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ORIGINAL PAPER

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Purification and characterization of laccase isozymes from the white-rot basidiomycete *Ganoderma lucidum*

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Abstract *Ganoderma lucidum*, a medicinal white-rot basidiomycete, produces many laccase isozymes in liquid culture. Three laccase isozymes (GaLc 1, 2, 3) have been purified 32.4-fold from the crude enzyme protein through anion exchange chromatography, preparative gel electrophoresis, and electroelution. Their estimated molecular weights are 65–68 kDa, and they contain 7–10% N-linked carbohydrates. The three isozymes have identical N-terminal amino acid sequences: G-I-G-P-T. The optimum pH and temperature both for each isozyme singly and the isozyme mixture are pH 3.5 and 20°C, respectively. One isozyme (GaLc 3) is quite stable at pH 4.0–10.0, and shows good stability when incubated at temperatures lower than 40°C. The K_m values of GaLc 3 for *o*-tolidine and 2,2'-azino-bis-(3-ethylthiazoline-6-sulfonate) (ABTS) are 401.6 μM and 3.7 μM respectively, and the V_{max} of GaLc 3 for these substrates is 0.0198 OD $min^{-1}unit^{-1}$ and 0.0142 OD $min^{-1}unit^{-1}$, respectively.

Introduction

White-rot fungi are very interesting eukaryotic microorganisms, not only because they can grow on dead trees by degrading cellulose and lignins, but also because they go through a fascinating developmental process: formation of the fruit body. Lignin degradation is important in carbon recycling and/or recalcitrant degradation (Field et al. 1992; Leonowicz et al. 1999), and fruit body formation is a very good model system to study regulation of gene expression during developmental stages (Wessels 1992; Horton et al. 1999). Several white-rot fungi, such as *Pleurotus ostreatus* and *Lentinula edodes*, are edible and are very important in the food market. The medicinal mushroom *Ganoderma lucidum*, which is also a white-rot fungus, has primarily been investigated for its pharmaceutical components (el-Mekkawy et al. 1998; Wasser and Weis 1999), while other basic research has been relatively poorly reported. In recent years, two papers on the molecular physiology of *G. lucidum* have been published, describing PCR cloning of a laccase gene fragment (D'Souza et al. 1996) and laccase isozyme induction (D'Souza et al. 1999).

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multicopper blue oxidases, which are widely distributed in many plants and fungi. Laccases catalyze the oxidation of a range of inorganic and aromatic substances by the removal of electrons with the concomitant reduction of O_2 to water (Youn et al. 1995; Xu 1996). Laccases are regulated during fungal development (Ross 1982; Choi 1987), and show diverse functions in different fungi such as controlling pathogenicity in *Cryptococcus neoformans* (Salas et al. 1996), and conidial pigment synthesis in *Aspergillus nidulans* (Clutterbuck 1972). Laccases from white-rot fungi are intimately involved in lignin- and recalcitrant-degradation (Roy-Arcand and Archibald 1991; Rodriguez et al. 1999). Therefore, there are reports on the purification and biochemical characterization of fungal laccases from several basidiomycetes: *Agaricus bisporus* (Perry et al. 1993), *P. ostreatus* (Garzillo et al. 1992; Palmieri et al. 1997), *Trametes versicolor* (Bourbonnais et al. 1995) and *T. villosa* (Yaver et al. 1996). D'Souza et al (1999) have reported the induction of the lignin-degrading enzymes: lignin peroxidase, manganese peroxidase and laccase by the addition of lignin components to *G. lucidum*. Here we describe the purification and biochemical characteristics of laccase isozymes of *G. lucidum* isolated in Korea, which show different electrophoretic banding patterns from those of the D'Souza group (D'Souza et al. 1999).

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Materials and methods

Fungal strains and enzyme activity

G. lucidum monokaryon (ASI 7071–9) was obtained from National Institute of Agricultural Science and Technology (NIAST) in

Korea. The fungus was maintained on semi-*Ganoderma* complete medium (SGCM) agar slant (glucose 4%, peptone 0.5%, yeast extract 0.5%, KH_2PO_4 0.046%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, agar 1.6%) at 4°C.

Laccase activity was assayed by modifying the Ross method (Ross 1982) using *o*-tolidine as the substrate; 3 ml of *o*-tolidine (2 mM) in an acetate buffer (0.1 M, pH 3.5) was mixed with enzyme solution (75 µl) for 10 min at 20°C, and the optical density (OD) was read at 590 nm. One enzyme unit was defined as the amount of enzyme which generated 0.1 OD under the above conditions. The protein concentration was determined by the method of Lowry et al. (1951).

Enzyme purification

The fungal strain was grown in SGCM liquid medium (100 ml) in a shaking incubator at 30°C by inoculating 20 pieces of fungal mycelia cut with a no. 1 cork borer (diameter 4 mm). Whole fungal cells were ground in a Waring blender and the homogenate was transferred (10%, v/v) to fresh SGCM liquid medium (300 ml in 1-l flask). When the extracellular laccase activity reached a maximum on day 2, the culture supernatant was collected by filtration through Whatman No.1 paper. The culture filtrate was mixed with cold ethanol (final concentration: 80%) to precipitate total protein, and the pellet was harvested by centrifugation at 6,000 *g* for further enzyme purification.

The crude protein pellet was dissolved in 30 ml of 20 mM Tris buffer (pH 7.0), and loaded on a DEAE-Sepharose CL-6B ion exchange column (4×40 cm). After the column was washed with the same buffer, the enzyme fraction was eluted with a linear concentration gradient of 0–0.5 M NaCl in the same buffer at a flow rate of 0.5 ml/min. The fractions showing laccase activity were collected and concentrated by ultrafiltration. The enzyme proteins were then separated by 10% preparative gel electrophoresis (Prep Cell, Bio-Rad), which revealed the presence of three laccase isozymes (designated GaLcs). The fastest moving laccase isozyme (GaLc 3) was purified through another run of Prep Cell, but the other two slow-moving isozymes were isolated from the native polyacrylamide gel by cutting out the active regions. The molecular weight of native protein was determined by gel-filtration chromatography through a Sephadex G-150 column. The column was calibrated with alcohol dehydrogenase (150 kDa), egg albumin (45 kDa) and cytochrome c (12.5 kDa). SDS-PAGE was performed on polyacrylamide gels (10%) (Laemmli 1970), with phosphorylase B (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa) as the standard marker proteins. The purified proteins were first denatured to remove glycosylations by treatment with N-glycosidase F and Endoglycosidase H (Boehringer Mannheim), following the supplier's protocols, and then their molecular weights were determined by SDS-PAGE analysis.

Biochemical characterization of purified laccase isozymes

Optimum pH was determined with different buffers: citrate-phosphate (pH 2.5–7.0) and phosphate (pH 6.5–8.0). pH stability was examined after the enzyme was incubated in buffers of different pH for 1 h. Tris buffer was used for the determination of stability from pH 7.0 to 10.0. Optimum temperatures of the purified laccases were determined within the range 10–50°C with *o*-tolidine dissolved in acetate buffer (pH 3.5). Temperature stability was measured using the same method with enzyme proteins incubated at various temperatures for 4 h.

K_m and V_{max} for *o*-tolidine (pH 3.5, 20°C, 0.2–1.0 mM, 590 nm) and 2,2'-azino-bis-(3-ethylthiazoline-6-sulfonate) (ABTS: pH 5.0, 20°C, 0.002–0.01 mM, 420 nm) were determined by measuring initial velocity at five different substrate concentrations. All kinetic studies were performed at least three times and the kinetic data were fitted to hyperbolae using the Michaelis-Menten equation.

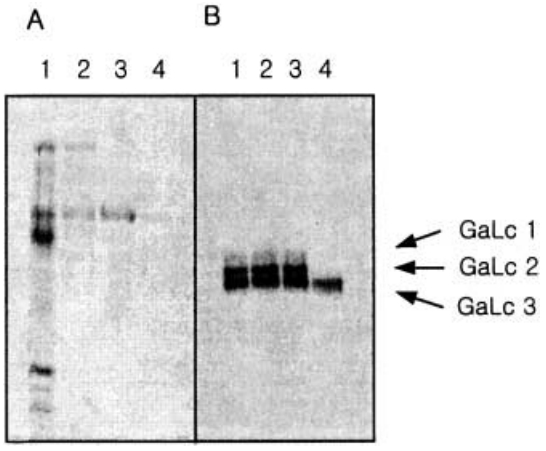

Fig. 1 **A** SDS-PAGE (10%) analysis of laccase isozymes of *Ganoderma lucidum* ASI 7071–9 by Coomassie blue R-250 staining. *1* Crude enzymes of alcohol precipitates, *2* enzymes partially purified by DEAE-Sepharose CL-6B, *3* three laccase isozymes (GaLcs) after preparative electrophoresis, *4* GaLc 3. **B** Non-denaturing, native PAGE (10%) with activity staining by *o*-tolidine. *Lanes 1–4* Same as Fig. 1A

N-Terminal amino acid sequences of the laccase isozymes were analyzed by using a Precise Protein Sequencing System (Applied Biosystem) at Korea Basic Science Institute, Seoul Branch.

Results

G. lucidum ASI 7071–9 monokaryon produced the highest laccase activity in liquid SGCM on day 2. When the laccase isozymes in the culture supernatant were separated by native PAGE, there were three bands which reacted with *o*-tolidine (GaLc 1, 2, 3, from highest to lowest molecular weight, respectively; Fig. 1). These three isozymes were purified by ion exchange chromatography and preparative electrophoresis as summarized in Table 1. The three laccase isozyme mixtures were purified 32.4-fold, with a recovery of 28.6%, while the yield of isozyme 3 (GaLc 3) was only 0.6%. GaLc 1 and 2 were purified by elution from native PAGE and the recovery yields were almost the same (0.55-0.6%) as that of GaLc 3. The specific activity of GaLc 3 was 1.58×10^4 U/mg protein.

The molecular mass of each isozyme was approximately 65–68 kDa as determined by Sephadex G-150 chromatography (data not shown) and by SDS-PAGE (Fig. 2), which meant that GaLcs were monomeric proteins. The N-terminal amino acid sequences of the three isozymes were identical as G-I-G-P-T. When GaLcs were treated with N-glycosidase F or Endoglycosidase H to remove carbohydrate moieties, the electrophoretic mobilities of the three isozymes were decreased and only one band appeared on SDS-PAGE (Fig. 2). The estimated molecular weight of the deglycosylated protein was 60.7 kDa, which meant GaLcs were glycoproteins containing 7–10% carbohydrates.

Table 1 Purification of a laccase isozyme (GaLc 3) from *Ganoderma lucidum*

Purification step	Total protein (mg)	Total enzyme activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification (fold)
Crude enzyme	164	8.00×10^4	4.88×10^2	100	1
DEAE-sepharose chromatography	46.4	5.99×10^4	1.29×10^3	74.9	2.6
Preparative electrophoresis I	1.452	2.29×10^4	1.58×10^4	28.6	32.4
Preparative electrophoresis II (GaLc 3)	0.031	4.89×10^2	1.58×10^4	0.6	32.4

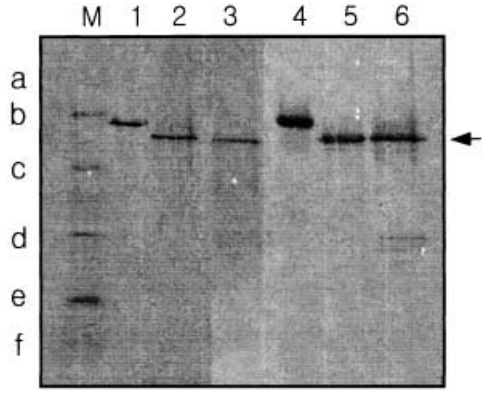

Fig. 2 SDS-PAGE (8–16%) of the *G. lucidum* GaLcs. Lanes: *M* Molecular weight marker (*a* phosphorylase b 97.4 kDa, *b* serum albumin 66.2 kDa, *c* ovalbumin 45 kDa, *d* carbonic anhydrase 31 kDa, *e* trypsin inhibitor 21.5 kDa, *f* lysozyme 14.4 kDa), *1* purified GaLc 3, *2* GaLc 3 treated with N-glycosidase F, *3* GaLc 3 treated with endoglycosidase H, *4* purified GaLcs, *5* GaLcs treated with N-glycosidase F, *6* GaLcs treated with endoglycosidase H

Table 2 Kinetic parameters of GaLc 3 of *G. lucidum* towards *o*-tolidine and ABTS

Substrate	K_m (mM)	V_{max} (OD/min)
o-Tolidine	0.4016	0.0198
ABTS	0.0037	0.0142

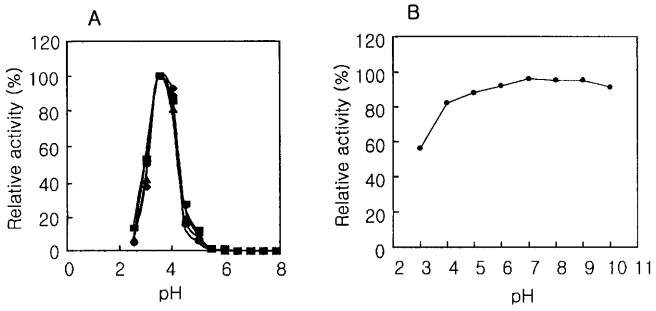

Fig. 3 **A** Optimum pH for GaLc 1 (◆), GaLc 2 (●), GaLc 3 (▲) and GaLcs (■). **B** pH stability of GaLc 3

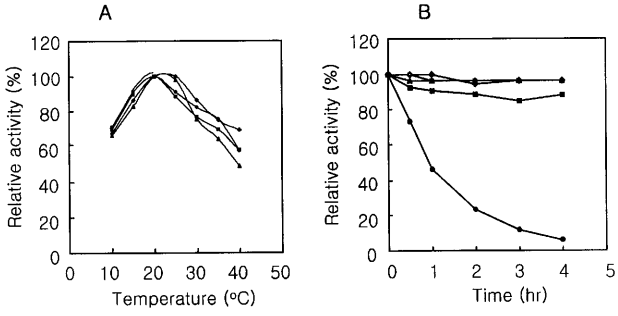

Fig. 4 **A** Optimum temperature for GaLc 1, GaLc 2, GaLc 3 and GaLcs. Legends are same as Fig. 3. **B** Thermal stability of GaLc 3 at 10°C (◆), 20°C (□), 30°C (▲), 40°C (■), 50°C (●)

The effect of pH on the laccase activity was examined at pH values ranging from 2.5 to 8.0. As shown in Fig. 3A, the optimum pH was estimated to be around 3.5. GaLc 3 was stable at 20°C for 1 h in the pH range from 4.0 to 10.0 (Fig. 3B). As shown in Fig. 4A, the optimum temperature for GaLcs activity was 20–25°C. Temperature stability was measured by incubation for 4 h at temperatures between 10°C and 50°C; GaLc 3 retained more than 80% activity after 4 h incubation at temperatures up to 40°C but activity decreased to lower than 50% after 1 h at 50°C (Fig. 4B).

K_m values of GaLc 3 for *o*-tolidine and ABTS were calculated from double reciprocal plots. As in Fig. 5A and B and Table 2, K_m values were 401.6 µM for *o*-tolidine and 3.7 µM for ABTS. However the V_{max} for *o*-tolidine was higher than that of ABTS.

Discussion

D'Souza et al (1999) have reported that a *G. lucidum* strain produces high amounts of laccase activity in high nitrogen medium, or using pine and poplar ground woods as the sole carbon and energy source. They reported two laccase isozymes, whose molecular weights were 40 kDa and 66 kDa, even though they were separated into five major protein bands by isoelectric focusing. In our case, three laccase isozymes (GaLcs) were produced in a complete medium without any specific induction. Furthermore, the Korean isolate had three laccase isozymes with a narrow range of molecular weights between 65 kDa and 68 kDa. The GaLcs showed very similar electrophoretical characteristics to one another since they moved at the same rates in native and SDS-

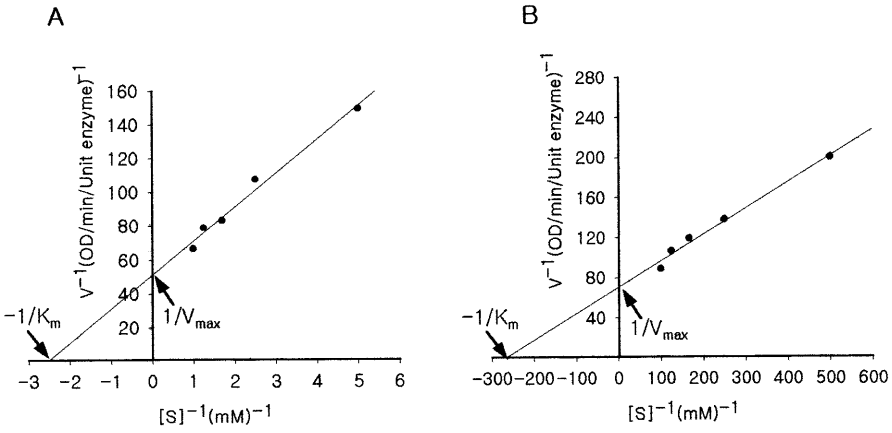

Fig. 5A, B Determination of K_m and V_{max} of GaLc 3. **A** *o*-Tolidine, **B** ABTS

PAGE, and even in the mild denaturing conditions of urea-PAGE (data not shown). Since we had difficulty in isolating each isozyme from the preparative gel, only the fastest moving GaLc 3 was purified from the early Prep Cell fractions, while GaLc 1 and 2 were purified from the active bands of a long, native PAGE.

Many fungal laccases have been purified and their biochemical characteristics analyzed. Their molecular weights are usually 55–90 kDa including carbohydrates (Peter and Wollenberger 1997). The molecular weights (65–68 kDa) of GaLc 1, 2 and 3 of *G. lucidum* ASI 7071–9 monokaryon are quite similar to other fungal laccases, and GaLcs show 7–10% glycosylation (Fig. 2). Glycoproteins usually lose activity when their carbohydrate moieties are removed. Since enzyme proteins must be denatured first to remove the carbohydrates from the laccases, it was impossible to measure the activities of the deglycosylated proteins. When N-glycosidase F (which removes N-glycosylation), or Endoglycosidase H (which removes all glycosylations) were applied to the GaLcs, only one protein band appeared on SDS-PAGE, which means GaLcs contain only N-glycosylation. When the carbohydrate moieties from GaLc 1, 2 and 3 were removed, each protein moiety moved as one identical band. Furthermore, their N-terminal amino acid sequences were the same (G-I-G-P-T). The N-terminal amino acid sequence of GaLcs is similar to that of other fungal laccases; *T. villosa* laccase isozyme I (*lcc1*; Yaver et al. 1996), and *T. versicolor lccII* (Bourbonnais et al. 1995) both begin with G-I-G-P-V, and *P. ostreatus* laccase 1 (*pox1*) with A-I-G-P-T (Giardina et al. 1995).

GaLcs showed maximum enzyme activity at pH 3.5, which is same as laccase II of *P. eryngii* (Munoz et al. 1997), and quite similar to many fungal laccases whose optimum pH range is 3.0–4.5 (Heinzkill et al. 1998). The optimum temperature of GaLcs was 20–25°C, which is same as the acidic laccase of *Coprinus congregatus* (Choi et al. 1995). However, other fungal laccases have optimum temperatures ranging from 55°C in laccase II of *P. eryngii* (Munoz et al. 1997) to 80°C in the laccase of PM1 (Coll et al. 1993). The three laccase isozymes from *G. lucidum* monokaryon (ASI 7071–9) showed almost identical properties. Therefore the internal peptide sequences and the gene sequences should be determined to clarify whether or not these three isozymes can be considered as one protein with three different amounts of glycosylation.

There are many reports about the functions of fungal laccases, and degradation of recalcitrants is widely investigated in many research labs. When *G. lucidum* was transferred to an acidic liquid SGCM (pH 4.1–4.5) instead of the neutral medium, 3–5 times more laccase activity was detected in the culture supernatant. In *C. congregatus*, an acidic laccase which is only induced under acidic conditions has been reported (Kim et al. 2001). In order to determine the functions of laccase isozymes in *G. lucidum*, their genes must be cloned.

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