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NOTE

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Purification of a novel extracellular laccase from solid-state culture of the edible mushroom *Lentinula edodes*

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Abstract The laccases (EC 1.10.3.2) secreted into solid-state culture by *Lentinula edodes* were analyzed. The fungus secreted at least two laccases in the solid-state culture. One laccase was purified to a homogeneous preparation using anion-exchange, hydrophobic, and size-exclusion chromatography. SDS-PAGE analysis showed that the purified laccase, Lcc6, was a monomeric protein of 58.5 kDa. The optimum pH for enzyme activity was about 3.5, and the laccase was most active at 40°C. The N-terminal amino acid sequence of Lcc6 did not correspond to the sequence of Lcc1, which was previously purified from *L. edodes*. Lcc6 had decolorization activity to some chemical dyes.

Key words Bioremediation · Laccase · *Lentinula edodes* · Lignin degradation

Many white-rot fungi secrete various isoforms of extracellular lignin-degrading enzymes, such as laccases (Lccs), manganese peroxidases (MnPs), and lignin peroxidases (LiPs) during the vegetative mycelial growth stage (Gold and Alic 1993). Generally, Lccs occur as a series of isozymes encoded by a family of genes. Many Lcc isozymes have been purified, and their encoding genes have been cloned from various white-rot basidiomycetes (Chen et al. 2004; Hoegger et al. 2004; Baldrian 2006). *Lentinula edodes* (Berk.) Pegler (Shiitake) is an important edible mushroom in Japan. Preparations of this fungus on potato dextrose agar plates have shown strong Lcc activity with no lignin peroxidase and weak MnP activities (Morisaki et al. 2001). Recently, Lccs have been attracted wide attention because of their ability to degrade phenolic compounds (Ullah et al. 2000; Fukuda et al. 2001; Schultz et al. 2001). The Lcc produced by this mushroom is advantageous for environmental bioremediation because the fungus is readily available, the mushroom and enzyme are edible and safe for human consumption, and the waste culture after harvesting the mushrooms can be used as an enzyme source.

We have purified two Lccs from *L. edodes* (Nagai et al. 2002, 2003). Although both Lccs have decolorization activity to several chemically different dyes (Nagai et al. 2004), the role of the two Lccs in lignin degradation was unclear. Lcc1 was purified from liquid culture that contained no lignin, and Lcc2 was an intracellular enzyme. Lccs secreted into solid-state culture that contains sawdust or lignin might be more useful for bioremediation than Lcc1 and Lcc2, because these two Lccs were thought to have an important role in lignin degradation. However, there are few studies of Lccs produced on solid-state culture. In this study, we purified and characterized an Lcc secreted by *L. edodes* into a solid-state culture that contained sawdust.

First, we analyzed the Lccs produced by three strains of *L. edodes* cultivated in solid-state culture. The commercially available dikaryotic strain of *L. edodes* Hokken 600 (H600; Hokken, Tochigi, Japan), an experimental dikaryotic strain SR-1 (Iwate Biotechnology Research Center, Iwate, Japan), and the wild-type strain (isolated in Yamada, Iwate, Japan) were used in this experiment. Mycelia were maintained in 0.25× MYPG agar medium containing 0.25% Bacto malt extract (Difco, Detroit, MI, USA), 0.1% Bacto yeast extract (Difco), 0.1% tryptone peptone (Difco), 0.5% glucose, and 1.5% agar. The solid-state culture was prepared with oak wood sawdust (10 mesh pass and 30 mesh on) and the nutrient supplement Baideru (Hokken). Specifically, 2.5 kg oak wood, 370 g Baideru, and 4.8 l water were mixed thoroughly; 20 g of the mixture was spread into a Petri dish (9 cm diameter) and autoclaved. As an inoculum, mycelial blocks (7 mm diameter) were cut from a 0.25

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× MYPG agar plate culture. One mycelial block was put on the solid-state culture plate, and the plates were then incubated at 25°C for 2 weeks. After incubation, the culture was mixed well, and the crude enzyme was extracted from 3 g of this mixture by adding 30 ml 10 mM sodium phosphate buffer (PB, pH 6.0) for 12 h at 4°C. After centrifugation (12 000 *g*, 10 min), the supernatant was dialyzed against PB and loaded onto a Mono Q HR 5/5 column (5 × 50 mm, Amersham Pharmacia Biotech, Uppsala) that had previously been equilibrated with PB. After the column was washed with PB, the adsorbed proteins were eluted by applying a linear concentration gradient of NaCl (20 ml, 0–500 mM) to the column at a flow rate of 0.5 ml/min. Lcc activity was analyzed by the method using 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Sigma Japan, Tokyo, Japan) as a substrate, described previously (Nagai et al. 2002).

The elution profiles of the crude enzymes from the three strains are shown in Fig. 1. Two Lcc peaks were observed for all three strains, which suggested that *L. edodes* produced at least two kinds of Lcc on this solid-state culture. We previously purified one Lcc (Lcc1) from the culture filtrate of *L. edodes* that had been cultivated on an MYPG liquid culture. Because Lcc1 was the only Lcc secreted into MYPG medium, some Lcc inducers may be present in this solid-state culture. The elution time of the second peak off the Mono Q column corresponded to Lcc1. Therefore, the first Lcc eluted off the column was characterized in this article.

We tested the distribution of the unknown Lcc on the solid-state culture using SR-1 strain. The mycelium was inoculated on the solid-state culture plate as already described. After a suitable incubation period, the culture from the center of the plate was cut into 5-mm-square pieces and the crude enzyme solutions were extracted with 10 times the volume of PB. Figure 2 shows the mycelial growth and the distributions of the Lcc and the protease activity on the culture after 15 days of incubation. The acid protease activity was analyzed by the method of Terashita et al. (1997) using Hammarsten casein (Nacalai Tesque, Kyoto, Japan) as the substrate. Analysis of mycelial growth and the bands of Lcc activity revealed a pattern of concentric circles. Throughout the incubation period, strong Lcc activity was detected at the near the tips of the mycelia. Lcc activity decreased after 17 days of incubation when the tips of the mycelia reached the edge of the plate (see Fig. 2). As shown in Fig. 2, acid protease activity was also detected on the culture. The protease activity of the culture continued to increase throughout the incubation periods (24 days). Thus, we speculated that the protease might be involved in the degradation of Lccs. Therefore, the crude enzyme was extracted from an Lcc-active area of the culture plate after 15 days of incubation so that the Lcc could be purified.

To purify Lcc, 60 plates were required. The cultures, which included the Lcc-active area from the SR-1 strain, were recovered from the plates and the crude enzyme was extracted. The crude enzyme solution (3 l) was concentrated to 200 ml using a stirred ultrafiltration cell (model 8400; Amicon, Beverly, MA, USA) and an ultrafiltration

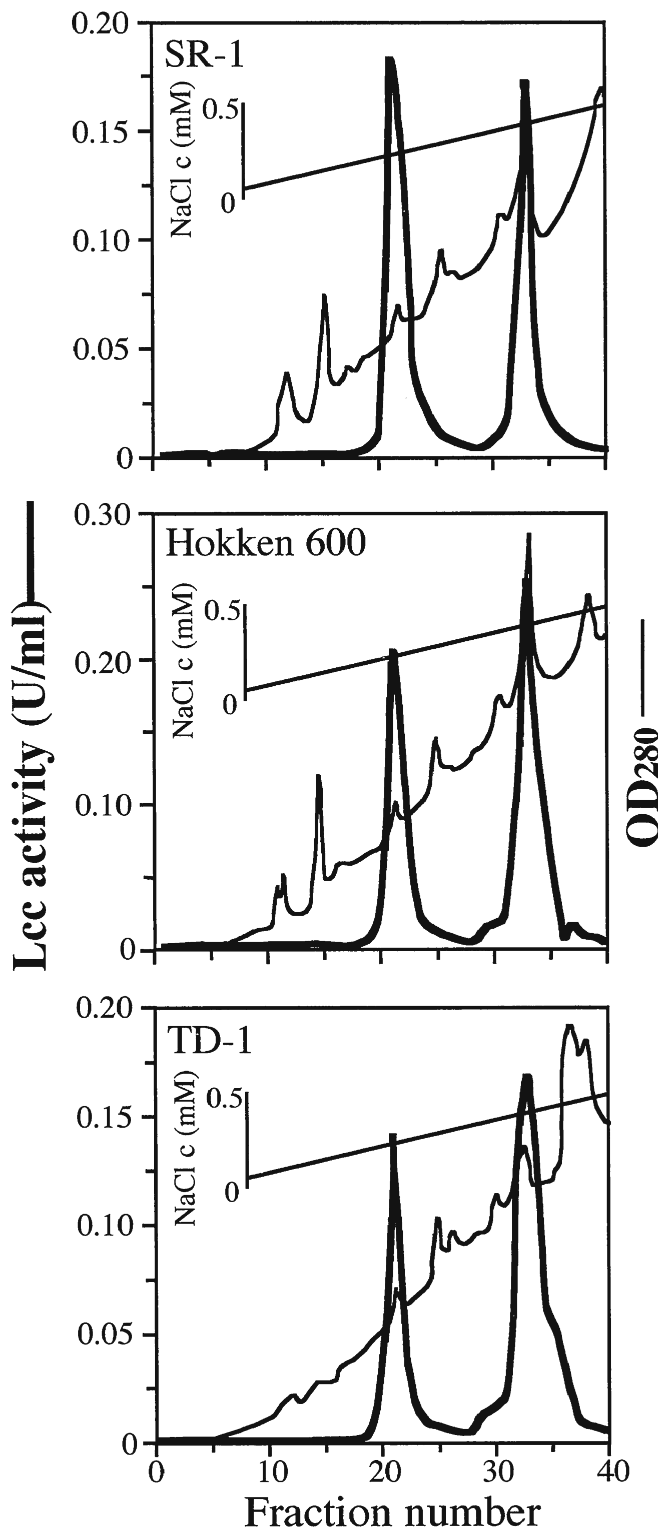

Fig. 1. Elution profiles of laccases from solid-state cultures of *Lentinula edodes*. The crude extracts from the sawdust cultures of three strains were applied to Mono Q column chromatography. *H600*, the commercially available dikaryotic strain; *SR-1*, an experimental dikaryotic strain; *TD-1*, the wild-type strain

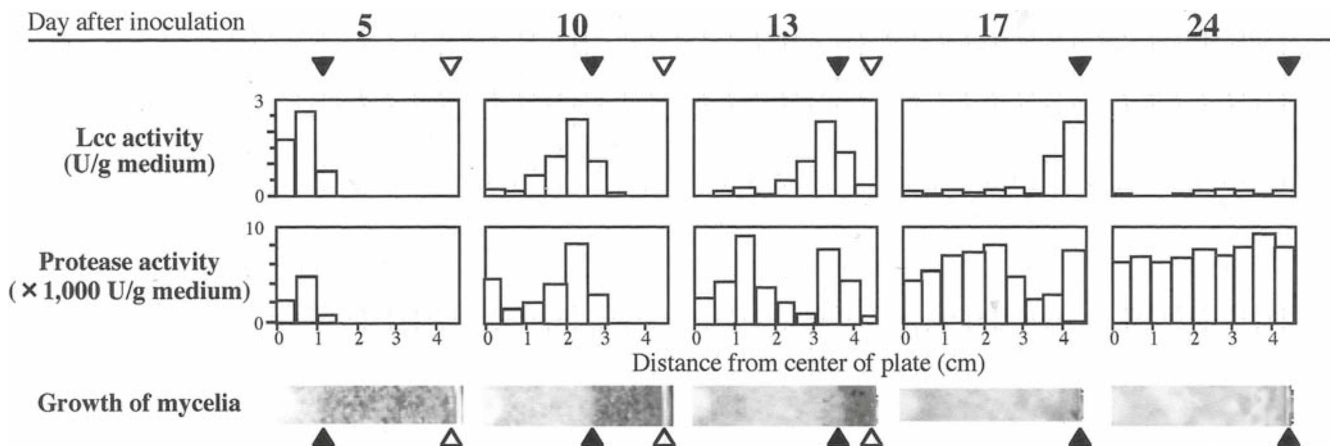

Fig. 2. Laccase and protease production on a solid-state culture from the SR1 strain of *Lentinula edodes*. Mycelial growth and distribution of the Lcc and protease activity on the solid-state culture are shown. *Open* and *closed arrowheads* indicate the edge of the plates and the tips of hyphae, respectively

Table 1. Purification of laccase (Lcc)6

Purification step	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Purification (fold)	Yield (%)
Culture filtrate	18 100	922	0.0510	–	100
Ultrafiltration	13 300	728	0.0547	1.07	79.0
TOYOPEARL DEAE-650M	37.6	86.4	2.30	45.1	9.37
TOYOPEARL Butyl-650M	1.50	55.5	37.0	725	6.01
Superdex 75 HR 10/30	0.285	36.3	127	2490	3.93
Mono Q HR 5/5	0.148	23.0	155	3040	2.49

Lcc activity was measured at pH 4.0

membrane with a molecular weight cutoff of 10 kDa (Millipore, Billerica, MA, USA). The concentrated solution was dialyzed against PB and loaded onto a TOYOPEARL DEAE-650 M column (25 × 100 mm; Tosoh, Tokyo, Japan) equilibrated with PB. After the column was washed with PB, the adsorbed proteins were eluted by a linear concentration gradient of NaCl (400 ml, 0–500 mM) at a flow rate of 1.5 ml/min. The Lcc-active fractions were pooled. Powdered ammonium sulfate was added to the enzyme solution to make it 40% saturated, and the resultant precipitate was removed by centrifugation at 12 000 *g* for 20 min. The supernatant was applied to a TOYOPEARL Butyl-650 M column (10 × 50 mm; Tosoh) equilibrated with PB containing 40% saturated ammonium sulfate. The column was washed with the same buffer, and adsorbed proteins were eluted by a linear concentration gradient of ammonium sulfate (40 ml, 40%–0% saturation) in PB at a flow rate of 0.5 ml/min. The Lcc-active fractions were pooled and concentrated to a volume of about 250 μl with Centriprep-30 and Centricon-30 ultrafiltration tubes with a molecular weight cutoff of 30 kDa (Amicon). The concentrated enzyme solution was applied to a Superose 12 HR 10/30 column (1.0 × 30 cm; Amersham Pharmacia Biotech) equilibrated with PB containing 100 mM NaCl. The enzyme was eluted with the same buffer at a flow rate of 250 μl/min. Finally, the fractions including Lcc activity were pooled, dialyzed against PB, and applied to a Mono Q HR 5/5 column (5 × 50 mm; Amersham Pharmacia Biotech) equilibrated with PB. After the column was washed with PB, the adsorbed proteins were eluted by a linear concentration gradient of NaCl (20 ml, 0–500 mM) at a flow rate of 0.5 ml/min.

The steps for purification of the enzyme are summarized in Table 1. The procedure yielded 148 μg purified enzyme from 3 l crude enzyme solution, and the total recovery of Lcc activity was 2.5%. The purified Lcc (which we designate as Lcc6) appeared as a single protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using methods described previously (Nagai et al. 2002) (Fig. 3). The molecular mass of Lcc6 was 58.5 kDa by SDS-PAGE and 48.8 kDa by gel filtration on Superdex 75. These results suggest that the enzyme is composed of a monomeric protein.

Lcc6 activity was tested against ABTS, and the maximum activity occurred at pH 3.5. The optimum temperature of Lcc6 was determined at pH 3.5 and was found to be 40°C. Purified Lcc6 was very unstable. The thermal stability of Lcc6 was assayed by incubating the enzyme at pH 6.0 for 30 min; Lcc6 was stable at this pH at 4°C. The activity of Lcc6 remained completely intact after incubation at 15°C, but there was a 10% or 76% loss of activity when Lcc6 was incubated at 20°C and 30°C, respectively. Some reports suggest that the extracellular enzyme purified from fungi is unstable (Rios et al. 1993; Diaz et al. 1996). Iwashita et al. (2001) reported that some extracellular enzymes from *Apsergillus kawachii* were stabilized by extracellular poly-

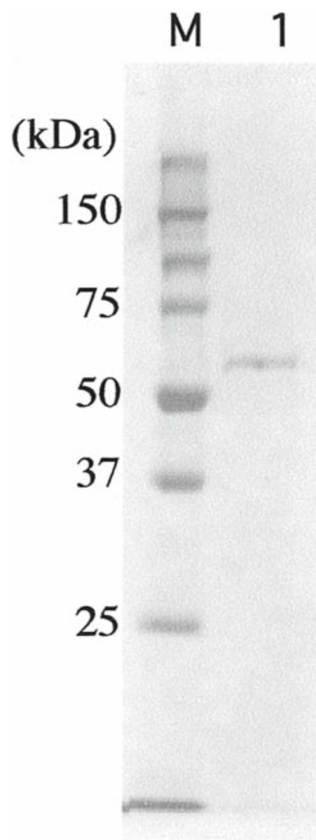

Fig. 3. SDS-PAGE of purified laccase. Purified Lcc6 stained with Coomassie brilliant blue (CBB). Lanes: *M*, molecular weight marker; *1*, purified laccase

saccharides produced by this fungus in solid-state culture but that these enzymes were made unstable by the purification process. There may be some stabilizer for Lcc6 that exists in the solid-state culture of *L. edodes* but separates from the enzyme during purification. The results shown in Fig. 2 suggest that Lcc6 may have been rapidly degraded by some protease in the solid-state culture, which may explain the instability of Lcc6 between cultivations.

To determine the N-terminal amino acid sequence of Lcc6, the purified Lcc6 (100 pmol) was loaded onto an SDS-PAGE gel. After electrophoresis, the protein was transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore) by electroblotting with a semidry blotter (AE-6677 HorizBlot; ATTO, Tokyo, Japan) and then stained with Coomassie brilliant blue R-250 (Wako, Osaka, Japan). The region on the PVDF membrane that contained the Lcc6 was excised and analyzed by an HP G1005A Protein Sequencing System (Hewlett-Packard, Palo Alto, C, USA). The N-terminal amino acid sequence of Lcc6 was AIGPVTDLHVVNKFIQP. The sequence showed high homology to the sequence of Lcc1 (AIGPVTDLHIVNSFIQP) but was not identical to Lcc1. The molecular mass of Lcc6 was smaller than that of Lcc1, and the optimum pH of the two Lccs was different. Based on these findings, we speculated that Lcc6 is one of the Lcc isozymes of *L. edodes*. The genetic analysis of Lcc6 is currently underway.

The substrate specificity of Lcc6 was similar to that of Lcc1. Lcc6 oxidized ABTS, *p*-phenylendiamine, pyrogallol, guaiacol, 2,6-dimethoxyphenol, and catechol, but not veratryl alcohol, tyrosine, or β-(3,4-dihydroxyphenyl) alanine. We reported that Lcc1 had decolorization activity to several chemically different dyes (Nagai et al. 2002). Therefore, we tested the decolorization activity of Lcc6 against several chemically different dyes by measuring the decrease in the maximum absorbance of each dye. Dye concentration in the reaction mixture was adjusted to 1.0 absorbance unit at the maximum wavelength in the visible spectrum (Table 2). Units for dye decolorization activity were estimated as the change in absorbance per minute (ΔA/min) at 30°C at each optimum pH, as described by previously (Nagai et al. 2004). Lcc2, the intracellular Lcc of *L. edodes* that have been extracted and purified from the fruit body (Nagai et al. 2003), was also used in this experiment. The results are shown in Table 2. Lcc6 was showed decolorization activity against some dyes, but the activity was relatively low compared to Lcc1 and Lcc2.

Table 2. Decolorization activity of the laccases

Dye (nm)	Lcc1		Lcc2		Lcc6	
	pH	U/mg	pH	U/mg	pH	U/mg
RBBR (600)	4.0	307	4.0	60.0	4.0	21.4
NBB (618)	4.0	59.2	N.D.	–	4.0	6.13
BPB (600)	4.0	74.0	4.0	4.04	4.0	1.26
CBB (560)	4.0	21.9	4.0	4.60	4.0	1.32

RBBR, Remazol Brilliant Blue R; NBB, Naphtol Blue Black; BPB, Bromophenol Blue; CBB, Coomassie Brilliant Blue R-250; N.D., not detected
Numbers in parentheses shows the maximum wavelength (γ_{max}) in the visible spectrum absorbance of each dye

In this article, we showed the existence of two Lccs in the solid-state culture of *L. edodes*, and we purified the novel Lcc, Lcc6, that was produced in the solid-state culture which included oak wood and that may be involved in lignin degradation. Because this enzyme was not produced in MYPG liquid culture, the expression of Lcc6 may be regulated by inducers such as lignin. In Japan, shiitake mushrooms are mainly produced by sawdust cultivation. Considering that Tsujiyama (2003) had reported that one of the lignin monomer, vanillin, induced Lcc production, these commercial sawdust cultures might include Lcc6 and Lcc1. The decolorization activity of Lcc6 suggests that waste cultures after mushroom harvesting would be useful as a source of enzyme for the bioremediation of phenolic substrate as dyes. Investigations into the Lcc activity of the waste cultures and the factors that regulate Lcc production by *L. edodes* are currently underway.

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