Important role of fungal intracellular laccase for melanin synthesis: purification and characterization of an intracellular laccase from Lentinula edodes fruit bodies

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A laccase (EC 1.10.3.2) was isolated from the fully browned gills of Lentinula edodes fruit bodies. The enzyme was purified to a homogeneous preparation using hydrophobic, cation-exchange and size-exclusion chromatography. SDS-PAGE analysis showed the purified laccase, Lcc 2, to be a monomeric protein of 58-0 kDa. The enzyme had an isoelectric point of around pH 6-9. The optimum pH for enzyme activity was around 3-0 against 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diamentium salt (ABTS), and it was most active at 40°C and stable up to 50°C. The enzyme contained 8·6% carbohydrate and some copper atoms. The enzyme oxidized ABTS, p-phenylenediamine, pyrogallol, guaiacol, 2,6-dimethoxyphenol, catechol and ferulic acid, but not veratryl alcohol and tyrosine. β-(3,4-Dihydroxyphenyl)alanine (L-DOPA), which was not oxidized by a laccase previously reported from the culture filtrate of L. edodes, was also oxidized by Lcc 2, and the oxidative product of L-DOPA was identified as L-DOPA quinone by HPLC analysis. Lcc 2 was able to oxidize phenolic compounds extracted from fresh gills to brown-coloured products, suggesting a role for laccase in melanin synthesis in this strain.

INTRODUCTION

The post-harvest preservation or mishandling during picking of Lentinula edodes fruit bodies causes a brown surface discolouration. This gill browning is commercially undesirable since it causes an unpleasant appearance and the concomitant development of an off-flavour, and it is considered to be due to melanin biosynthesis as a result of a stress response. Melanin is known to protect fungi from environmental stresses, such as UV radiation, elevated temperatures, antimicrobial agents and lytic enzymes (Bell & Wheeler, 1986). In general, fungal melanins are classified into four types: β-(3,4-dihydroxyphenyl)alanine (DOPA) melanin derived from tyrosine, γ-glutaminyl-3,4-dihydroxybenzene (GDHB) melanin derived from γ-glutaminyl-4-hydroxybenzene (GHB), catechol melanin derived from catechol and dihydroxynaphthalene (DHN) melanin derived from pentaketide (Bell & Wheeler, 1986). In all cases, these phenolic compounds are oxidized enzymically to quinones, which polymerize by non-enzymic means to form the melanin pigments. Oxidation of these phenolic compounds is commonly catalysed by tyrosinase (Tyr; EC 1.14.18.1).

The mechanisms of mushroom browning have been investigated extensively in Agaricus bisporus (Burton, 1998; Espíñ et al., 1999). Browning in this species is mainly due to DOPA and GDHB melanins (Jolivet et al., 1998), and Tyr seems to play the most important role in their synthesis (Turner, 1974). Burton (1988) reported that epidermal tissues of A. bisporus had a greater activity of non-latent Tyr and a greater concentration of phenols than did the flesh. Previously, we also reported that Tyr activity of L. edodes fruit bodies increased during post-harvest storage and that gill browning increased with increasing Tyr activity (Kanda et al., 1996a).

Laccases (Lcc; EC 1.10.3.2), catalyse the single-electron oxidation of phenols or aromatic amines to form different products via various pathways. Recently, Castro-Sowinski et al. (2002) reported that a strain of Sinorhizobium meliloti with an intracellular Lcc synthesized different types of melanin compared with strains without this Lcc. Ikeda et al. (2002) also reported a correlation between melanin synthesis and intracellular Lcc in Cryptococcus neoformans. The melanins of Aspergillus conidia are formed from L-DOPA and DHN, and an Lcc gene of Aspergillus nidulans is specifically expressed in the conidia (Aramayo & Timberlake, 1990). In addition, Clutterbuck (1972)

Abbreviations: ABTS, 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt; DHN, dihydroxynaphthalene; L-DOPA, β-(3,4-dihydroxyphenyl)alanine; GDHB, γ-glutaminyl-3,4-dihydroxybenzene; GHB, γ-glutaminyl-4-hydroxybenzene; Lcc, laccase; Tyr, tyrosinase; PB, 10 mM sodium phosphate buffer.
showed that yellow-spored mutants of *Aspergillus nidulans* are deficient in Lcc. Although there are many reports dealing with extracellular Lccs produced by white-rot basidiomycetes (Leonowicz et al., 2001), there are few studies of the intracellular Lccs produced by these fungi (Burke & Cairney, 2002; Schlosser et al., 1997; Roy-Arcand & Archibald, 1991). In *A. bisporus*, the biological significance of intracellular Lccs is considered to be very limited because of their low levels (Turner, 1974), but their significance remains unclear.

In this paper, we continue in our attempt to clarify the relationship between gill browning and Lcc activity in *L. edodes* through the isolation and characterization of an Lcc from the mature gills and through the comparison of some properties of the purified enzyme with those of an extracellular Lcc (Lcc 1) purified previously from *L. edodes* (Nagai et al., 2002). To our knowledge, this is the first report of the purification of intracellular Lcc from a basidiomycete.

**METHODS**

**Chemicals.** Unless otherwise stated, all chemicals were certified reagent grade purchased from Wako Pure Chemicals. Lcc 1, an extracellular Lcc, was purified from the culture filtrate of *L. edodes* SR-1 as described by Nagai et al. (2002). Tyr was purified from the gills of *L. edodes* strain Hokken 600 (H 600) as described by Kanda et al. (1996b).

**Organisms and culture conditions.** A commercial dikaryotic strain of *L. edodes*, strain H 600, was obtained from Hokken Sango and was used throughout this study. Mycelia were maintained on 1.5 % agar plates (diam. 90 mm) with 0.25 x YM medium containing 0.25 % Bacto malt extract (Difco), 0.1 % Bacto yeast extract (Difco), 0.1 % tryptone peptone (Difco) and 0.5 % glucose.

For production of fruit bodies, mycelia were cultivated for 50 days in sawdust medium containing 3.7 kg sawdust, 1.3 kg Baidera (a nutrient supplement for mushroom production; Hokken Sango) and 7.6 l water according to the method of Matsumoto (1988). Fruit bodies were harvested immediately after the veil had broken.

**Preparation of crude extract from fruit bodies.** Fruit bodies were separated into caps (pigmented rind and flesh), stipes and gills and frozen by liquid nitrogen. The frozen tissue was suspended in 10 times its volume of 10 mM sodium phosphate buffer (PB), pH 6.0, and homogenized using an Excel Auto Homogenizer (Nihon Seiki) at 10 000 r.p.m. for 1 min. The homogenate was centrifuged at 12 000 g for 20 min, after which the supernatant was collected as the crude extract.

**Purification of Lcc from the browned gills.** All steps were carried out at 4 °C. Column chromatography was operated with a gradient controller (AC-5900 GradiCon II; ATTO) or with an FPLC system (Pharmacia).

A crude extract was prepared from 40 g sliced, fully browned gills. Powdered ammonium sulfate was then added to the extract to achieve 30 % saturation and the resulting precipitate was removed by centrifugation at 12 000 g for 20 min.

The supernatant was applied to a TOYOPEARL Butyl-650 M (Tosoh) column (25 x 80 mm) equilibrated with PB containing 30 % saturated ammonium sulfate. The column was washed with the same buffer and adsorbed proteins were eluted by a linear concentration gradient of ammonium sulfate (300 ml, 30–0 % saturation) in PB, at a flow rate of 2 ml min-1. The fractions containing Lcc activity were collected, dialysed against 20 mM sodium acetate buffer, pH 4.0, and applied to a TOYOPEARL CM-650 M (Tosoh) column (10 x 50 mm) equilibrated with 20 mM sodium acetate buffer, pH 4.0. After washing the column with the same buffer, the adsorbed proteins were eluted by a linear concentration gradient of NaCl (40 ml, 0–500 mM) at a flow rate of 1 ml min-1. The Lcc active fractions were pooled and concentrated to about 250 μl by ultrafiltration using a Centricon-30 concentrator (30 kDa cut-off; Amicon). The concentrated enzyme solution was applied to a Superdex 75 HR 10/30 column (1 x 30 cm; Pharmacia) equilibrated with PB containing 100 mM NaCl. The enzyme was eluted with the same buffer at a flow rate of 250 μl min-1.

Fractions exhibiting Lcc activity were pooled and dialysed against PB, and powdered ammonium sulfate was added to achieve 20 % saturation. The enzyme solution was then applied to a Phenyl Superose HR 5/5 column (5 x 50 mm, Pharmacia) equilibrated with PB containing 20 % saturated ammonium sulfate. After washing the column with the same buffer, the final elution was with a linear concentration gradient of ammonium sulfate (20 μl, 20–0 % saturation) in PB, at a flow rate of 500 μl min-1.

**Enzyme assay.** To determine Lcc activity, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma) was used as the substrate. The reaction mixture for the standard assay contained 1 mM ABTS, McIlvaine buffer (pH 3–0) and the enzyme solution in a total volume of 100 μl. After incubation at 30 °C for 20 min, the reaction was stopped by adding 100 μl 5 % trichloroacetic acid. The formation of the cation radical was detected by measuring the absorbance increase at 420 nm (ε270=36 000 M–1 cm–1). One unit of Lcc activity was defined as the amount of enzyme that catalysed the oxidation of 1 μmol ABTS in 100 μl reaction mixture at 30 °C in 1 min. Tyr activity was measured at pH 6–0 using 1 mM catechol as the substrate. The formation of the cation radical was detected by measuring the absorbance increase at 450 nm (ε270=2211 M–1 cm–1). One unit of Tyr activity was defined as the amount of enzyme that catalysed the oxidation of 1 μmol catechol in 100 μl reaction mixture at 30 °C in 1 min.

**Protein assay.** Protein concentration was measured with BCA Protein Assay Reagent (Pierce) using BSA (Sigma) as the standard. During Lcc purification steps, protein concentration was monitored spectrophotometrically by A280.

**Electrophoresis.** Native PAGE was performed according to the method of Davis (1965) using a 5–20 % polyacrylamide gradient gel (NPG-520L PAGEL; ATTO) and Premixed 10 × Tris/Glycine Buffer (Bio-Rad). SDS-PAGE was performed according to the method of Laemmli (1970) using a 10 % polyacrylamide gel (NPU-10L PAGE; ATTO) and Premixed 10 × Tris/Glycine/SDS Buffer (Bio-Rad). The samples were boiled in 2 % SDS and 5 % 2-mercaptoethanol for 10 min and then applied to the gel. The isoelectric point (pl) of the enzyme was measured in an isoelectric focusing gel between pH 3.5 and 9.5 (Ampholine PAG plate; Pharmacia) and Multiphor II system (Pharmacia) using an isoelectric focusing calibration kit, pH 3.5–9.3 (Pharmacia). Proteins were stained with Coomassie brilliant blue R 250 (PAGE Blue 83; Daichi Chemicals). Activity staining was carried out by incubating the gel after native PAGE at room temperature in Mcllvaine buffer (pH 3–0) with 1 mM ABTS.

**Estimation of molecular mass.** The molecular mass of the enzyme was estimated by two methods: (1) gel filtration on a Superdex 75 HR 10/30 column with Gel filtration standards (Bio-Rad); and (2) SDS-PAGE as described above with Precision Protein Standards (Bio-Rad). The molecular mass of the enzyme was calculated from the mobility versus molecular mass plots of the marker proteins.
Determination of carbohydrates. Carbohydrate molecules in the purified Lcc were determined by endoglycosidase treatment. The purified enzyme (1 µg) was boiled with 5 % 2-mercaptoethanol for 10 min, then incubated with 5 mM Endoglycosidase-H (Roche Diagnostics) in PB at 37°C for 16 h. After this treatment, the molecular mass of the protein was calculated by SDS-PAGE.

Effect of pH and temperature on Lcc activity and stability. The effect of pH on Lcc activity was examined at pH values from 1·0 to 6·0, using 0·1 M KCl/HCl buffer at pH values from 1·0 to 2·0, 0·1 M Glycine/HCl buffer at values from 2·0 to 4·0 and McIlvaine buffer at values from 4·0 to 6·0.

The effect of pH on enzyme stability was investigated by measurement of the activity remaining after incubation for 16 h at 30°C in various buffers with 50 µg BSA ml⁻¹. The buffers were 0·1 M KCl/HCl (pH 1·0–2·0), 0·1 M Glycine/HCl (2·0–4·0), McIlvaine buffer (4·0–6·0), 0·1 M sodium acetate (5·0–7·0), 0·1 M Tris/HCl (7·0–8·0) and CAPS (8·0–10·0). The effect of temperature on enzyme activity was determined at pH 3·0, with reactions performed by incubating at each temperature and pH 3·0 for 10 min. The thermal stability of Lcc was investigated by incubating preparations in PB with 50 µg BSA ml⁻¹ for 30 min at various temperatures. After incubation, the activity remaining was determined.

Substrate specificity. Spectrophotometric measurement of substrate oxidation by purified Lcc was carried out in a 100 µl reaction mixture containing the test substrates in McIlvaine buffer (pH 3·0–6·0). Activity against ABTS, p-phenylenediamine, 2,6-dimethoxyphenol, catechol, guaiacol, ferulic acid and L-DOPA was assayed at concentrations of between 0·1 and 1 mM. Activity against pyrogallol was assayed between 1 and 10 mM. All reactions were conducted at 30°C for 10 min. The rate of substrate oxidation was determined by measuring the absorbance increase, with the molar extinction coefficient (ε) obtained from the literature (Eggert et al., 1996; Shin & Lee, 2000). Michaelis constants (Km) were calculated from Lineweaver–Burk plots at the optimum pH in each case.

The oxidative products of L-DOPA and catechol were also analysed by HPLC. The analysis was carried out using a reverse phase HPLC cartridge and a TSK gel ODS-8TM (150 mm x 4·6 mm i.d.; Tosoh), radially compressed by a separations module (Waters 2960) at 25°C. The mobile phases (flow rate 1 ml min⁻¹) consisted of 5 ml PIC B8 (Waters 84283) in 1 1·0·05 % acetic acid for L-DOPA, and CH₃CN and 5 % acetic acid (12·88) for catechol. Detection was performed between 220 and 400 nm with a photodiode array detector (Waters 996) connected to a Millennium Chromatography Manager (Waters).

In vitro gill browning experiments. Gills (4 g) were cut from the fruit bodies of L. edodes, frozen and homogenized in an Excel Auto Homogenizer at 10000 r.p.m. for 1 min with 10 ml McIlvaine buffer, pH 4·0. Tyr (0·1 U ml⁻¹), Lcc 1 (0·4 U ml⁻¹) or Lcc 2 (0·4 U ml⁻¹) was added to 80 µl of the supernatant of the homogenate and the reaction mixture was incubated at 30°C for 60 min. An absorbance increase at 400 nm, showing the synthesis of L-DOPA quinone, was measured at the end of the incubation period.

RESULTS

Purification of Lcc 2

We tested Lcc activity to study the production of Lcc during post-harvest preservation. Fruit bodies were preserved in a desiccator at 25°C and 80 % humidity. Some brown spots appeared on the gills after 2 days preservation, and after 3 days gills were coloured dark brown (Fig. 1a).

Lcc activities of the gill, cap and stipe increased over the preservation period. The highest Lcc activity was obtained in the homogenate of gills preserved for 4 days (Fig. 1b), and Lcc was therefore purified from gills at this stage.

Before enzyme purification, the enzyme stability was tested at 4°C for 16 h. The Lcc was stable at a pH range from 3·0 to 7·0. Thus, the purification was done at this pH range. Enzyme yields during purification steps are summarized in Table 1. The procedure yielded 604 µg of the purified enzyme from 40 g gill tissue, and recovery of total Lcc activity was 23·5 %. The purified Lcc, which we designate as Lcc 2, showed as a single protein band on native PAGE and was identified by having the same location as the band stained for activity in a gel run simultaneously (Fig. 2a).

Homogeneity, molecular mass, spectroscopy and pl

The purified enzyme appeared as a single band in SDS-PAGE (Fig. 2b). The molecular mass of Lcc 2 was estimated as 58 kDa by SDS-PAGE and 53 kDa by gel filtration. These
results suggest that the enzyme is a monomeric protein. When the enzyme was treated with Endoglycosidase-H, a clear and smaller (53 kDa) protein band was obtained (data not shown), indicating that Lcc 2 was a glycoprotein with 8·6% glycosylation. Spectrophotometric analysis of Lcc 2 showed a peak absorption at around 610 nm, typical for a type-I copper signal, with a shoulder at 320 nm, typical for a type-II binuclear copper signal (data not shown; Hanna et al., 1988). Isoelectric focusing indicated that Lcc 2 had a pI of around 6·9.

Effect of pH and temperature

The pH profile for Lcc 2 activity against ABTS showed a single peak of maximum activity at pH 3·0 (Fig. 3a). When the effect of pH on enzyme stability was tested at 30°C for 16 h, the enzyme was stable over a pH range of 4·0–7·0 (Fig. 3b). The optimum temperature of Lcc 2, determined at pH 3·0, was 40°C (Fig. 4a). The thermal stability of Lcc 2 was determined by incubating the enzyme at pH 6·0 (Lcc 2 was stable at 30°C for 16 h at this pH) for 30 min. No loss of activity was observed after incubation at 50°C, but incubation at 60°C resulted in a 33% loss of Lcc activity (Fig. 4b). Thus, we concluded that Lcc 2 is stable up to 50°C.

Effects of metal ions

The effects of metal ions on Lcc activity were tested using ABTS as the substrate (Table 2). The enzyme was strongly inhibited by 1 mM Hg²⁺ (32·9% inhibition) and 1 mM Sn²⁺ (31·1% inhibition), and slightly by 1 mM Mn²⁺ (16·7% inhibition). Neither 1 mM nor 10 mM Cu²⁺ affected its activity.

### Table 1. Purification of Lcc 2

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units mg⁻¹)</th>
<th>Purification (-fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>3080</td>
<td>783</td>
<td>0·254</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>30% Ammonium sulfate fraction (sup.)</td>
<td>2770</td>
<td>730</td>
<td>0·264</td>
<td>1·04</td>
<td>93·2</td>
</tr>
<tr>
<td>TOYOPEARL Butyl-650M</td>
<td>217</td>
<td>703</td>
<td>3·24</td>
<td>12·8</td>
<td>89·8</td>
</tr>
<tr>
<td>TOYOPEARL CM-650M</td>
<td>4·24</td>
<td>246</td>
<td>58·0</td>
<td>228</td>
<td>31·4</td>
</tr>
<tr>
<td>Superdex 75 HR 10/30</td>
<td>0·977</td>
<td>210</td>
<td>215</td>
<td>846</td>
<td>26·8</td>
</tr>
<tr>
<td>Phenyl Superose HR 5/5</td>
<td>0·604</td>
<td>184</td>
<td>305</td>
<td>1200</td>
<td>23·5</td>
</tr>
</tbody>
</table>

Fig. 2. (a) Native-PAGE and (b) SDS-PAGE of purified laccase (Lcc 2). (a) Lanes: 1, purified Lcc 2 (1 µg) stained with Coomassie brilliant blue; 2, purified Lcc 2 (1 µg) stained for activity with ABTS. (b) Lanes: M, molecular mass markers; 1, purified Lcc 2 (1 µg) stained with Coomassie brilliant blue.

Fig. 3. Effect of pH on enzyme (a) activity and (b) stability. (a) The enzyme reaction was performed at 30°C for 10 min. (b) Activity remaining was measured after incubation with 50 µg BSA ml⁻¹ at various pH values at 30°C for 16 h. Buffers: open circles, 0·1 M KCl/HCl; closed circles, 0·1 M Glycine/HCl; open triangles, Mcllvaine; closed triangles, 0·1 M sodium acetate; open squares, 0·1 M Tris/HCl; closed squares, CAPS.
Substrate specificities of two Lccs from *L. edodes*

Specificities of purified Lcc 2 for various substrates were determined at the optimum pH for each substrate and compared with those of Lcc 1. As shown in Table 3, the conventional substrates of Lccs, such as ABTS, guaiacol and 2,6-dimethoxyphenol, were oxidized by Lcc 2. Both enzymes showed relatively low \( K_m \) values for these substrates. \( K_{cat}/K_m \) values were also determined. As shown in Table 3, significant differences between Lcc 1 and Lcc 2 were found in the activities against 2,6-dimethoxyphenol, catechol, ferulic acid and L-DOPA.

L-DOPA, which is the precursor for DOPA melanin synthesis, was not oxidized by Lcc 1 (Nagai *et al.*, 2002), but was oxidized by Lcc 2. Spectrophotometric analysis of the products of Tyr- or Lcc 2-mediated oxidation of L-DOPA and catechol showed identical patterns of absorbance spectra (data not shown). These products of L-DOPA and catechol were also analysed by HPLC. The oxidative products of L-DOPA had retention times of 2:86 min (by Tyr) and 2:87 min (by Lcc 2, Fig. 5). Fig. 6 shows the HPLC profiles of catechol oxidation products mediated by Tyr and Lcc 2. Oxidation by Tyr resulted in three peaks at 3:24, 5:58 and 6:89 min, and that by Lcc 2 resulted in three peaks at 3:23, 5:57 and 6:86 min. All corresponding peaks obtained by Tyr and Lcc 2 reactions showed identical patterns of absorbance spectra (data not shown). Thus, we concluded that Tyr and Lcc 2 produced the same oxidative products of L-DOPA and catechol.

Table 3. Substrate specificities of two purified Lccs from *L. edodes*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Optimum pH*</th>
<th>( K_m ) (mM)</th>
<th>( K_{cat}/K_m )</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Lcc 1</td>
<td>Lcc 2</td>
<td>Lcc 1</td>
</tr>
<tr>
<td>ABTS</td>
<td>4:0</td>
<td>3:0</td>
<td>0:108</td>
</tr>
<tr>
<td>Guaiacol</td>
<td>4:0</td>
<td>4:0</td>
<td>0:917</td>
</tr>
<tr>
<td>2,6-Dimethoxyphenol</td>
<td>4:0</td>
<td>3:0</td>
<td>0:557</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>ND</td>
<td>4:0</td>
<td>–</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>5:0</td>
<td>5:0</td>
<td>2:86</td>
</tr>
<tr>
<td>Catechol</td>
<td>4:0</td>
<td>3:0</td>
<td>2:24</td>
</tr>
<tr>
<td>p-Phenylenediamine</td>
<td>5:0</td>
<td>4:0</td>
<td>0:256</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>4:0</td>
<td>3:0</td>
<td>0:417</td>
</tr>
<tr>
<td>Veratryl alcohol</td>
<td>ND</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>ND</td>
<td>ND</td>
<td>–</td>
</tr>
</tbody>
</table>

*ND, Not detected.

\( ^\d K_{cat} (10^{-3} \times \text{mol min}^{-1} \text{ mol}^{-1}) \).
In vitro gill browning by Lcc 2

The results of the in vitro gill browning experiments by purified enzymes are shown in Fig. 7. When gill extracts were incubated with Lcc 1 or without enzymes, the colour changed only slightly. However, Lcc 2 and Tyr clearly browned the gill extract. During the early stages of incubation, 0.4 U Lcc 2 ml⁻¹ browned the extract faster than 0.1 U Tyr ml⁻¹ and the absorbance increase in the presence of Lcc 2 reached a maximum (about 0.3) at 30 min and changed little thereafter. Ultimately, Tyr was more effective in causing browning, with a maximum absorbance of about 0.65. When both enzymes were added to the same reaction mixture, an early synergistic effect was observed.

DISCUSSION

Generally, Lccs of basidiomycetes are secreted extracellularly from the vegetative mycelia, increase during fruiting and decrease when the fruit bodies mature (Wood, 1980; Leonard, 1971; Leonard & Phillips, 1973). Ohga et al. (2001) reported that the level of Lcc gene transcripts in L. edodes was maximal during the mycelial growth stage and then rapidly decreased at the fruiting stage. Thus, it is thought that extracellular Lccs degrade lignin and have a role in nutrient uptake to support the developing fruit bodies. Lcc activity in the fruit bodies of L. edodes was investigated by Leatham & Stahmann (1981) who observed highest activity in the pigmented rind. Zhao & Kwan (1999) studied the expression of two Lcc genes of L. edodes and showed that both of these genes (lac1 and lac2) were highly expressed in the caps and that high Lcc activity was detected from the caps when the blocks of fresh fruit bodies were used as enzyme source. These results suggest that Lcc also has a role in pigment synthesis in the rind of caps. We speculated that the synthesis of melanin in the gill during post-harvest preservation (gill browning) might be catalysed...
by Lcc 2 as well as by Tyr. As shown in Fig. 1, Lcc activity of the gill homogenate increased over the preservation period and was proportional to the intensity of gill browning. This result corresponds to that of Tyr activity reported previously (Kanda et al., 1996a).

The purified intracellular Lcc, Lcc 2, was characterized and compared with an extracellular Lcc, Lcc 1, previously purified from the culture filtrate of \textit{L. edodes}. The molecular mass of Lcc 2 (58 kDa) was lower than Lcc 1 (72-2 kDa) and other Lccs so far characterized from basidiomycetes (Thurston, 1994). The molecular masses of both Lccs after treatment with endoglycosidase-H were also determined. The molecular mass of deglycosylated Lcc 2 (53 kDa) was lower than that of Lcc 1 (55 kDa). The isoelectric point (pI) of Lcc 2 (pH 6-9) was higher than that of Lcc 1 (around pH 3-0). To our knowledge, the pI values of most Lccs secreted in culture media are acidic (Eggert et al., 1996; Dedeyan et al., 2000; Galhaup et al., 2002; Saparrat et al., 2002), and the pI of Lcc 2 is the highest of all Lccs reported previously, except for an Lcc from \textit{Coriolus hirista} (pH 7-4; Shin & Lee, 2000). Thus, the neutral pI of Lcc 2 might be very specific. Although the N-terminal amino acid sequence of Lcc 2 was analysed by the methods described previously (Nagai et al., 2002), the clear sequence was not detected because the N terminus was probably blocked. The facts that the molecular mass of the deglycosylated Lcc 2 was lower than that of Lcc 1 and that the ion charge of the surface of these proteins was different, suggest that the two Lccs are encoded individually.

When the effect of metal ions on enzyme activity was tested, 10 mM Cu$^{2+}$ activated Lcc 1, but not Lcc 2 (Table 2; Nagai et al., 2002). The absorption spectrum of Lcc 1 showed a peak absorption at around 610 nm, suggesting the existence of type-I copper ions. However, no secondary peak absorption at around 310 nm was observed to indicate a type-II copper signal. Thus, we speculated that the activation of Lcc 1 by Cu$^{2+}$ may be due to the filling of type-II copper-binding sites with copper ions (Nagai et al., 2002). In this study the absorption spectrum of Lcc 2 showed a peak for a type-II copper signal as well as the principle peak at 610 nm indicating a type-I copper signal.

When substrate specificities of Lcc 2 were tested, the optimum pH of Lcc 2 for each substrate was different (Table 3). Such differences have been observed by several authors and have been ascribed to variable degrees of substrate protonation under different pH conditions (D’Annibale et al., 1996; Fukushima & Kirk, 1995). The optimum pHs of Lcc 2 for many substrates were lower than those of Lcc 1. Schlosser et al. (1997) have also reported that the optimum pH of the intracellular Lccs from \textit{Trametes versicolor} is lower than that of the extracellular Lccs. In addition, they have reported that the substrate specificity of the Lcc contained in the culture filtrate was different from the Lcc contained in the crude extract of the mycelia.

The $K_m$ values of both enzymes for typical Lcc substrates – ABTS, $p$-phenylene diamine, 2,6-dimethoxyphenol, ferulic acid or guaiacol – were similar, but those of Lcc 2 for catechol and L-DOPA (1·72 and 1·22 mM, respectively), were noticeably lower than those of Lcc 1 (22·4 mM for catechol and zero oxidation for L-DOPA). Also, the activities ($K_{cat}/K_m$ values) of Lcc 2 against catechol and L-DOPA were significantly higher than those of Lcc 1. Because dihydroxyphenols are known to be oxidized by Tyr for synthesis of melanin \textit{in vivo} (Bell & Wheeler, 1986), we analysed the activities of Tyr and Lcc 2 for oxidation of L-DOPA and catechol. As shown in Figs 5 and 6, both enzymes oxidized L-DOPA and catechol to generate the same products. Both enzymes generated three products from catechol. It is established that catechol is oxidized by Tyr to \textit{o}-benzoquinone and three types of semiquinone radical, and that these quinones are polymerized to catechol dimers \textit{in vivo} (Bell & Wheeler, 1986). The three peaks obtained by HPLC analysis in the present study probably correspond to these quinones or catechol dimers. Thus, Lcc 2 appears to oxidize L-DOPA and catechol to the same products as generated by Tyr.

Lcc 2 had oxidative activity for the phenolic compounds extracted from the gill of \textit{L. edodes} fruit bodies (Fig. 7). The slight browning that occurred during incubation of gill extracts without enzymes may have been caused by native enzyme activity or by non-enzymic oxidation reactions. Although the addition of Lcc 1 to the extract did not effect browning, addition of Lcc 2 and Tyr did, suggesting that the phenolic compounds (perhaps especially dihydroxyphenols) in the gills of \textit{L. edodes} fruit bodies can be oxidized by Lcc 2 but not by Lcc 1. Because the maximum absorbance change was obtained by the addition of Tyr, we speculated that Tyr can oxidize a wider variety of compounds than Lcc 2. However, increased oxidation following the addition of Lcc 2 to the Tyr reaction mixture, especially in the early stages, suggests either synergistic interactions between Tyr and Lcc 2 or perhaps the existence of phenolic compounds which can be oxidized by Lcc 2 but not Tyr. In this study, the reactions were carried out with a ratio of 4 Lcc 2 : 1 Tyr. The corresponding enzyme ratio in gill homogenates prepared from the fruit bodies of \textit{L. edodes} preserved for 3 days was 22 : 1 (data not shown). Thus, we conclude that Lcc 2 has an important role in gill browning.

Jolivet et al. (1995) reported that the amount of phenols (tyrosine, GHB and GDHB) in the epidermal tissue was related to susceptibility to browning in \textit{A. bisporus}. Kanda et al. (1996a) showed a dependency of gill browning in \textit{L. edodes} on \textit{de novo} synthesis of a latent-type Tyr and increasing Tyr activity. Our results suggested that not only Tyr, but also an intracellular Lcc (Lcc 2) is produced during post-harvesting preservation and functions in the oxidation of phenolic compounds to turn gills brown. Considering the fact that purified Lcc 2 oxidized both L-DOPA and catechol, Lcc 2 might have a strong activity against dihydroxyphenols. Investigations into the phenol
content of the gills of L. edodes fruit bodies are currently under way. We are also analysing the Lcc 2 gene with a view to its repression or disruption to construct a strain which can be preserved for longer periods without gill browning.

REFERENCES


