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Purification and characterization of laccase from *Ganoderma* sp. 03

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Abstract

Laccases are enzymes that catalyze oxidation of a wide range of substrates. The enzyme shows promising applications in various fields such as toxic substance degradation, organic synthesis, and biosensors. In this study, we purified and characterized laccase from *Ganoderma* sp. Three different *Ganoderma* sp. found in the northeast Thailand were studied for laccase activity. When cultured in media containing rice bran and rice husk, *Ganoderma* sp. 03 showed high laccase activity. The laccase produced by this strain was purified using Phenyl Sepharose fast flow (FF) chromatography and quaternary amine (Q) Sepharose chromatography. Estimated molecular weight of the purified laccase was 39.81 kDa as determined by the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The purified laccase had an optimal potential hydrogen (pH) of 3.5, showed high stability at pH 3.0-5.0 using 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as a substrate, and exhibited an optimal temperature of 40.0-55.0°C. The best temperature stability was observed at 30°C. Effect of different substances on laccase activity showed that *p*-coumaric acid and dithiothreitol (DTT) strongly inhibited laccase activity. Especially, DTT could inhibit 100% of laccase activity, even at a concentration as low as 0.1 mM. All the tested metal ions, except $ZnSO_4$, decreased laccase activity. This information may be useful for further studies and applications of this enzyme.

Keywords: Laccase, Protein purification, Biocatalysis, *Ganoderma* sp

1. Introduction

Laccases (EC 1.10.3.2; benzenediol: oxygen oxidoreductase) are enzymes that catalyze the oxidation of a wide range of phenolic and nonphenolic substrates via reduction of molecular oxygen to water. Typically, laccases contain four copper (Cu^{2+}) ions at their active sites. Based on their spectroscopic characteristics, these copper ions are classified into one type-1 copper ion (T1), one type-2 (T2) copper ion, and two type-3 copper ions (T3) in which three copper ions form a trinuclear cluster [1]. The copper ion at the T1 position is first reduced by the substrates, after which the electron is transferred to the trinuclear center (copper ions at T2 and T3 sites) where molecular oxygen (O_2) is reduced to water [2]. Several substances have been reported to act as substrates for laccase. Polyphenols, aminophenols, para- and ortho-diphenols, aryl diamines, polyamines, lignins, as well as some inorganic ions can be catalyzed by laccases [3]. Laccases are found in bacteria, insects, plants, and fungi. These enzymes have diverse functions in living organisms-both anabolic and catabolic [4]. The most studied laccases are found in fungi, in which their major function is related to lignin degradation. Several fungi have been reported as laccase sources, such as *Lentinus edodes*, *Pleurotus ostreatus*, *Pycnoporus cinnabarinus* [5], *Lentinus polychrous* [6], and *Grifola frondosa* [7]. Laccases can use a wide variety of substrates, rendering them to be suitable for several applications. Laccase can be used to catalyze the conjugation of polysaccharides and proteins, which broadens the applications of proteins in the food industry [8,9]. In addition, it can be used for wine and beer stabilization [10]. Bleaching of hair by laccase, owing to its use as an oxidizing agent instead of hydrogen peroxide, has been previously reported [10]. Laccase-based biosensors have been developed to detect *p*-coumaric

acid [11] and epinephrine [12]. Furthermore, toxic substances can be degraded by laccases [13], and various investigators have used laccases for construction of biofuel cells [14,15]. Nevertheless, new laccases with properties suitable for specific applications are needed. Therefore, sources of laccase have been investigated to identify enzymes with desired properties for various applications.

Ganoderma lucidum (*G. lucidum*) is a well-known mushroom with various medicinal properties. Although laccases from this species have been reported [16,17] in Thailand, especially in the northeastern area, little is known about these enzymes from this mushroom. Therefore, in this study, we aimed to purify a laccase from *Ganoderma* species found in the northeastern area of Thailand and to investigate some of its properties. Interesting properties of such new laccases can be applied in various fields in the future.

2. Materials and methods

2.1 Media and culture conditions for growth of fungi

Ganoderma sp. 03 maintained in potato dextrose agar (PDA) media at 4°C was grown in a solid substrate containing rice husk and rice bran (15 g husk: 30 g bran) and 50 mL distilled water and incubated at room temperature (30 ± 3°C) for 1 month. The enzyme was extracted using distilled water at a ratio of 1 g of the cultured substrate per 10 mL of distilled water. After centrifugation at 6,000 × g for 20 min, the supernatant was used as a crude enzyme.

2.2 Assay for laccase activity and protein content analysis

Laccase activity was assayed by measuring the oxidation product of 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) at 30°C, as described by Sarnthima et al [6]. Briefly, the assay mixture contained ABTS at a final concentration of 0.1 mM and sodium acetate buffer (0.1 M, potential hydrogen (pH) 4.5). The oxidized ABTS was analyzed by spectrophotometry at 420 nm (ε = 36,000 M^{-1}/cm^{-1}). One unit of enzyme activity was defined as the amount of enzyme required to catalyze transformation of 1 µmol of the substrate to the product per minute under certain assay conditions. Protein content analysis was performed by the Bradford method [18], using bovine serum albumin (BSA) as a standard protein.

2.3 Laccase purification

The crude enzyme at a volume of 350 mL was precipitated with 85% saturated ammonium sulfate. After centrifugation at 10,000 × g, the precipitate was dissolved in 100 mL distilled water. It was then passed through a Phenyl Sepharose fast flow (FF) column (General Electric (GE) Healthcare, Sweden) equilibrated with 50 mM sodium acetate buffer pH 4.5 (buffer A) run on an AKTAprime plus system (Sweden). After washing the column with buffer, A, the bound protein was eluted using a linear concentration gradient of 2-0 M $(NH_4)_2SO_4$ in buffer A at a flow rate of 3 mL/min. The fractions showing laccase activity were pooled, and the pooled fraction was subsequently passed through a Q Sepharose column (GE Healthcare, Sweden), which was equilibrated with 20 mM Tris-HCl buffer pH 7.5 (buffer B). Bound proteins were eluted employing a linear concentration gradient of 0-1 M NaCl in buffer B at a flow rate of 3 mL/min, and 3 mL fractions were collected. The fractions with laccase activity were pooled. Purity of the enzyme was checked using the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.4 Effects of pH and temperature on laccase activity

The effect of pH on laccase activity was tested using ABTS as a substrate in a pH range of 2.5-7.0 (citric acid-Na_2HPO_4 buffers). The effect of pH on enzyme stability was determined by incubating the enzyme at the tested pH and 30°C; the enzyme solution was then withdrawn for laccase assay every 30 min for 120 min. The effect of temperature on purified laccase activity was determined by analyzing laccase activity at different temperatures ranging from 30 to 65°C. The temperature stability was determined by incubating the enzyme samples at various temperatures (30, 40, and 55°C) for different time intervals (15-180 min). All the measurements were performed in triplicate.

2.5 Effects of selected substances and selected ions on laccase activity

The effects of selected substances and selected ions on laccase activity were assessed by an assay of laccase activity in the presence of various concentrations of selected substances and selected ions.

2.6 Statistical analysis

All data were derived from triplicates and means ± standard deviation (SD). Differences between means were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test using GraphPad Prism 7. $p< 0.05$ was considered as statistically significant.

3. Results and discussion

3.1 Screening and purification of laccase

Three different fruiting bodies of *Ganoderma* sp. mushrooms obtained from northeast Thailand were isolated until pure cultures were obtained; these were designated as G01, G02, and G03 (Figure 1). These three strains of the *Ganoderma* sp. were observed for laccase production based on their ability to produce green ABTS radicals (Figure 1C). The three strains were then cultured in solid-state substrates. Strain G03 exhibited the highest laccase activity. Therefore, this strain was selected to be used for laccase production.

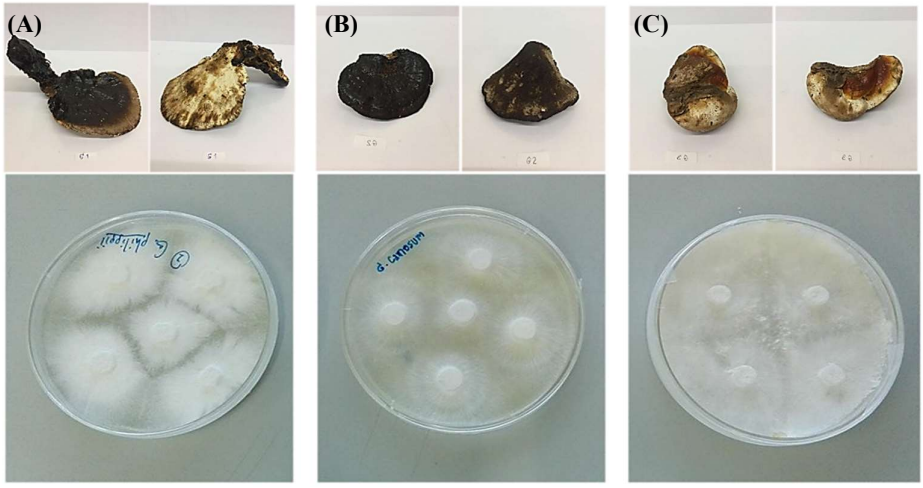

Figure 1 *Ganoderma* sp., G01 (A), G02 (B), and G03 (C).

Crude laccase (350 mL; 0.45 U/mL or 0.26 mg/mL protein) was precipitated with 85% saturated ammonium sulfate and re-dissolved in buffer A. The enzyme solution was purified on a Phenyl FF Sepharose column (Figure 2A). After washing, the bound enzyme was eluted by decreasing the linear gradient 2-0 M $(NH_4)_2SO_4$ in buffer A. Fractions containing laccase activity were pooled (6.035 U, 5.1 mg protein). The bound fraction obtained following purification on the Phenyl FF column was passed through a Q Sepharose column; its chromatogram is shown in Figure 2A. However, the unbound fraction obtained from the Phenyl FF Sepharose column contained higher laccase activity (designated as UbP, 73.3 U, 7.5 mg protein) (Table 1). Therefore, this fraction (UbP) was used for further purification on the Q Sepharose column; the chromatogram is shown in Figure 2B.

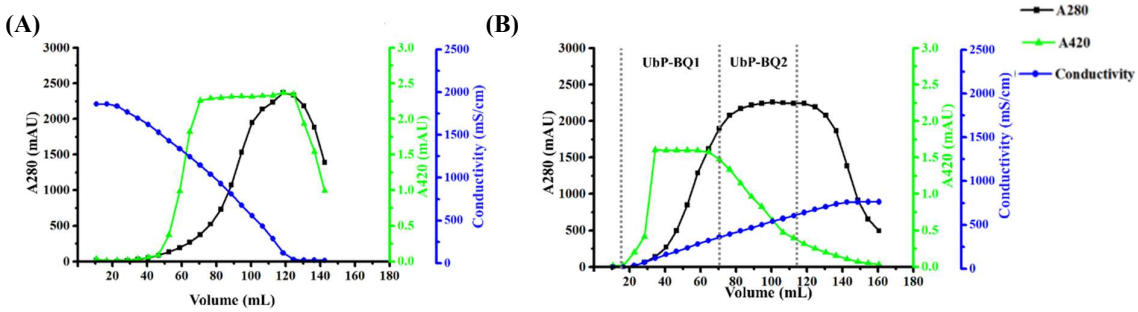

Figure 2 Chromatograms of crude laccase obtained using (A) Phenyl FF Sepharose hydrophobic chromatography and (B) employing Q Sepharose column of an unbound fraction obtained following purification on the Phenyl Sepharose column.

Based on laccase activity observed on the Q Sepharose chromatogram, two pooled fractions were obtained (designated as UbP-BQ1, fraction 15-71.9 mL; UbP-BQ2, fraction 72-114 mL). The UbP-BQ1 fraction exhibited dramatically higher laccase activity than the UbP-BQ2 fraction, as summarized in Table 1. Up to approximately 11.7 fold purified enzyme was obtained with a final yield of 33.4% compared to the crude enzyme. The specific activity of this purified laccase was 20.2 U/mg using ABTS as substrate.

Table 1 Purification of laccase from *Ganoderma* sp. 03.

Purification steps	Volume (mL)	Activity (U/mL)	Protein (mg/mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold	Yield (%)
Crude enzyme	350	0.45	0.26	157.50	91.00	1.73	1.00	100
0-85% $(NH_4)_2SO_4$	100	0.35	0.16	35	16	2.19	1.26	22.22
Phenyl FF Sepharose--UbP	250	0.29	0.03	73.25	7.50	9.77	5.64	46.51
Q sepharose--UbP-BQ1	52	1.01	0.05	52.62	2.60	20.24	11.69	33.41
Q sepharose--UbP-BQ2	39	0.02	0.12	0.86	4.60	0.18	0.11	0.54

3.2 SDS-PAGE analysis and zymograms

UbP-BQ1 was analyzed by SDS-PAGE. A dominant protein band with an estimated molecular weight of approximately 39.81 kDa was observed (Figures 3A, 3C). In addition, native PAGE of this laccase fraction was performed, and the activity was determined using laccase substrates, ABTS and syringaldazine. Zymograms confirmed that the purified enzyme was laccase, as it was shown to be positive upon staining for laccase activity (Figures 3B and 3C).

Genome analysis of *G. lucidum* revealed 16 laccase genes [19]. The molecular mass of *G. lucidum* isozymes is reported to be in the range of 40-66 kDa [20]. These laccase isozymes show different expression and regulation depending on the type of media and culture conditions [17]. Laccase with a molecular mass of 38.3 kDa obtained from *G. lucidum* having the *Glac3* gene [21] and that from *G. lucidum*-CDBT1 with a MW of 43 kDa have been reported [21,22]. The purified laccase obtained by our group showed a similar range of the molecular sizes mentioned in these reports.

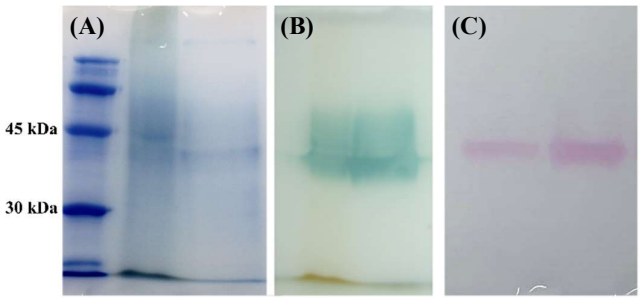

Figure 3 Crude laccase and purified laccase (UbP-BQ1) shown on a 12% gel using SDS-PAGE (A). Zymograms of the purified laccase (UbP-BQ1) using ABTS (B) and syringaldazine (C) as substrates.

3.3 Optimal pH and pH stability

The purified laccase showed an optimal pH at pH 3.5 with ABTS as the substrate; however, when a pH higher than 4.0 was used, the laccase activity decreased gradually (Figure 4A). A similar optimal pH for laccase from *G. lucidum* has been reported [23], including recombinant laccase of *G. lucidum* expressed in *Pichia pastoris* GS115 [24]. Other optimal pH values for *G. lucidum* laccases, such as laccases obtained from *G. lucidum* strain MDU-7 [25] and strain CBS229.93, with ABTS as a substrate have also been reported, which had the same values at pH 5.0 [21]. In our study, the purified laccase was highly stable at pH values ranging from 3 to 5. Especially, at pH 5, the enzyme retained activity of more than 80% after incubation for 120 min (Figure 4B). A similar pH stability of laccase from *G. lucidum* has been reported [22].

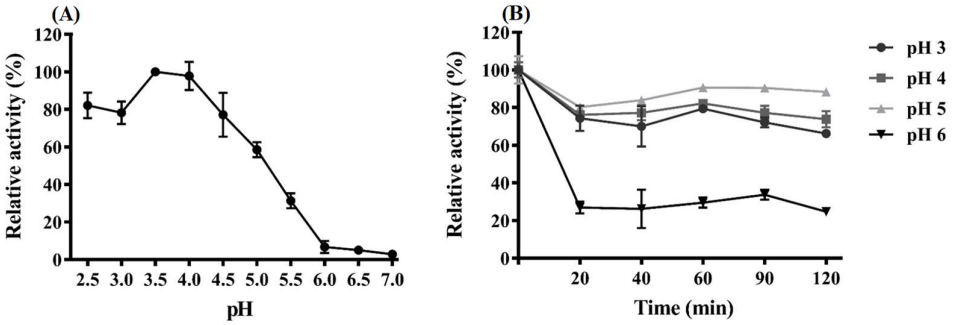

Figure 4 Optimal pH (A) and pH stability (B) of the purified laccase (UbF-BQ1).

3.4 Optimal temperature and temperature stability

The purified laccase showed a broad optimal temperature between 40.0-55.0°C (Figure 5A); among the temperatures tested, the purified laccase showed the highest stability at 30.0°C (Figure 5B). A study reported that laccase from *G. lucidum*-CDBT1 showed an optimum temperature of 30°C, and more than 80% remaining laccase activity was observed [22] after 180 min. This result is similar to that obtained in our study.

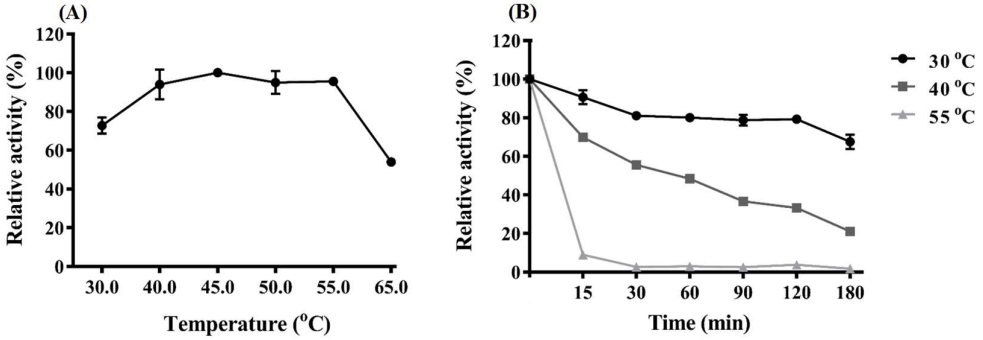

Figure 5 Optimal temperature (A) and temperature stability (B) of the purified laccase (UbP-BQ1).

3.5 Substrate specificity

Three substrates were used in this study: ABTS, 2,6-DMP (2,6-Dimethoxyphenol), and syringaldazine. Each substrate was assayed at an individual optimal pH. ABTS was found to be the most specific substrate for the purified laccase (UbP-BQ1) (Table 2). Our results were similar to those of a previous report [26], which also showed that the best substrate was ABTS among the three substrates tested (ABTS, 2, 6-DMP, and guaiacol). However, *G. lucidum* 77002 (Glac15) laccase tended to use syringaldazine slightly better than ABTS [27]. This difference might be due to different isozyme actions.

Table 2 Substrate specificity of purified laccase (UbP-BQ1).

Substrate	Optimal pH	Relative activity (%)
0.1 mM ABTS	3.5	100.0
0.1 mM 2,6-DMP	4.0	20.6
0.1 mM Syringaldazine	5.5	2.3

3.6 Effects of selected substances and selected ions on laccase activity

Among the substances tested, at 0.1 mM, only *p*-coumaric acid and dithiothreitol (DTT) showed the ability to inhibit laccase (Figure 6A). Especially, DTT at a concentration of only 0.1 mM could inhibit 100% of laccase activity. Reducing agents such as DTT and cysteine, which contain a sulfhydryl group, can affect laccase activity by reducing the oxidized substrate [28]. EDTA could decrease laccase activity by chelating Cu^{2+} ions present at the type 1 copper center; therefore, laccase activity could be inhibited by this substance [29]. Ionic surfactants have been reported to inhibit and stimulate laccase activity [30]. Our results showed that when SDS concentration increases up to 10 mM, laccase activity was inhibited by approximately 60%. This result is similar to that of a previous study [31]. *p*-coumaric acid has been reported as a laccase substrate [32]; therefore, this chemical may compete with the ABTS substrate to bind to the active site of the enzyme, resulting in a decrease in laccase activity.

All metal ions were tested; it was found that when their concentration was increased to 10 mM, they showed the ability to inhibit laccase activity, except for $ZnSO_4$ (Figure 6B). In the presence of 10 mM, almost all types of metal ions resulted in a laccase activity of approximately 70%. $MnCl_2$ seemed to decrease more laccase activity (about 60% remaining laccase activity). It can therefore be speculated that the enzyme laccase obtained from *Ganoderma* sp. displayed moderate stability in the presence of Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, and Cu^{2+} ions, which makes it possible to be applied in the treatment of wastewater containing heavy metals, as well as in pulp and paper industry [29].

Application of laccase on large scale are limited by the cost of production and efficiency of the enzyme [33]. Production of large amounts of laccase can be achieved at lower costs with the use of recombinant protein expression technology [13,34]. Enzyme activity and stability can be improved by protein engineering [35, 36]. Therefore, laccase production would be feasible for commercial use toward a wide range of industrial applications.

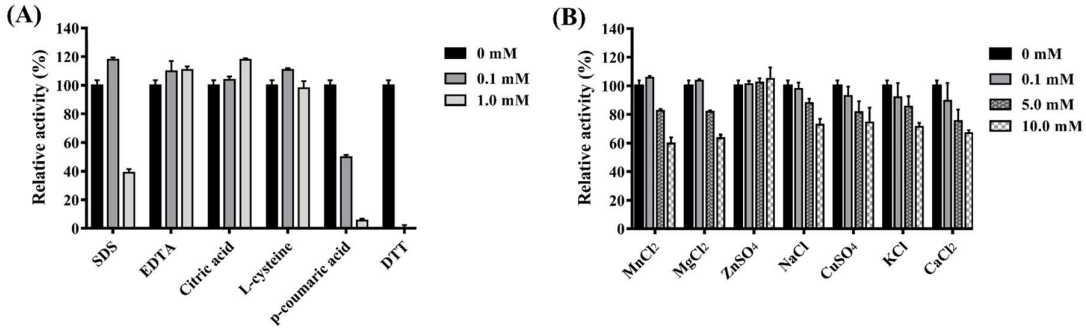

Figure 6 Effects of (A) selected substances and (B) selected ions on laccase activity.

4. Conclusion

Laccase from *Ganoderma* sp.03, grown on a solid substrate comprising rice husk and rice bran, was purified using Phenyl FF Sepharose and Q Sepharose columns. The purified enzyme had a molecular weight of 39.81 kDa which falls within the molecular weight of other reported laccases from *G. lucidum*. The purified enzyme had an optimal pH at pH 3.5 and an optimal temperature between 40.0-55.0°C. The laccase activity was strongly inhibited by DTT and *p*-coumaric acid. However, the enzyme tolerated metal ions at concentrations of up to 10 mM, especially $ZnSO_4$ which had no effect on laccase activity.

5. Acknowledgements

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