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A laccase from the medicinal mushroom *Ganoderma lucidum*

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Abstract A protein demonstrating laccase activity and potent inhibitory activity towards human immunodeficiency virus (HIV)-1 reverse transcriptase (IC_{50} 1.2 μM) was isolated from fresh fruiting bodies of the medicinal mushroom *Ganoderma lucidum*. The laccase had a novel N-terminal sequence and a molecular mass of 75 kDa, which is higher than the range (55–56 kDa) reported for most other mushroom laccases. It was isolated by sequential chromatography on DEAE-cellulose and Affi-gel blue gel and adsorption on Con A-Sepharose. Unlike some of the previously isolated laccases, it was adsorbed only on Con A-Sepharose. The enzyme required a pH of 3–5 and a temperature of 70°C to exhibit maximal activity. Minimal activity was detected at pH 6 and 7. Activity was undetectable at pH 8 and 9 and after exposure to 100°C for 10 min.

Introduction

A variety of proteins have been isolated and characterized from mushrooms and fungi including lectins (Yagi et al. 1997; Wang et al. 1995, 1996, 2004), ribonucleases, ribosome-inactivating proteins (Wang and Ng 2000a, 2001a,b), anti-fungal proteins (Lam and Ng 2001; Wang and Ng 2004d, 2006b), laccases (Wang and Ng 2004b,c,e, 2006b) and ubiquitin-like peptides (Wang and Ng 2000b). Some of these proteins exhibit anti-proliferative/anti-tumour (Wang et al. 1995, 1996), anti-microbial (Lam and Ng 2001; Wang and Ng 2004b,c) and human immunodeficiency virus (HIV)-1 reverse transcriptase (RT) inhibitory (Lam and Ng 2001, Wang and Ng 2001a,b) activities.

From the medicinal fungus *Ganoderma lucidum*, polysaccharides designated ganoderans A and B, which elicited hypoglycaemia in diabetic mice, have been isolated (Hikino et al. 1985). A polysaccharide peptide with anti-tumour and anti-angiogenic activities has been reported (Cao and Lin 2004). However, little is known about the protein constituents of *Ganoderma* spp. other than lectins (Tanaka et al. 1989; Kawagishi et al. 1997; Ngai and Ng 2004), an anti-fungal protein (Wang and Ng 2004d,2006a) and a ribonuclease (Wang et al. 2003).

Laccases are ligninolytic enzymes (Evans et al. 1994), but they find applications in biosensors, pulping, textile dyes, detoxification of polluted water and other biotechnological procedures (Palmieri et al. 1993; Brenna and Bianchi 1994; Reid and Paice 1994; Ghindilis et al. 1995; Martirani et al. 1996). The objective of the present study was to isolate a laccase from *G. lucidum*. The results would add to the existing knowledge about this medicinal mushroom. Hitherto laccases have been isolated from a number of mushrooms (Eggert et al. 1996; Munoz et al. 1997; Cambria et al. 2000; Dedeyan et al. 2000; Shin and Lee 2000; Garzillo et al. 2001; Rogalski et al. 2001; Wang and Ng 2006a,b), mostly from their mycelia, but very few from medicinal mushrooms, e.g. that from *Trametes versicolor*, also known as *Coriolus versicolor* (Milsten et al. 1989) and renowned for its polysaccharide peptide (Sakagami et al. 1991; Ng 1998). The present investigation disclosed that the fruiting bodies of the prized medicinal mushroom *G. lucidum* produce a laccase with some unique characteristics, including a high molecular mass, a novel N-terminal sequence, a fairly high thermostability, a pH optimum ranging from pH 3 to 5 and non-adsorption on various chromatographic media except Con A-Sepharose.

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Materials and methods

Isolation of laccase Fresh fruiting bodies (800 g) of the mushroom *G. lucidum* were used. They were extracted with distilled water (3 ml/g) in a Waring blender. The homogenate was centrifuged (13,000×*g*, 20 min), and the supernatant was saved. Tris–HCl buffer (1 M, pH 7.6) was added to the supernatant until the concentration of Tris attained 10 mM. The supernatant was then passed through a column (5×20 cm) of DEAE-cellulose (Sigma) in 10 mM Tris–HCl buffer (pH 7.4). Unadsorbed proteins eluted with the starting buffer were collected as fraction D1, while adsorbed proteins eluted with 0.8 M NaCl added to the starting buffer were collected as fraction D2. Laccase activity was concentrated in D1. D1 was next subjected to affinity chromatography on an Affi-gel blue gel (Bio-Rad) column (2.5×20 cm). Unadsorbed proteins were washed off the column with 10 mM Tris–HCl buffer (pH 7.4) and collected as fraction B1. Adsorbed proteins were eluted with 10 mM Tris–HCl buffer containing 2 M NaCl and collected as fraction B2. Fraction B1 was applied to a 2.5×20 cm column of Con A-Sepharose (Amersham Biosciences). Unadsorbed proteins were eluted into fraction Con A1 with 50 mM Tris–HCl buffer (pH 7.4) containing 0.5 M NaCl, 10 mM $CaCl_2$ and 10 mM $MgCl_2$. Adsorbed proteins were desorbed with buffer B, i.e. 0.4 M α-methyl-D-glucopyranoside added to the starting buffer and collected as fraction Con A2. Fraction Con A2 was dialyzed, lyophilized and then further fractionated by fast protein liquid chromatography (FPLC) on a gel filtration Superdex 75 HR 10/30 column (Amersham Biosciences) using an AKTA Purifier System (Amersham Biosciences). The first eluted peak represented purified laccase.

Assay of laccase activity Laccase activity was assayed by measuring the oxidation of 2,7′-azinobis(3-ethylbenzothiazolone-6-sulfonic acid) diammonium salt (ABTS). A modification of the method of Shin and Lee (2000) was used. An aliquot of the enzyme solution was incubated in 1.3 ml of 67 mM sodium acetate buffer (pH 4.5) containing 1.54 mM ABTS at 30°C. One unit of enzyme activity was defined as the amount of enzyme required to produce an absorbance increase at 405 nM of one per minute per millilitre of reaction mixture under the aforementioned condition.

Molecular mass determination by sodium dodecyl sulphate polyacrylamide gel electrophoresis and by FPLC-gel filtration Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in accordance with the procedure of Laemmli and Favre (1973), using a 12% resolving gel and a 5% stacking gel. At the end of electrophoresis, the gel was stained with Coomassie brilliant blue. FPLC-gel filtration was carried out using a Superdex 75 column, which had been calibrated with molecular mass standards (Amersham Biosciences).

Analysis of N-terminal amino acid sequence Amino acid sequence analysis was carried out using an HP G1000A Edman degradation unit and an HP1000 HPLC system (Lam and Ng 2001).

Assay for HIV-1 RT inhibitory activity The assay for HIV-1 RT inhibitory activity was carried out according to instructions supplied with the assay kit from Boehringer Mannheim (Germany). The assay takes advantage of the ability of RT to synthesize DNA, starting from the template/primer hybrid poly(A) oligo(dT)15. The digoxigenin- and biotin-labelled nucleotides in an optimized ratio are incorporated into one of the same DNA molecule, which is freshly synthesized by the RT. The detection and quantification of synthesized DNA as a parameter for RT activity follows a sandwich ELISA protocol. Biotin-labelled DNA binds to the surface of microtitre plate modules that have been precoated with streptavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase, binds to the digoxigenin-labelled DNA. In the final step, the peroxidase substrate is added. The peroxidase enzymes catalyze cleavage of the substrate, producing a coloured reaction product. The absorbance of the samples at 405 nm can be determined using a microtitre plate (ELISA) reader and is directly correlated to the level of RT activity. A fixed amount (4–6 ng) of recombinant HIV-1 RT was used. The inhibitory activity of the isolated protein was calculated as percent inhibition as compared to a control without the protein (Lam and Ng 2001; Wang and Ng 2001a,b; Ye and Ng 2002).

Results

When the fruiting body extract was chromatographed on DEAE-cellulose, laccase activity was confined to fraction D1 (Table 1). D1 was separated on Affi-gel blue gel into an unadsorbed fraction B1 with much higher laccase activity and an adsorbed fraction B2 with much lower activity (Table 1). Con A-Sepharose was capable of adsorbing essentially all of the laccase activity found in B1 (Table 1), although the unbound fraction Con A1 was much larger

Table 1 Yields and laccase activities of chromatographic fractions

Fraction	Yield (mg)	Laccase activity (U/mg)	Purification fold	% Recovery of activity
Extract	2,870	0.69	1	100
D1	960	1.27	1.84	61.5
D2	1,040	<0.1	–	–
B1	583	1.56	3.26	45.9
B2	164	0.34	–	–
Con A1	337.1	<0.1	–	–
Con A2	54.8	11.33	16.4	31.4
S1	22.8	17.55	25.4	20.2
S2	14.1	1.15	–	–

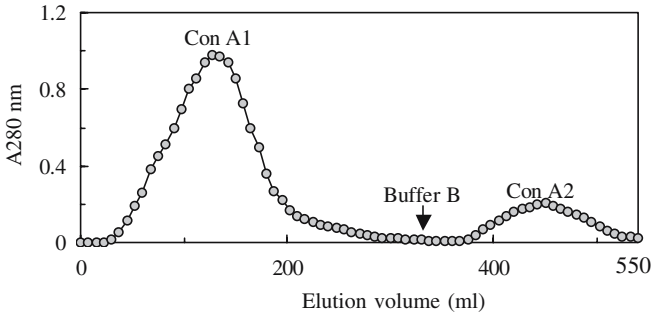

Fig. 1 Affinity chromatography on Con A-Sepharose. Sample: *Ganoderma lucidum* fruiting body extract unadsorbed successively on DEAE-cellulose, Affi-gel blue gel and then CM-cellulose. Column dimensions: 2.5×20 cm. Starting buffer for eluting fraction Con A1: 50 mM Tris–HCl (pH 7.4) containing 0.5 M NaCl, 10 mM $CaCl_2$ and 10 mM $MgCl_2$. Buffer B for eluting fraction Con A2: 0.4 M α-methyl-D-glucopyranoside in starting buffer

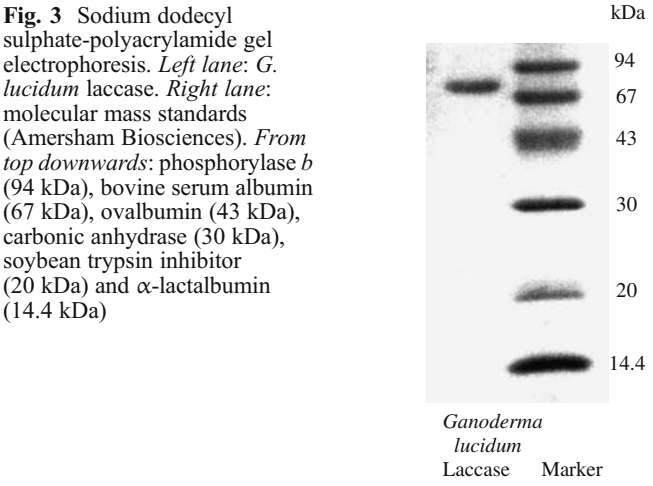

Fig. 3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. *Left lane*: *G. lucidum* laccase. *Right lane*: molecular mass standards (Amersham Biosciences). *From top downwards*: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa)

than the bound fraction designated as Con A2 (Fig. 1). Superdex 75 separated Con A2 into two fractions, S1 and S2. S2 was slightly smaller (Fig. 2). The bulk of laccase activity was retained in S1 (Table 1). S1 appeared as a single band with a molecular mass of 75 kDa in SDS-PAGE (Fig. 3). Its molecular mass as estimated by gel filtration on Superdex 75 was also 75 kDa (Fig. 2). The laccase showed very little resemblance to other mushroom laccases in N-terminal sequence. Some of the other mushroom laccases, e.g. those from *Trametes veriscolor*, *Coriolus hirsutus*, Basidiomycete PM1, *Cariporiopsis subvermispora*, *Phlebia radiata* and *Pycnoporus cinnabarinus* exhibited considerable sequence homology to each other (Table 2). The activity of the purified laccase underwent a 80% increase when the temperature was raised from 20 to 70°C. When the temperature was increased to 80°C, there was a small drop in activity (Fig. 4). The activity of the enzyme was totally destroyed after exposure to 100°C for 10 min. It stayed at a high level when the pH was varied from 3 to 5. When the pH was increased to 6, a significant decline in enzyme activity occurred. The activity remained low at pH 7 and further decreased to an undetectable level at pH 8 and 9 (Fig. 5). The enzyme inhibited HIV-1 RT with an IC_{50} of 1.2 μM.

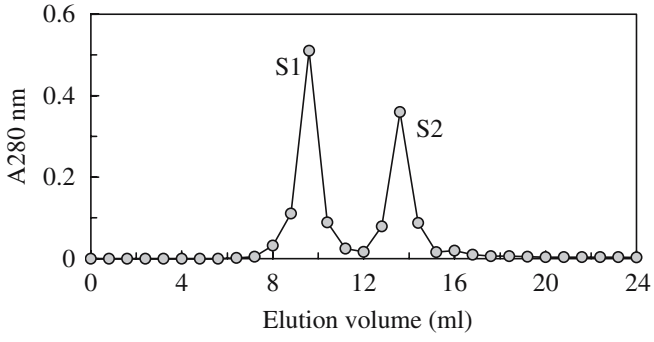

Fig. 2 Gel filtration by fast protein liquid chromatography on a Superdex 75 HR 10/30 column using an AKTA Purifier System (Amersham Biosciences). Sample: fraction Con A2. Eluent: 0.2 M NH_4HCO_3 buffer (pH 8.5). Flow rate: 0.4 ml/min. Fraction size: 0.8 ml

Discussion

In the present study, a laccase was successfully isolated from the fruiting bodies of the medicinal mushroom *G. lucidum*. Ion-exchange chromatography on DEAE-cellulose, affinity chromatography on Affi-gel blue gel and Con A-Sepharose and gel filtration on Superdex 75 were used successively to remove inactive proteins from the laccase-containing chromatographic fraction. Each of the aforementioned chromatographic media was effective in yielding a laccase-enriched fraction separable from a fraction with little or no laccase activity. In contradistinction to laccases from the mushrooms *Pleurotus eryngii* (Wang and Ng 2006b), *C. hirsutus* (Shin and Lee 2000) and *Rigidoporus lignosus* (Cambria et al. 2000), which are adsorbed on DEAE-Sepharose and Q-Sepharose, *G. lucidum* laccase is unadsorbed on DEAE-cellulose. *G. lucidum* laccase is also unadsorbed on Affi-gel blue gel. Con A-Sepharose was thus selected as an adsorption

Table 2 N-terminal sequence comparison of laccases from *Ganoderma lucidum* and other mushrooms

Ganoderma lucidum laccase	GQNGDAVP
Trametes versicolor laccase I	AIGPVASLVV
Trametes versicolor laccase II	GIGPVADLTI
Trametes versicolor laccase III	GIGPVADLTI
Coriolus hirsutus laccase	GIGTKANLVI
Coriolus hirsutus laccase	AIGPTADLTI
Basidiomycete PM1 laccase	SIGPVADLTI
Cariporiopsis subvermispora laccase	AIGPVTDLEI
Phlebia radiata laccase	SIGPVTDFHI
Pycnoporus cinnabarinus laccase	AIGPVADLTL
Pleurotus ostreatus lacase	AIGPDGNMYI
Rigidoporus lignosus laccase	ATVALDLHTLN
Pleurotus eryngii laccase I	AXKKLDFHIIN
Pleurotus eryngii laccase II	ATKKLDFHIIN
Agaricus bisporus laccase I	KTRTFDFDLVN
Agaricus bisporus laccase II	DTKTFNFDLVN

Sequence data of laccases other than that from *G. lucidum* are from Shin and Lee (2000) and Cambria et al. (2000)

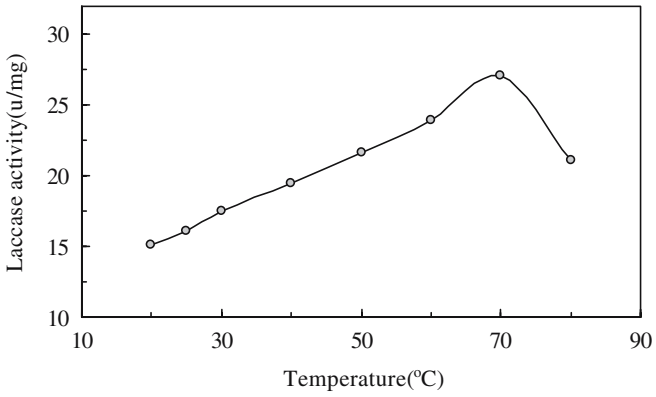

Fig. 4 Dependence of *G. lucidum* laccase activity on temperature

media for the laccase which is presumably a glycoprotein. Superdex 75 was used in the final purification step and also for molecular mass estimation.

Many mushroom laccases demonstrate marked similarity in N-terminal sequence (Table 2). On the other hand, *G. lucidum* laccase exhibits a dissimilar N-terminal sequence. The molecular mass of *G. lucidum* laccase (75 kDa) is higher than the range of molecular masses (55–65 kDa) reported for previously isolated laccases (Geiger et al. 1986; Eggert et al. 1996; Munoz et al. 1997; Cambria et al. 2000; Dedeyan et al. 2000; Shin and Lee 2000; Garzillo et al. 2001; Rogalski et al. 2001). It is remarkable that the laccase has a temperature optimum at a high temperature (70°C). The activity of the laccase increases progressively as the ambient temperature is raised from 20 to 70°C. The increase in enzyme activity is about 80%. Nevertheless, the enzyme is susceptible to thermal denaturation at 100°C, and all enzyme activity vanishes after 10 min. On the other hand, *C. versicolor* laccase manifests a temperature optimum at 45°C (Shin and Lee 2000).

Another distinctive feature of *G. lucidum* laccase is its need of an acidic pH (3–5) for activity. Between pH 5 and 6, there is a sudden drop in activity. At pH 6 and 7, there is only residual activity. At pH 8 and 9, activity is indiscernible. The laccases reported in the literature have an optimal pH of 2–3 (Shin and Lee 2000; Garzillo et al. 2001).

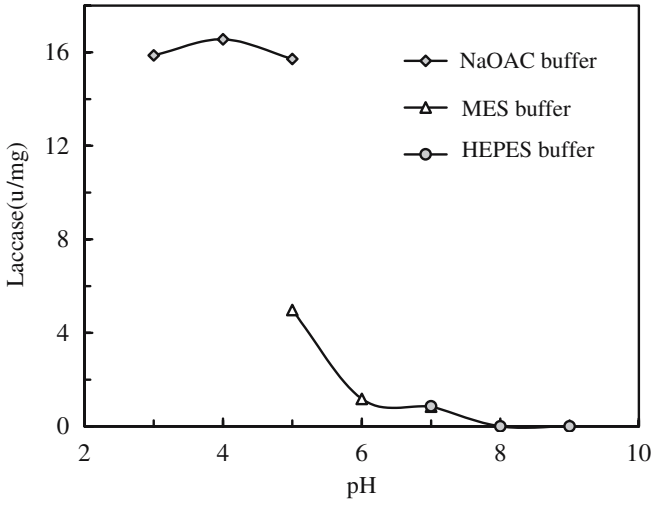

Fig. 5 Dependence of *G. lucidum* laccase activity on pH

G. lucidum laccase is capable of inhibiting HIV-1 RT, probably by protein–protein interaction. HIV-1 RT inhibitory activity has been reported for some laccases (Wang and Ng 2004a–e), ribosome-inactivating proteins (Lam and Ng 2001; Wang and Ng 2000a, 2001b), ubiquitin-like proteins (Wang and Ng 2000b) and lectins (Wang and Ng 2004a) of mushroom origin. The inhibitory potency of *G. lucidum* laccase is at the upper end of the range demonstrated by natural products (Ng et al. 1997) and the range demonstrated by mushroom laccases. *P. eryngii* laccase (Wang and Ng 2006b), *Tricholoma giganteum* laccase (Wang and Ng 2004e), *Hericium erinaceum* laccase (Wang and Ng 2004b) and *Albatrella dispansus* laccase (Wang and Ng 2004c) inhibit the HIV enzyme with an IC_{50} of 1.2, 2.2, 9.5 and 16 μM, respectively. Some mushroom laccases, e.g. *Cantharellus cibarius* laccase (Ng and Wang 2004), are devoid of this activity (Wang and Ng 2004a–c,e).

D'Souza et al. (1999) reported that a strain (Karsten FP-58537-Sp) of *G. lucidum* in high-nitrogen culture and in cultures containing both poplar and pine produced a large amount of laccase. SDS-PAGE revealed two bands with molecular mass of 40 and 66 kDa, respectively, while isoelectric focusing disclosed five bands with p*I* of 3.0, 4.25, 4.5, 4.8 and 5.1, respectively. Ko et al. (2001) detected three laccase isozymes in the mycelia of *G. lucidum* ASI 7071-9. These mycelial laccases differ from the fruiting body laccase isolated in the preset study in several aspects. The molecular mass (65–68 kDa), N-terminal sequence (GIGPT), optimum pH (3.5), optimum temperature (20°C) and chromatographic behaviour on DEAE-cellulose (adsorption) of the mycelial laccases (Ko et al. 2001) are different from the fruiting body laccase. This is reminiscent of the differences between fruiting body and mycelial lectins isolated from *Tricholoma mongolicum* (Wang et al. 1995, 1998).

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