not oxidized. This substrate specificity is similar to that of laccase in wild-type *N. crassa* as reported by Froehner and Eriksson (5). Accordingly, we deduced that laccase activity is responsible for the h^+ phenotype of the *lah-1* mutant.

TABLE 2. Mapping of the *lah-1* gene in *N. crassa*

Zygote type (recombination %)			No. of progenies of parental genotype	No. of progenies of recombinant genotype[a]		Total no. of progenies (% germination)
1		2		Region 1	Region 2	
+	*leu-3*	*a*	103	10	6	222 (56)
lah-1	+	*A*	103			
(4.5)		(2.7)				
nit-2	+	*a*	129	5	73	333 (94)
+	*lah-1*	*A*	126			
(1.5)		(21.9)				

[a] There were no double (region 1 and 2) recombinant progenies.

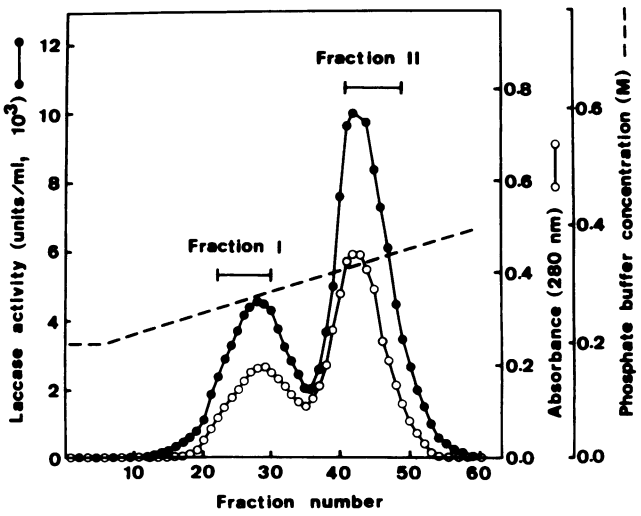

FIG. 2. Hydroxylapatite column chromatography of extracellular laccase from the *lah-1* mutant of *N. crassa*. Elution of the enzyme was performed with a linear gradient of 0.2 to 0.4 M sodium phosphate buffer (pH 6.0). The bars indicate the fractions pooled.

Purification of extracellular laccase in the *lah-1* mutant. Laccase from the culture of the *lah-1* mutant showed two activity peaks when eluted from a hydroxylapatite column (Fig. 2). This elution profile is similar to that of the wild-type laccase reported by Froehner and Eriksson (5). Only fraction II obtained by their method was further concentrated to approximately twofold purification (Table 3). In SDS-PAGE of the purified protein, laccase migrated as one major polypeptide band with a molecular mass of approximately 66 kilodaltons (kDa) (Fig. 3, Lane a), which is in good agreement with that reported by Froehner and Eriksson (5). SDS-PAGE of the crude *lah-1* mutant culture medium showed a major band at 66 kDa (Fig. 3, lane B), but there was no 66-kDa band in the SDS-PAGE pattern of the crude culture medium of the wild-type strain grown without cycloheximide (Fig. 3, lane C). The extracellular laccase produced by the *lah-1* mutant without cycloheximide was one of the major proteins in the crude *lah-1* culture medium (Fig. 3).

Comparison of extracellular laccase produced by the *lah-1* mutant with that produced by the cycloheximide-induced wild-type strain. (i) K_m value. The K_m values of extracellular laccase in crude culture medium from the *lah-1* mutant and the wild-type strain were measured with *N,N*-dimethyl-*p*-phenylenediamine as the substrate, and both were estimated to be 70 μM (data not shown).

(ii) Immunological characterization. In a double-immunodiffusion study, the polyclonal antiserum raised in rabbits immunized with laccase purified from the culture medium of

TABLE 3. Purification of extracellular laccase from the *lah-1* mutant of *N. crassa*

Purification step	Vol (ml)	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Yield (%)	Fold purification
Culture filtrate	4,500	1,240,000			100	
Ammonium sulfate	143	712,000	54.1	13,200	58	1.0
Hydroxylapatite	5.0	249,000	10.1	24,700	20	1.9
Sephadex G-100	6.0	168,000	6.5	25,900	14	2.0

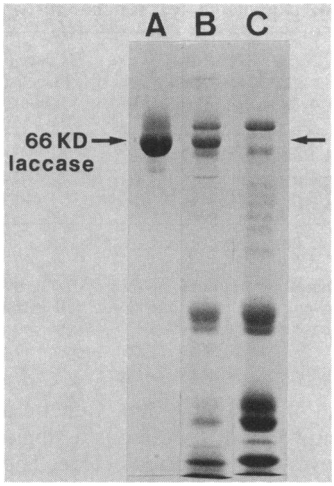

FIG. 3. SDS-PAGE pattern of polypeptides from culture filtrates of the *lah-1* mutant and wild-type *N. crassa*. Lanes: A, purified *lah-1* mutant laccase; B, extracellular proteins from the *lah-1* mutant (H12-1) grown without cycloheximide; C, extracellular proteins from the wild-type strain grown without cycloheximide. The position of laccase (66 kDa) is indicated. Proteins were stained with Coomassie brilliant blue R.

the *lah-1* mutant formed a single precipitation band with the extracellular laccases from the *lah-1* mutant and the cycloheximide-induced wild-type strain (data not shown). Fraction I eluted from a hydroxylapatite column (Fig. 2) also formed a single precipitation band with the antiserum and showed immunological identity to the laccase from fraction II. Extracellular polypeptides from the *lah-1* mutant and the cycloheximide-induced wild-type strain were subjected to SDS-PAGE and immunoblot analysis with antiserum to the *lah-1* mutant laccase (Fig. 4).

As expected, a single band at the 66-kDa position was detected in the crude culture media of both the *lah-1* mutant (lane B) and the cycloheximide-induced wild-type strain (lane C). These data suggest that the *lah-1* mutant and the cycloheximide-induced wild-type strain both produce a single laccase protein which appears to be indistinguishable when polyclonal antiserum specific for the laccase from the *lah-1* mutant is used.

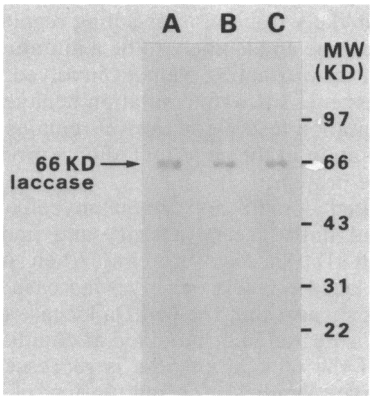

FIG. 4. Immunoblot analysis of extracellular proteins from the *lah-1* mutant and wild-type *N. crassa*. Extracellular proteins were subjected to SDS-PAGE, transferred to a nitrocellulose sheet, and probed with polyclonal antibodies to laccase purified from the *lah-1* mutant. Lanes: A, purified *lah-1* mutant laccase; B, extracellular proteins from the *lah-1* mutant (H12-1) grown without cycloheximide; C, extracellular proteins from the cycloheximide-induced wild-type strain. MW, Molecular weight.

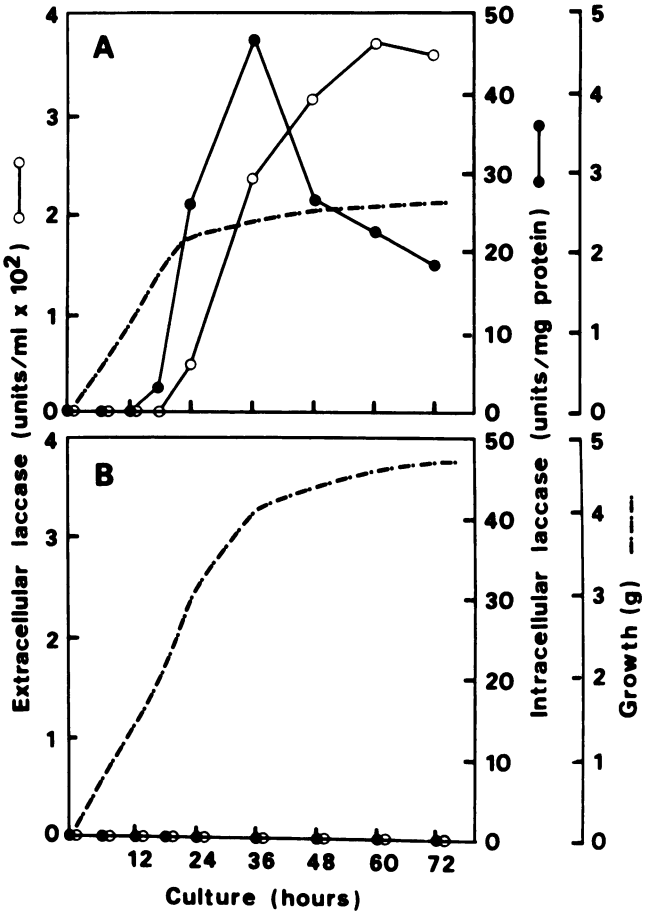

FIG. 5. Time course of the increase in intra- and extracellular laccase activities in the *lah-1* mutant and wild-type *N. crassa* during vegetative culture. Culture filtrates and crude cell extracts were prepared from vegetative cultures and assayed for laccase activity at the times indicated. (A) *lah-1* mutant (H12-1). (B) Wild-type strain.

Time course of intra- and extracellular laccase activities in the *lah-1* mutant and the wild-type strain during vegetative culture. To determine the stage of vegetative growth at which production of laccase occurs in the *lah-1* mutant, both intra- and extracellular laccase activities were measured at various times during vegetative culture. As a control, both laccase activities in the wild-type strain were measured in the same manner. In the *lah-1* mutant, neither intra- nor extracellular laccase activity was detected in conidia, germinated conidia, or young mycelia up to hour 12 (Fig. 5A). Intra- and extracellular laccase activities were detected at hours 18 and 24 of culture, respectively, and thereafter increased to a maximum at hours 36 and 60, respectively. After reaching its maximum, intracellular activity decreased rapidly, while extracellular activity did so slowly. In the wild-type strain, neither intra- nor extracellular laccase activity was detected over a period of 72 h (Fig. 5B).

Changes in laccase protein level detected immunologically in the *lah-1* mutant and the wild-type strain during vegetative culture. As described above, laccase activity was not detected in conidia, germinated conidia, or young mycelia of the *lah-1* mutant. It is possible that laccase protein exists at

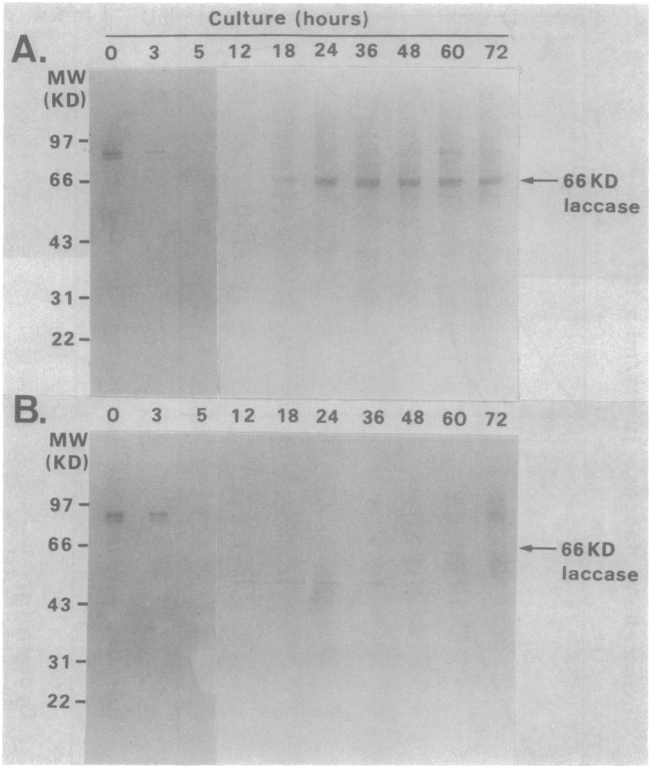

FIG. 6. Time course of increase in intracellular laccase protein in the *lah-1* mutant and wild-type *N. crassa* during vegetative culture. Crude cell extracts were prepared from conidia, germinated conidia, and mycelia grown in vegetative cultures and assayed for laccase protein at the times indicated. Equal amounts of protein (10 μg) were immunoblotted as described in the legend to Fig. 4. (A) *lah-1* mutant (H12-1). (B) Wild-type strain. MW, Molecular weight.

these stages in inactive forms. This possibility was tested by immunoblot analysis of the protein samples collected at these stages (Fig. 6A). Lanes containing conidial and young mycelial extracts did not show laccase protein at 66 kDa. The 66-kDa protein was first detected in the lane containing extracts from mycelia at hour 18 of culture. Thereafter, the amount of the 66-kDa protein increased for the rest of the culture period (up to hour 72). In the wild-type strain, no 66-kDa laccase proteins were detected over the entire period (Fig. 6B). Thus, the 66-kDa laccase protein level is closely correlated with laccase activity. Unexpectedly, in both the *lah-1* and wild-type strains at least two proteins that cross-reacted with anti-laccase serum were detected at the 86 and 82-kDa positions in the lanes of conidia and germinated conidia up to hour 5 (Fig. 6A and B).

Recessiveness of the *lah-1* mutation. To determine whether the *lah-1* mutation is recessive or dominant, we constructed a forced heterokaryon between *lah-1 pan-2 A* and *nic-2 A*. Neither intra- nor extracellular laccase activity was detected in the heterokaryon (Table 4). Therefore, the *lah-1* mutation is recessive.

TABLE 4. Dominance test for the *lah-1* mutation[a]

Strain	Genotype	Extracellular laccase (U/ml)	Intracellular laccase (U/mg of protein)
H8-17A	*A lah-1 pan-2*	310	35
M80-C1-12A	*A nic-2*	0	0
Heterokaryon	*A lah-1 pan-2* + *A nic-2*	0	0

[a] Conidia from two homokaryotic strains, H8-17A and M80-C1-12A, and the heterokaryotic strain constructed from H8-17A and M80-C1-12A were collected and inoculated into culture medium. After growth for 72 h under standard conditions, culture filtrates and crude cell extracts were prepared and assayed for laccase activity.

DISCUSSION

Neurospora laccase is repressed in vegetative cultures but can be induced by treatment with low concentrations of cycloheximide in the culture medium. In this report, we describe the isolation and characterization of a derepressed mutant that produces and secretes laccase into the medium without addition of cycloheximide. Genetic analysis of this *lah-1* mutant showed that it contains a single mutation in a gene that has never been reported before. The new mutation was recessive in a forced heterokaryon.

The *lah-1* mutation increased laccase production appreciably but did not increase levels of other secretory polypeptides (Fig. 3). These properties made it easy to purify laccase from the culture medium of the *lah-1* mutant. Severalfold purification of laccase from the *lah-1* mutant made it pure enough to migrate as a single major band on SDS-PAGE (Table 3). On the other hand, several hundredfold purification was required to purify wild-type laccase (5). Thus, the *lah-1* mutant strain is useful for production and purification of laccase protein.

Extracellular laccase from the *lah-1* mutant may be heterogeneous; at least two peaks of laccase activity were detected in the profile eluted from a hydroxylapatite column, although they could not be distinguished by SDS-PAGE, double immunodiffusion, and substrate specificity (data not shown). A similar phenomenon has been observed in the purification of laccase from the cycloheximide-induced wild-type strain (5, 14).

Several lines of biochemical and immunological evidence suggest that the laccase produced by the *lah-1* mutant in vegetative cultures without cycloheximide is identical to that of the cycloheximide-induced wild-type strain. Therefore, both laccases must be products of the same structural gene. One may therefore conclude that *lah-1* is not a mutation in the structure gene for laccase itself. Moreover, the *lah-1* mutation is unlikely to be in a *cis*-acting regulatory element of the laccase gene and is likely to be a mutation that affects production of a *trans*-acting element involved in repression of the laccase gene, since this mutation behaved recessively in complementation tests. However, it remains possible that *lah-1* is a mutation in the gene that affects processing of the mRNA or the protein.

Ungerminated conidia and young mycelia of the *lah-1* mutant lacked both laccase activity and immunologically detectable 66-kDa laccase protein. When mycelia were grown, both laccase levels began to increase coincidently. These results suggest that the 66-kDa laccase protein of the *lah-1* mutant is degraded or modified at conidiation and that expression of the 66-kDa enzyme is repressed in conidia. Derepression of synthesis or maturation of this enzyme occurs during the vegetative mycelial growth phase. Therefore, expression of laccase by the *lah-1* mutant is still developmentally regulated. This regulation, in which expression of laccase is repressed at conidiation, is opposite to that of wild-type *Aspergillus nidulans*, in which expression of laccase occurs at conidiation (2, 11). It should be noted that conidia and germinated conidia of both the *lah-1* mutant and

the wild-type strain contained large molecules (Fig. 6) that cross-reacted with anti-laccase serum. These molecules were not found in vegetative mycelia. It is unlikely that they are related to the 66-kDa laccase itself, since their molecular weights are considerably higher than that estimated on the basis of the open reading frame of the cloned laccase gene (7) and they do not possess laccase activity. Accordingly, these molecules are likely to be the proteins whose molecular structure has some homology with the 66-kDa laccase. However, we cannot exclude the possibility that these proteins are inactive forms of laccase with longer carbohydrate chains or other modifications.

The *lah-1* mutant may contribute to the identification and isolation of genes that regulate expression of the laccase gene in *N. crassa*. A possible approach that can be taken is isolation of revertants which suppress the h^+ phenotype of the *lah-1* mutant. Such revertants would be postulated to carry mutations in *cis*- and *trans*-acting genes and mutations in the structural gene itself. We are using this approach in further studies of laccase production in *N. crassa*.

ACKNOWLEDGMENTS

We thank Toshio Ogawa for patient and expert instruction on antiserum generation. We are also grateful to David F. Callen, George R. Hoffmann, and Sadao Ichikawa for critical reading of the manuscript.

This work was supported in part by a grant to H.I. from the Fisheries Agency of Japan.

LITERATURE CITED

1. **Boucherie, H., C. H. Dupont, and J. Bernet.** 1981. Polypeptide synthesis during protoplasmic incompatibility in the fungus *Podospora anserina*. Biochem. Biophys. Acta **653**:18–26.
2. **Clutterbuck, A. J.** 1972. Absence of laccase from yellow-spored mutants of *Aspergillus nidulans*. J. Gen. Microbiol. **70**:423–435.
3. **Davis, R. H., and F. J. de Serres.** 1971. Genetic and microbiological research techniques for *Neurospora crassa*. Methods Enzymol. **17**:79–143.
4. **Froehner, S. C., and K. E. Eriksson.** 1974. Induction of *Neurospora crassa* laccase with protein synthesis inhibitors. J. Bacteriol. **120**:450–457.
5. **Froehner, S. C., and K. E. Eriksson.** 1974. Purification and properties of *Neurospora crassa* laccase. J. Bacteriol. **120**: 458–465.
6. **Germann, U. A., and K. Lerch.** 1986. Isolation and partial nucleotide sequence of the laccase gene from *Neurospora crassa*: amino acid sequence homology of the protein to human ceruloplasmin. Proc. Natl. Acad. Sci. USA **83**:8854–8858.
7. **Germann, U. A., G. Müller, P. E. Hunziker, and K. Lerch.** 1988. Characterization of two allelic forms of *Neurospora crassa* laccase. J. Biol. Chem. **263**:885–896.
8. **Gratzner, H., and D. N. Sheehan.** 1969. Neurospora mutant exhibiting hyperproduction of amylase and invertase. J. Bacteriol. **97**:544–549.
9. **Inoue, H., R. C. Harvey, D. F. Callen, and F. J. de Serres.** 1981. Mutagenesis at the *ad-3A* and *ad-3B* loci in haploid UV-sensitive strains of *Neurospora crassa*. Mutat. Res. **84**:49–71.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
11. **Law, D. J., and W. E. Timberlake.** 1980. Developmental regulation of laccase levels in *Aspergillus nidulans*. J. Bacteriol. **144**:509–517.
12. **Leatham, G. F., and M. A. Stahmann.** 1981. Studies on the laccase of *Lentinus edodes*: specificity, localization and association with the development of fruiting bodies. J. Gen. Microbiol. **125**:147–157.
13. **Lee, D. B., and S. J. Free.** 1984. Isolation and characterization of *Neurospora* mutants affected in invertase synthesis. Genetics **106**:591–599.
14. **Lerch, K., J. Deinum, and B. Reinhammer.** 1978. The state of copper in *Neurospora* laccase. Biochem. Biophys. Acta **534**: 7–14.
15. **Levy, H. B., and H. S. Sober.** 1960. A simple chromatographic method for preparation of gamma globulin. Soc. Exp. Biol. Med. Proc. **103**:250–252.
16. **Marbach, I., E. Harel, and A. M. Mayer.** 1983. Inducer and culture medium dependent properties of extracellular laccase from *Botrytis cinerea*. Phytochemistry **22**:1535–1538.
17. **Marbach, I., E. Harel, and A. M. Mayer.** 1984. Molecular properties of extracellular *Botrytis cinerea* laccase. Phytochemistry **23**:2713–2717.
18. **Mayer, A. M.** 1987. Polyphenol oxidases in plants—recent progress. Phytochemistry **26**:11–20.
19. **Ross, I. K.** 1982. The role of laccase in carpophore initiation in *Coprinus congregatus*. J. Gen. Microbiol. **128**:2763–2770.
20. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
21. **Vogel, H. J.** 1964. Distribution of lysine pathways among fungi: evolutionary implications. Am. Nat. **98**:435–446.