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Purification and characterization of extracellular laccase from *Pleurotus ostreatus*

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Laccase (EC 1.10.3.2) from the culture filtrate of a strain of white rot basidiomycete *Pleurotus ostreatus* was purified using DEAE-Toyopearl 650M and butyl-Toyopearl 650M column chromatographies and Superdex 75 HR 10/30 fast protein liquid chromatography. Molecular weight of the purified laccase was about 55,000, and the isoelectric point was 3.0. The optimum pH for enzyme activity was 6.5, and the optimum temperature was 50°C. This enzyme contained 7.4% sugar and two copper atoms per molecule. The substrate specificity was similar to those of other fungal laccases. Comparison of the N-terminal amino acid sequence of the *P. ostreatus* laccase with those from *Pleurotus ostreatus* Florida, *Coriolus hirsutus*, *Phlebia radiata*, basidiomycete PM1 (CECT 2971), *Trametes villosa*, *Pycnoporus cinnabarinus*, *Ceriporiopsis subvermispora*, and *Agaricus bisporus* showed 95, 65, 60, 55, 55, 55, 50, and 35% similarity, respectively, in the first 20 residues. No similarity in this region was detected with laccases from *Neurospora crassa*, *Aspergillus nidulans*, and *Cryptococcus neoformans*.

Key Words——basidiomycete; laccase; *Pleurotus ostreatus*.

Microorganisms with degradation systems of complex lignocellulosics are potentially applicable for detoxification of various organic compounds that cause environmental pollution. Among the ligninolytic enzymes of basidiomycetes, such as laccase, lignin peroxidase, and manganese peroxidase, we have studied laccases, which are found in major white-rot fungi capable of degrading lignin. Extracellular laccases have been purified and characterized from such fungi as *Trametes versicolor* (Cheung and Marshall, 1969; Fåhraeus and Reinhammar, 1967; Mosbach, 1963; Rogalski et al., 1990), *Ganoderma lucidum* (Kumari and Sirsi, 1972), *Neurospora crassa* (Froehner and Eriksson, 1974), *Agaricus bisporus* (Wood, 1980), *Aspergillus nidulans* (Kurtz and Champe, 1982), *Schizophyllum commune* (de Vries et al., 1986), *Lentinus edodes* (Kofujita et al., 1991), *Panus tigrinus* (Maltseva et al., 1991), *Armillaria mellea* (Rehman and Thurston, 1992), basidiomycete PM1 (CECT 2971) (Coll et al., 1993), *Cryptococcus neoformans* (Williamson, 1994), *Trametes villosa* (Yaver et al., 1996), and *Pycnoporus cinnabarinus* (Eggert et al., 1996). Though these laccases have similar molecular weights and similar substrate specificities, they exhibit considerably different amino acid sequences.

In our diagnostic studies on phenoloxidase (laccase) activities of various edible basidiomycetes, 24 strains of *Pleurotus ostreatus* (*hiratake* in Japanese) all showed remarkably high activities, in agreement with the report on European mushrooms (Bollag and Leonowicz, 1984).

A monokaryotic *P. ostreatus* line, K16-2, which we isolated from the protoplasts of a wild dikaryotic strain in Japan, was found to be one of the most active strains with laccases. In our previous study, we acquired a *Coprinus cinereus* strain with high laccase activity from an original *C. cinereus* strain with no significant activity by insertion of DNA fragments from *P. ostreatus* K16-2 (Okamoto et al., 1995). Subsequently, we have attempted to clone the laccase genes of K16-2 to elucidate the expression mechanism.

Although the laccase from *P. ostreatus* 'Florida' has been purified and characterized (Palmieri et al., 1993), we need to know in further detail the precise nature of *P. ostreatus* laccases to identify the laccase genes. *P. ostreatus* 'Florida', now treated as a variety of *P. ostreatus*, was formerly classified as a different species, *P. florida* (Eder and Wünsch, 1991; Platt et al.,1984). However, comparisons of the similarities and differences in the properties of these strains, especially their enzymes, have not been reported.

In this paper, we purified and characterized the extracellular laccase from *P. ostreatus* K16-2, and compared its properties with those of reported laccases, especially that from *P. ostreatus* 'Florida'.

Materials and Methods

Organisms and cultures *Pleurotus ostreatus* K16-2 is a

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monokaryotic strain screened from the protoplasts of a dikaryotic wild strain K16 collected in Kanagawa prefecture. It was maintained on 1.5% agar slants of a medium containing 1% malt extract (Difco), 0.4% yeast extract (Difco) and 0.4% glucose (MYG medium, pH 6.3). For production of laccase, the mycelium grown on an agar slant was suspended in 9 ml of MYG medium, transferred to a 500-ml Erlenmeyer flask containing 70 ml of MYG medium, and incubated for 8 d at 28°C without shaking.

Purification of laccase All steps were carried out at 4°C. Proteins in 38 L of culture filtrate were precipitated by addition of ammonium sulfate (80% saturation). The precipitate was collected by centrifugation (51,200 g × 20 min) and dissolved in 50 mM potassium phosphate buffer, pH 6.5 (buffer A). The enzyme solution was dialyzed overnight against buffer A, then loaded onto a DEAE-Toyopearl 650M column (3.2 × 43 cm, Tosoh) previously equilibrated with buffer A. The column was washed with buffer A and eluted with 2,000 ml of 0 to 1.0 M linear gradient of NaCl in buffer A at a flow rate of 45 ml/h. Fractions containing laccase activity were collected. Ammonium sulfate was added to the active fractions to a final concentration of 30% saturation, and the solution was loaded onto a butyl-Toyopearl 650M column (1.6 cm × 25 cm) equilibrated with buffer A, containing 30% saturation of ammonium sulfate (buffer B). The column was washed with buffer B, and eluted with 400 ml of 30 to 0% linear gradient of ammonium sulfate in buffer A at a flow rate of 30 ml/h. Fractions with laccase activity were pooled and dialyzed overnight against buffer A, then loaded onto a DEAE-Toyopearl 650M column (2 × 44 cm), previously equilibrated with buffer A. The column was washed with buffer A and eluted with 1,000 ml of 0 to 0.5 M linear gradient of NaCl in buffer A at a flow rate of 25 ml/h. The active fractions (67 ml) were collected and concentrated to 5 ml by ultrafiltration with a Centriprep-3 (3 kDa cut-off, Amicon). The concentrated supernatant was loaded onto a Superdex 75 HR10/30 column equilibrated with buffer A, containing 100 mM NaCl in a fast protein liquid chromatography (FPLC) system (Pharmacia). The elution was carried out repeatedly with buffer A at a flow rate of 45 ml/h. A total of 40 ml of laccase-active fractions were collected and concentrated to 2 ml with a Centricon-3. Finally, the concentrated supernatant was loaded onto a Superdex 75 column again as described above. At this step, laccase activity was eluted as a single peak correspondong to a peak of absorbance at 280 nm. The purified and concentrated enzyme was kept frozen at −20°C without significant loss of activity for several months.

Laccase assay Laccase activity was assayed spectrophotometrically using guaiacol as a substrate. The reaction mixture for the standard assay contained 1 mM guaiacol, 50 mM potassium phosphate buffer (pH 6.5), and enzyme in a total volume of 1.0 ml. One unit of the activity was defined as the amount of enzyme that caused an increase of 1.0 in absorbance at 470 nm per minute at 30°C.

Protein assay Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. During laccase purification after the first anion-exchange chromatography, protein concentration was monitored by the light absorbance at 280 nm.

Electrophoresis Polyacrylamide gel electrophoresis (native-PAGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were done with a 10% gel, as described by Davis (1965) and Laemmli (1970), respectively. Proteins were stained with Coomassie Brilliant Blue G-250 (Quick CBB kit; Wako). Activity staining was carried out by incubating the gel after native-PAGE at room temperature in 50 mM potassium phosphate buffer (pH 6.5) with 1 mM guaiacol. Glycoprotein was assayed by the periodic acid Schiff (PAS) staining method (Leach et al., 1980).

Estimation of molecular mass Molecular mass was estimated by mass spectrometry and gel filtration chromatography. The purified enzyme was dissolved in sinapinic acid solution containing trifluoroacetic acid and CH_3CN, and the sample was applied to a Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) mass spectrometer KOMPACT MALDI I (Shimadzu/Kratos). Gel filtration chromatography was done on a Superdex 75 HR10/30 column (FPLC system, Pharmacia) at a flow rate of 45 ml/h with the LMW protein standard calibration kit (Pharmacia).

Isoelectric focusing An isoelectric focusing gel, pH 4.0–6.5 (Ampholine PAG plate; Pharmacia) was used. Gels were run and stained according to the manufacturer's manual. The isoelectric point of the enzyme was estimated with an isoelectric focusing calibration kit, pH 2.5–6.5 (Pharmacia).

Sugar and copper contents Sugar content of the purified protein was determined by the phenol-sulfuric acid method (Dubois et al., 1956) using glucose as a standard. Copper content was determined with an atomic absorption spectrophotometer (model Z-8270; Hitachi).

Absorption spectrum Absorption spectra of laccase solutions were measured with a spectrophotometer (model UV-2200A; Shimadzu).

Amino acid analysis The purified enzyme was hydrolyzed *in vacuo* at 110°C with conc. HCl, and analyzed with an amino acid analyzer (model L-8500; Hitachi).

Effects of pH and temperature on activity To investigate optimum pH, activity was assayed at various pHs (McIlvaine's buffer, 50 mM, for pHs 3–8) after reaction for 30 min. To investigate optimum temperature, the reaction was performed in 50 mM potassium phosphate buffer, pH 6.5 at various temperatures for 30 min.

Effects of pH and temperature on stability To examine the effect of pH, enzyme was incubated at 30°C for 20 h in various buffers (McIlvaine's, Tris-HCl, and 3-cyclohexylaminopropanesulfonic acid-NaOH, 50 mM, for pHs 3–8, 7–9, and 9–12, respectively) containing 50 μg/ml bovine serum albumin, and the residual activity was assayed. To examine the effect of temperature, the enzyme was incubated at various temperatures for

30 min in 50 mM potassium phosphate buffer, pH 6.5, containing 50 μg/ml bovine serum albumin, and the residual activity was assayed.

Substrate specificity The rate of oxidation of various substrates catalyzed by the purified enzyme was determined by the standard assay, measuring the absorbance increase at 470 nm ($\varepsilon_{470}=1.2\times10^4\ M^{-1}\ cm^{-1}$) for guaiacol, 525 nm ($\varepsilon_{525}=6.5\times10^4\ M^{-1}\ cm^{-1}$) for syringaldazine, 415 nm ($\varepsilon_{415}=3.6\times10^4\ M^{-1}\ cm^{-1}$) for ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)], 460 nm ($\varepsilon_{460}=1.2\times10^4\ M^{-1}\ cm^{-1}$) for L-tyrosine, and 310 nm ($\varepsilon_{310}=9.3\times10^3\ M^{-1}\ cm^{-1}$) for veratryl alcohol. Michaelis constants (K_m) were calculated from the Lineweaver-Burk (Lineweaver and Burk, 1934) plots.

Determination of N-terminal amino acid sequence Ten micrograms of the purified protein was blotted on a polyvinylidine difluoride membrane (Nippon Bio-rad Laboratories) previously washed with methanol. The membrane was washed again with methanol. Proteins were sequenced with a protein sequencer PSQ-1 (Shimadzu) with the PSQ-1 standard program version 2.0. Phenylthiohydantoin amino acid derivatives were separated by use of a reverse-phase column (Wakosil PTH, 4.6× 250 mm; Wako) and identified.

Results and Discussion

Production of laccase Higher laccase activity was found in the medium of static cultures than in those of shaken cultures of *P. ostreatus* K16-2. The time course of laccase activity in static cultures with MYG medium is shown in Fig. 1. The laccase activity paralleled mycelial growth in the early phase of culture phase, and peaked at day 8, before the mycelial growth reached the stationary phase. The culture filtrate of day 8 was used as a source of laccase for purification.

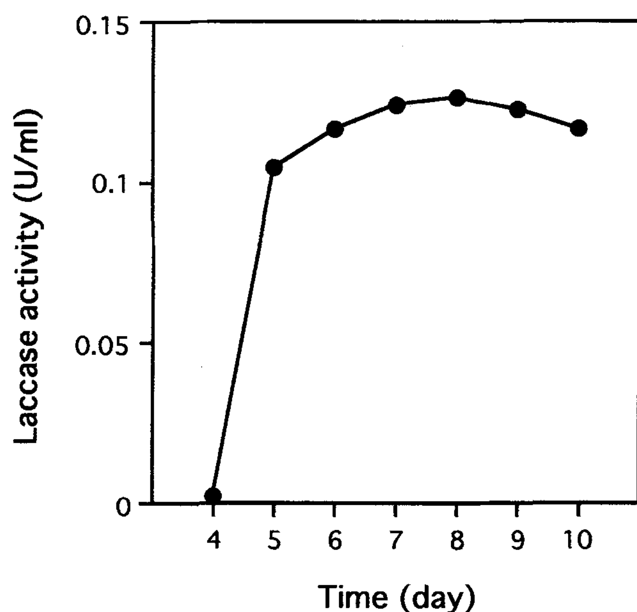

Fig. 1. Laccase activity in a liquid culture of *P. ostreatus* K16-2.

Purification and structural properties of laccase The purification of laccase consisted of six steps, including ammonium sulfate precipitation, and anion exchange, hydrophobic, and gel filtration chromatographies. Hydrophobic chromatography on a butyl-Toyopearl 650M column proved to be effective for removing large amounts of pigment from the enzyme solution. The target protein peak (A_{280}) was then fractionated by the subsequent anion exchange chromatography. Finally, the activity was eluted as a single peak that corresponded to a peak in the absorbance at 280 nm in the second gel filtration. The purified enzyme showed single protein band on native-PAGE. This band coincided with the band stained for activity in a gel run simultaneously (Fig. 2).

Many laccases have been reported to be excreted extracellularly as several isozymes (Fåhraeus and Reinhammar, 1967; Cheung and Marshall, 1969; Morohoshi et al., 1987; Rehman and Thurston, 1992; Coll et al., 1993). However, the laccase of *P. ostreatus* K16-2 appeared as a single activity peak during all chromatography steps. One reason might be that K16-2 is a monokaryotic strain. The purification procedure is summarized in Table 1. The yield of purified enzyme was 23% in activity, and the specific activity was purified 1,500-fold.

An attempt to estimate the molecular mass of the purified protein by SDS-PAGE failed because the protein did not migrate normally as a single band, even upon changing the denaturing conditions, such as heat treatment, or the concentrations of mercaptoethanol and sodium dodecyl sulfate. This phenomenon seems to be typical of glycoproteins in SDS-electrophoresis (Shirahama et al., 1974; Leach et al., 1980; Wood, 1980; Germann, 1988). When the electrophoresed gel was stained by the PAS method, a glycoprotein band was found. Sugar

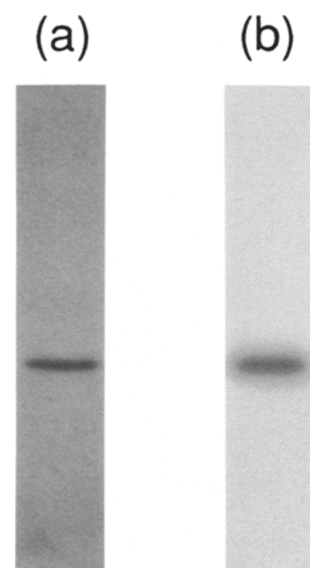

Fig. 2. Polyacrylamide gel electrophoresis of purified laccase from *P. ostreatus* K16-2. (a) stained for protein (CBB); (b) stained for activity (guaiacol).

Table 1. Summary of purification of laccase from *P. ostreatus* K16-2.

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Culture filtrate	4,400	21,000	0.2	—	100
Ammonium sulfate	4,100	10,000	0.4	2	93
1st DEAE-Toyopearl	2,900	900	3.2	16	66
butyl-Toyopearl	2,300	190	12	60	52
2nd DEAE-Toyopearl	1,800	18	100	500	41
1st Superdex	1,300	4.9	270	1,300	30
2nd Superdex	1,000	3.4	290	1,500	23

content of the purified enzyme, determined by the phenol-sulfuric acid method, was 7.4%. Most of the extracellular laccases isolated from fungi were reported to be glycoproteins (Fähraeus and Reinhammar, 1967; Wood, 1980; Germann et al., 1988; Coll et al., 1993; Perry et al., 1993; Eggert et al., 1996; Yaver et al., 1996).

The molecular mass of the purified enzyme was measured by MALDI-TOF mass spectrometry and Superdex gel-filtration. By mass spectrometry, the protein gave an abundant $[M+H]^+$ ion at *m/z* 55,034 (Fig. 3). The molecular weight estimated by gel-filtration was about 56,000. These results indicated that laccase from *P. ostreatus* was monomeric in structure. The isoelectric point was about 3.0, as determined by isoelectric focusing.

The concentrated enzyme fractions in the purification step after the first gel filtration chromatography showed a blue color, which suggested the presence of the type I copper. Difference absorption spectrum of the *P. ostreatus* laccase showed a peak at 610 nm, typical of type I copper, which is responsible for the deep blue color of the enzyme (Hanna et al., 1988). The enzyme contained 2.7 μg copper per mg enzyme, determined by atomic absorption spectrometry, which corresponded to 2.3 copper atoms per molecule. This copper content was similar to that of the laccases from *Agaricus bisporus* (Wood, 1980) and *Schizophyllum commune* (de Vries et al., 1986).

As shown in Table 2, the amino acid composition of the purified enzyme exhibited high contents of Asp(+Asn), Ala, Gly, and Pro. In this feature of amino acid composition, the enzyme was similar to the laccases from other fungi, such as *Polyporus versicolor* (Fähraeus

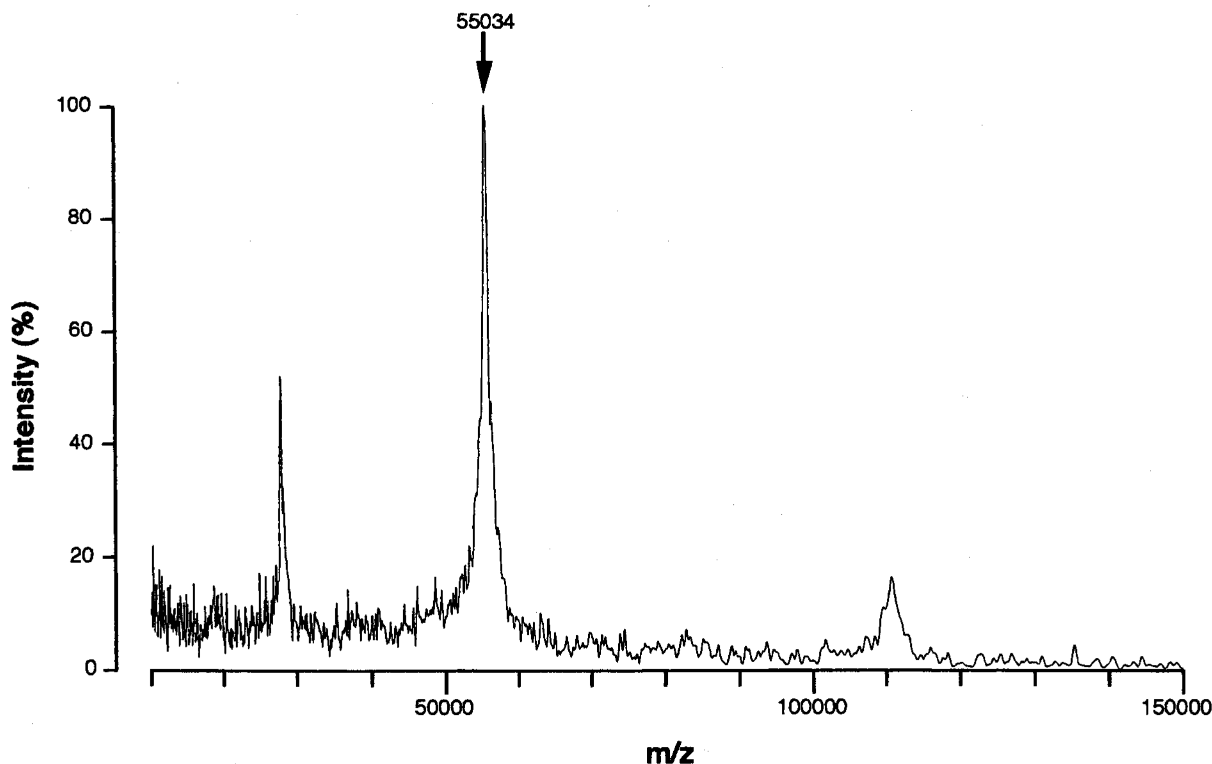

Fig. 3. Mass spectrum of purified laccase from *P. ostreatus* K16-2.

Table 2. Amino acid composition of laccase from *P. ostreatus* K16-2.

Amino acid	Amount (mol %)
Aspartic acid / Asperagine	14.33
Threonine	7.77
Serine	7.92
Glutamic acid / Glutamine	5.52
Proline	9.16
Glycine	8.84
Alanine	9.22
Cysteine	0.73
Valine	6.97
Methionine	0.92
Isoleucine	5.95
Leucine	7.78
Tyrosine	2.84
Phenylalanine	5.04
Lysine	1.40
Histidine	2.65
Tryptophan	ND[a]
Arginine	2.94

[a] ND, not detected.

and Reinhammar, 1967), *Neurospora crassa* (Froehner and Eriksson, 1974), and *Agaricus bisporus*.

Functional properties, kinetics, effects of metal ions and inhibitors The optimum pH for enzyme activity was 6.5 in 50 mM McIlvaine's buffer. At pH 5 and pH 8, the enzyme exhibited approximately 59% and 27% of maximum activity, respectively. The optimum temperature for activity was 50°C during 30-min assay, and the activity was approximately 1.5-fold higher than at 30°C. After incubating the enzyme in several buffers at 30°C for 20 h, the residual activity was assayed. The enzyme was stable in the pH range from 9 to 11 (Fig. 4A). After incubation for 30 min at 30, 40, 50, 60, and 70°C, the residual activities were measured under the standard conditions. The enzyme was stable (maintained more than 85% of maximum activity) below 50°C and inactivated at 70°C (Fig. 4B).

The effects of metal ions and inhibitors on the laccase activity are shown in Table 3. Ca^{2+}, Co^{2+}, Mg^{2+}, Zn^{2+}, PCMB, and iodoacetic acid did not affect the enzyme activity, whereas Ag^{2+}, Fe^{2+}, Hg^{2+}, EDTA, and thioglycolic acid showed varying inhibitory effects. In addition, the activity was slightly inhibited by Cu^{2+}, though the enzyme is a copper-containing protein. This phenomenon was also reported in the laccase from *Lentinus edodes* (Kofujita et al., 1991). The excess supply of Cu^{2+} ions might cause the change of the laccase structure in some groups.

The enzyme oxidized the typical substrates of laccase, of which syringaldazine was oxidized at the highest rate calculated from molar extinction coefficient and K_m. Tyrosine and veratryl alcohol were not oxidized. This result clearly proves that the purified enzyme is a laccase. The K_m values for syringaldazine, ABTS, and guaiacol as the substrate were 3, 15, and 180 μM, respectively. Those reported for *P. ostreatus* 'Florida' were 15, 280,

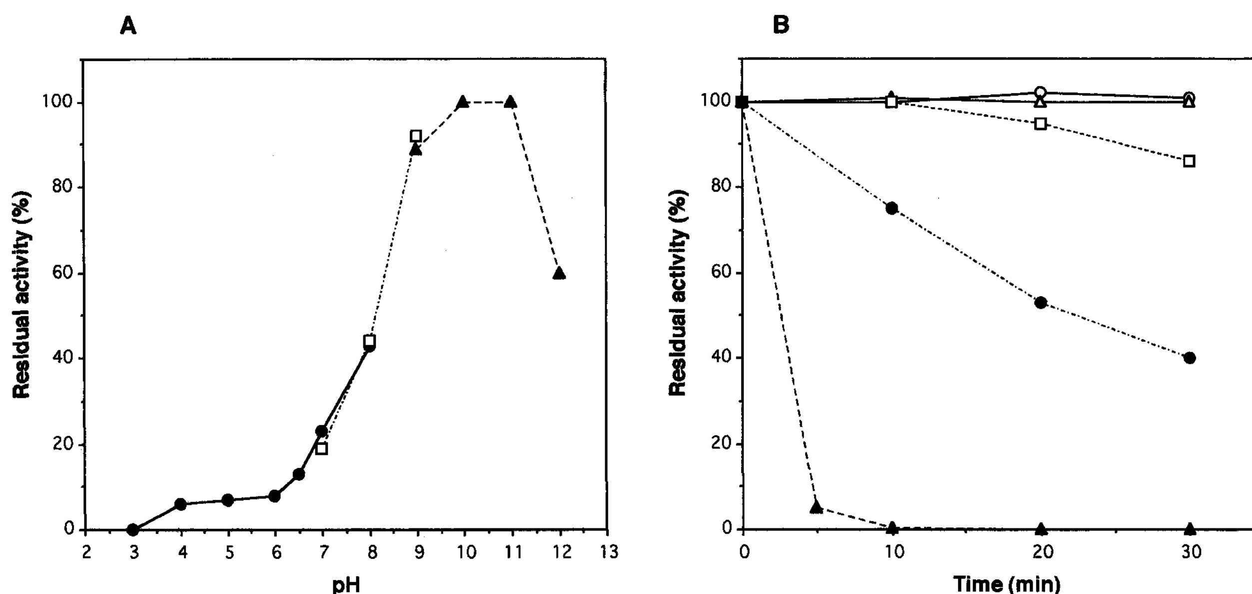

Fig. 4. Effect of pH and temperature on stability of laccase. (A) pH stability. The purified enzyme was incubated for 20 h at 30°C in various buffers, McIlvaine's for pH 3 to 8 (●), Tris-HCl for pH 7 to 9 (□), and CAPS-NaOH for pH 9 to 12 (▲), containing 50 μg/ml bovine serum albumin. Residual activity was expressed as the percentage of the activity of the untreated control, taken as 100%. (B) Thermal stability. The purified enzyme in 50 mM potassium phosphate buffer (pH 6.5) containing 50 μg/ml bovine serum albumin was incubated at each temperature, 30°C (○), 40°C (△), 50°C (□), 60°C (●), and 70°C (▲), for various intervals. Residual activity was expressed as the percentage of the activity of the untreated control.

Table 3. Effects of metal ions and inhibitors on the enzyme activity.

Metal ions and inhibitors (1 mM)	Relative activity (%)
None	100
$MgCl_2$	110
$CaCl_2$	100
$FeCl_2$	4
$HgCl_2$	6
$AgNO_3$	2
$CuCl_2$	80
$CuSO_4$	85
$ZnCl_2$	110
$CoCl_2$	100
EDTA[a]	55
Thioglycolic acid	0
PCMB[b]	100
Iodoacetic acid	100

a) EDTA: ethylenediaminetetraacetic acid.
b) PCMB: *p*-(chroromercuri)benzoic acid.

Source of enzyme	N-terminal amino acid sequence
Pleurotus ostreatus K16-2	A I G P T G N M Y I V N E D V S P D G F
Pleurotus ostreatus 'Florida'	A I G P A G N M Y I V N E D V S P D G F
Coriolus hirsutus	A I G P T A D L T I S N A E V S P D G F
Phlebia radiata	S I G P V T D F H I V N A A V S P D G F
Basidiomycete PM1	S I G P V A D L T I S N G A V S P D G F
Trametes villosa	G I G P V A D L T I T N A A V S P D G F
Pycnoporus cinnabarinus	A I G P V A D L T L T N A A V S P D G F
Ceriporiopsis snbvermispora	A I G P V T D L E I T D A F V S P D G P
Agaricus bisporus	D T K T F N F D L V N T R L A P D G F
Neurospora crassa	G G G G G C N S P T N R Q C W S P G F N
Aspergillus nidulans	M Y L S T V L F P L L A L N L G L S H A
Criptococcus neoformans	M R G L A K L F F L S C S F V S L V S S
	5 10 15 20

Fig. 5. Comparison of N-terminal amino acid sequences of the purified laccase with those from several fungi. Identical amino acid residues are boxed.

and 650 μM, respectively. These results show that the purified enzyme of *P. ostreatus* K16-2 is slightly different from that of *P. ostreatus* 'Florida' (Table 4).

N-terminal analysis and comparison with other laccases The N-terminal amino acid sequence of the purified laccase from *P. ostreatus* K16-2 was Ala-Ile-Gly-Pro-Thr-Gly-Asn-Met-Tyr-Ile-Val-Asn-Glu-Asp-Val-Ser-Pro-Asp-Gly-Phe. These 20 residues were compared with the data reported for laccases from other fungi, as shown in Fig. 5. The highest homology of 95% was observed with the laccase from *P. ostreatus* 'Florida' (Palmieri, 1993). Similarity was also found with the laccases from other basidiomycetes, such as *Coriolus hirsutus* (65% homology) (Kojima et al., 1990), *Phlebia radiata* (60%) (Saloheimo et al., 1991), basidiomycete PM1 (CECT 2971) (55%) (Coll et al., 1993), *Trametes villosa* (55%) (Yaver et al., 1996), *Pycnoporus cinnabarinus* (55%) (Eggert et al., 1996), *Ceriporiopsis subvermispora* (50%) (Fukushima and Kirk, 1995), and *Agaricus bisporus* (35%) (Perry et al., 1993). In contrast, there was no homology with the N-terminal sequences of laccases from *Neurospora crassa* (Germann et al., 1988), *Aspergillus nidulans* (Aramayo and Timberlake, 1990), and *Cryptococcus neoformans* (Williamson, 1994). In comparison with the N-terminal amino acid sequences of reported laccases, the laccase from *P. ostreatus* K16-2 clearly conserves the character of basidiomycetes, especially in the sequence I-G-P and V-S-P-D-G-F. It is remarkable that Japanese wild *P. ostreatus* strain K16-2 and *P. ostreatus* 'Florida' differ by only one amino acid in this sequence, though the enzymes differ somewhat in their properties (Table 4).

Table 4. Comparison of properties of laccase from *P. ostreatus* K16-2 and *P. ostreatus* 'Florida'.

Properties	K16-2	Florida[a]
Molecular Weight	55,034	59,000
Thermostability	<50	<60
pH stability	pH 6–10	pH 6–7
Active optimum at pH	50°C	50°C
Active optimum at temperature	pH 6	pH 6
Sugar content (%)	7.4	—
Copper content (per molecule)	2 atoms	—
K_m values		
Syringaldazine	3 μM	15 μM
ABTS	15 μM	280 μM
Guaiacol	180 μM	650 μM
Effect of metal ions (%)		
1 mM $CuCl_2$	80	—
1 mM $CuSO_4$	85	—
Effect of inhibitors (%)		
1 mM EDTA	55	—
1 mM Thioglycolic acid	0	—
1 mM PCMB	100	—
1 mM Iodoacetic acid	100	—

a) Palmieri et al., 1993.

To our knowledge, there have been no reports on laccase from wild strains of *P. ostreatus* collected in Japan. Here we have reported the detailed characterization of such a purified enzyme and its comparison with other laccases described previously. To determine the laccase homologies between *P. ostreatus* and *Coprinus cinereus* and develop a method for high level production of laccase, molecular cloning of the enzyme from *P. ostreatus* is now being undertaken.

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