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Role of *N*-glycosylation on the specific activity of a *Coprinopsis cinerea* laccase Lcc9 expressed in *Pichia pastoris*

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Laccase Lcc9 from *Coprinopsis cinerea* heterologously expressed in *Pichia pastoris* (rLcc9) displayed different molecular weight and specific activity from the native laccase (nLcc9). Glycosylation may play a role in regulating the Lcc9 specific activity. To elucidate this hypothesis, in this study, firstly we demonstrated that rLcc9 and nLcc9 were glycoproteins, and then enzymatically deglycosylated them. The obtained drLcc9 and dnLcc9 showed an apparent decrease in their specific activities. Three putative *N*-glycosylation sites (N293, N313, and N454) were then predicted in Lcc9 and substituted to evaluate their roles in its specific activity. Molecular weight analysis on those mutants suggested that glycosylation should have occurred on N313 and N454 whereas not on N293 in rLcc9. Comparison of catalytic properties of those mutants revealed that glycosylation at N313 and N454 in rLcc9 could affect the binding affinity to substrates and the catalytic rate, respectively. In addition, the glycosylation could also affect the thermal stability of rLcc9 and nLcc9 since deglycosylation of those Lcc9s resulted in decreases in their thermal stability to some extent. These results will help us to understand the effect of glycosylation on biochemical characteristics of fungal laccases, and provide us support for the improvement of fungal laccase activity based on *N*-linked glycosylation modification.

[**Key words:** *N*-glycosylation; Laccase; Specific activity; *Pichia pastoris*; *Coprinopsis cinerea*]

Fungal laccases (benzenediol: oxygen oxidoreductases, EC1.10.3.2) are a family of blue multicopper oxidases. They are proved to be able to oxidize more than 200 different types of substrates, including aromatic compounds, non-phenolic compounds, polycyclic aromatic hydrocarbons, and dye pollutants, with or without the help of mediators. Simultaneously, the molecular oxygen acts as the sole electron acceptor and is reduced to water, which is the only by-product, during the catalytic process (1,2). Thus, fungal laccases are regarded as one of the most promising green enzymes in many processes such as lignocellulosic compounds delignification, textile colorants transformation, wastewater treatment, as well as work as additives in the food industries (3,4).

Fungal laccases court considerable industrial interests, resulting in a real need of producing prolific quantities of low-cost enzymes for distinct industrial applications. However, achieving this goal is generally impeded by the poor expression of fungal laccases in homologous hosts (5). In comparison, the heterologous expression is considered as one of the efficient ways to obtain fungal laccases. Regarding eukaryotic hosts, *Pichia pastoris* is an excellent expression system that has been successfully used for over 20 years for fungal laccase expression, owing to its high-protein-secretion capability, post-translational modifications, and easy of manipulation (2). More than 40 fungal laccase genes have been successfully expressed in *P. pastoris*. Some of them were expressed in a large amount. For example, expression of a *Moniliophthora roreri* laccase Mrl2 in *P. pastoris* resulting in productivity of 1.05 g protein per liter in an optimized fed-batch process (6).

On the other hand, it should be noted that most fungal laccases especially that from basidiomycetes are glycoproteins carrying small, high-mannose *N*-glycans (7) on 3–10 glycosylation site of conserved Asn-X-Thr/Ser (8). Glycosylation is supposed to play a role in the fine tuning of activity, secretion, copper retention, and thermal stability of laccases (9–11). Fungal laccases heterologously expressed in *P. pastoris* can also be *N*-glycosylated by mannose oligosaccharide. However, *P. pastoris* showed different glycosylation patterns compared to that of basidiomycetes during the last several steps. For instance, the laccases expressed in *P. pastoris* were specifically modified in the endoplasmic reticulum, with a Man_{8}-$GlcNAc_2$ (Man, mannose; GlcNAc, *N*-acetylglucosamine) core structure further processed in the Golgi complex (12,13). The different glycosylation strategies of laccases between the basidiomycetous fungi and *P. pastoris* may cause biochemical property changes in fungal laccases. Actually, compared to the native fungal laccases, some of the fungal laccases obtained by heterologous expression in *P. pastoris* exhibit different biochemical characteristics such as optimal pH and thermal stability (3). However, little

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information has gained on the exact roles of glycosylation on heterologously expressed fungal laccases.

Previously, we obtained a fungal laccase, Lcc9, from basidiomycete *Coprinopsis cinerea* Okayama 7 (#130) (14). To put this enzyme into more practical applications, *lcc9* was cloned and expressed in *P. pastoris* and systematically investigated. The results showed that the recombinant laccase Lcc9 (rLcc9) was over-glycosylated in *P. pastoris*. Furthermore, the specific activity of the rLcc9 was about 3.4 times that of native Lcc9 (nLcc9) (15). As a result, it is reasonable that the different glycosylation between nLcc9 and rLcc9 may responsible for the changes. In this study, the details about the affluence of glycosylation on Lcc9 specific activity were evaluated based on bioinformatics analysis, site-directed mutagenesis, and biochemical characterization comparison. Our results demonstrated that each glycosylation site plays a different role in defining the catalytic activity and physical properties of Lcc9.

MATERIALS AND METHODS

Strains, chemicals, and culture media *C. cinerea* Okayama 7 (#130) (ATCC No. MYA-4618) was a gift from Dr. Patricia J. Pukkila (University of North Carolina). *Gongronella* sp. w5 was from China Center for Type Culture Collection (CCTCC No. AF2012004) and stored at the School of Life Sciences of Anhui University. *P. pastoris* GS115 and the expression vector pPIC9K were purchased from Invitrogen (Carlsbad, CA, USA). Syringaldazine (SGZ), 2,6-dimethoxyphenol (2,6-DMP), and 2,2′-azino-bis (3-ethylbenzothazoline-6-sulfonate) (ABTS) were from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade unless otherwise indicated. Yeast extract peptone dextrose medium (YPD), buffered glycerol-complex medium (BMGY) and buffered minimal methanol medium (BMM) were prepared according to the manual of manufactures (Invitrogen). BMM medium containing 0.1 mM $CuSO_4$ (BMMC) was used as an induction medium for recombinant laccase expression.

Site-directed mutagenesis Mutation of Asn to Gln at positions N293, N313, and N454 in *lcc9* were conducted based on site-directed mutagenesis by using the overlap PCR primers (Table 1) and the plasmid pPIC9K-*lcc*9 that contains the *lcc9* cDNA as the template (15). Positive *P. pastoris* strains (N293Q, N313Q, N454Q, and N313Q/N454Q) harboring the mutant genes were transformed and screened as described by Xu et al. (15) and verified by sequencing.

Expression and purification of rLcc9 and mutant proteins N293Q, N313Q, N454Q, and N313Q/N454Q were cultured and purified according to Xu et al. (15). In brief, cells were cultivated in 250-mL flasks containing 50 mL BMGY medium at 28°C and 220 rpm until the cell destiny of OD_{600} reached 2.0–6.0. Cells were then harvested by centrifugation at 3000 ×*g* and 4°C for 5 min and resuspended in 150 mL BMMC medium in 500-mL flasks at a final cell density of OD_{600} = 1.0. To induce extracellular laccase production, these cultures were incubated at 28°C and 220 rpm with daily addition of 0.5% (v/v) methanol for 9 days. Then supernatants were collected by centrifugation at 6000 ×*g* for 30 min and concentrated to 40 mL in a Minitab Ultrafiltration System with a low-binding regenerated cellulose membrane (Millipore, Bedford, MA, USA). The concentrated supernatant was dialyzed overnight against the citrate-phosphate buffer (20 mM, pH 6.5), followed by centrifugation as described before. Then the supernatant was applied to a DEAE-Sepharose FF column (10 × 200 mm, Amersham Pharmacia, Uppsala, Sweden) pre-equilibrated with citrate-phosphate buffer. The column was eluted with a linear gradient of $(NH_4)_2SO_4$ (0–0.3 M in the citrate-phosphate buffer, with a flow rate of 1.0 mL min^{-1}).

The homogeneity of target proteins was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 12% polyacrylamide gel. The protein concentration was assayed using the Bradford method at 595 nm, with bovine serum albumin as a standard (Sangon Biotech, China). Activity staining of laccases was performed according to Pan et al. (14) in 100 mM citrate–phosphate buffer (pH 4.0) after PAGE using 15 mM ABTS as the substrate.

Deglycosylation assay Purified nLcc9 was prepared according to Pan et al. (14). The deglycosylation reactions of nLcc9 and rLcc9 using Endo H or PNGase F were carried out according to the manufacturer's instructions (New England BioLabs, Ipswich, MA, USA). Briefly, 20 μg of purified nLcc9 or rLcc9 was incubated with 1000 unit of Endo H or PNGase F at 37°C for overnight to generate dnLcc9 or drLcc9. The completion of the deglycosylation reactions was confirmed by SDS-PAGE (12%) and native-PAGE, respectively. The dnLcc9 and drLcc9 were then purified as described above.

Laccase activity assay The assay mixture consisted of 10 μL of appropriately diluted laccase stock and 990 μL of 50 mM citrate-phosphate buffer (pH 4.0) containing 0.5 mM ABTS (ε_{420} = 36,000 M^{-1} cm^{-1}). The reaction was initiated by adding the enzyme into the assay solution. Then the absorbance was measured at 420 nm and 30°C for 3 min. Alternative substrate for the measurement of laccase activity were 100 μM syringaldazine (ε_{525} = 65,000 M^{-1} cm^{-1}). Reactions with heat-treated laccase were used as controls. One activity unit (U) was defined as the amount of enzyme required for oxidizing 1 μmol of substrate per minute (14). All experiments were performed in three independent experiments and in triplicate for each test case.

TABLE 1. Nucleotide sequences of PCR primers.

Primer	Oligonucleotide sequence
lcc9-F	GAATTCATGCAAATCCTTGGCCCGA
lcc9-R	GCGGCCGCTTAAGGAGTTGGGGACAA TTTGG
N293Q-F	AATCTCCCA*CAA*TTCTCTTCTGGAGGT
N293Q-R	AGAAGAGAA*TTG*TGGGAGATTGTTGCT
N313Q-F	CCCAATGCC*CAA*CCCACCAGCACTCCT
N313Q-R	GCTGGTGGG*TTG*GGCA TTGGGAGCGCC
N454Q-F	GCAAGCGAC*CAA*GTCACCATTCGCTTC
N454Q-R	AATGGTGAC*TTG*GTCGCTTGCACCTCC

The underlined line is the mutation site.

Characterization of laccase The effect of pH on protein activity was examined from pH 2.5 to 8.5 at 60°C in 50 mM citrate-phosphate buffer. The effect of temperature on protein activity was measured by incubating proteins within the temperature range of 4–80°C at the optimum pH for every substrate. The enzyme stabilities against pH and temperature were determined by incubating proteins at various temperatures and different pH values, and then the residual activities were determined as described above.

Enzyme kinetics The kinetics and the specificity of proteins toward substrates were measured at the optimum conditions of each protein (Fig. 2). The Michaelis–Menten constants, turnover numbers, and catalytic efficiency (K_m, k_{cat}, and k_{cat}/K_m) were determined by incubating protein with various concentrations of ABTS. The kinetic parameters were then calculated by non-linear regression to the Michaelis–Menten equation using software Origin 8.0.

Molecular mass determination The molecular masses of rLcc9 and mutant proteins were determined by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS). About 0.2 μg protein sample was loaded and dry naturally according to instructions of equipment. The molecular weight of the sample was then measured by a linear method in a positive ion mode. The raw data generated by the 5800 MALDI-TOF/TOF (AB Sciex, Foster City, CA, USA) were exported and analyzed by the 4000 Series Explorer V3.5 software (AB Sciex).

Bioinformatics analysis The potential *N*-glycosylation sites of Lcc9 were predicted by NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). A model of the three-dimensional structure of Lcc9 was obtained using Swiss-model (http://swissmodel.expasy.org/) and nLcc4 from *Lentinus* sp. as the template (PDB #3X1B). The sequence identity between Lcc9 and Lcc4 is 53.36%.

RESULTS

Deglycosylation of rLcc9 and nLcc9 with Endo H Compared to nLcc9 from *C. cinerea* that showed a specific activity of 92.9 U/mg, our previous results showed that rLcc9 expressed in *P. pastoris* showed a specific activity of 315.3 U/mg. The hyper-glycosylation of rLcc9 may play a role in promoting its specific activity (15). To elucidate this hypothesis, drLcc9 and dnLcc9 were generated and their biochemical properties were analyzed and compared with rLcc9 and nLcc9, respectively. However, treated nLcc9 and rLcc9 with PNGase F resulted in no change in their molecular weight as shown on SDS-PAGE, even incubated at 37°C for overnight (Figs. S1 and S2). In comparison, after treated with Endo H, drLcc9 and dnLcc9 migrated faster than rLcc9 and nLcc9 both on SDS-PAGE and native-PAGE (Fig. 1), suggested that both rLcc9 and nLcc9 were glycoproteins. Because the molecular weight of PNGase F (36 kDa) is heavier than Endo H (29 kDa), it was suggested that the steric hindrance of the proteins in their active form may cause the result.

As shown on native-PAGE, drLcc9 showed significantly lower specific activity than rLcc9, while dnLcc9 was slightly lower than that of nLcc9 (Fig. 1). Further experiment showed that the specific activity of drLcc9 was 119.9 U/mg, which is about 2.63 times lower

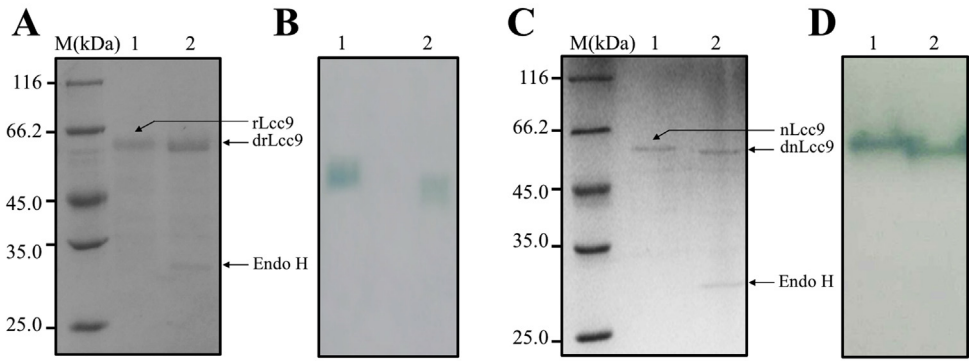

FIG. 1. Analysis of molecular weight and enzymatic activity of native and Endo H deglycosylated Lcc9(s) by 12% SDS-PAGE and native-PAGE. (A) Unheated laccase samples in lysis buffer, in which the enzymes were not denatured and remained active, were separated in SDS-PAGE. M, protein marker; lane 1, recombinant Lcc9 (rLcc9); lane 2, rLcc9 treated with Endo H for overnight. (B) Unheated laccase samples in lysis buffer, in which the enzymes were not denatured and remained active, were separated in native-PAGE and analysis using ABTS substrate. Lane 1, recombine Lcc9 (rLcc9); lane 2, rLcc9 treated with Endo H for overnight. (C) Unheated laccase samples in lysis buffer, in which the enzymes were not denatured and remained active, were separated in SDS-PAGE. M, protein marker; lane 1, native Lcc9 (nLcc9); lane 2, nLcc9 treated with Endo H for overnight. (D) Unheated laccase samples in lysis buffer, in which the enzymes were not denatured and remained active, were separated in native-PAGE and analysis using ABTS substrate. Lane 1, native Lcc9 (nLcc9); lane 2, nLcc9 treated with Endo H for overnight.

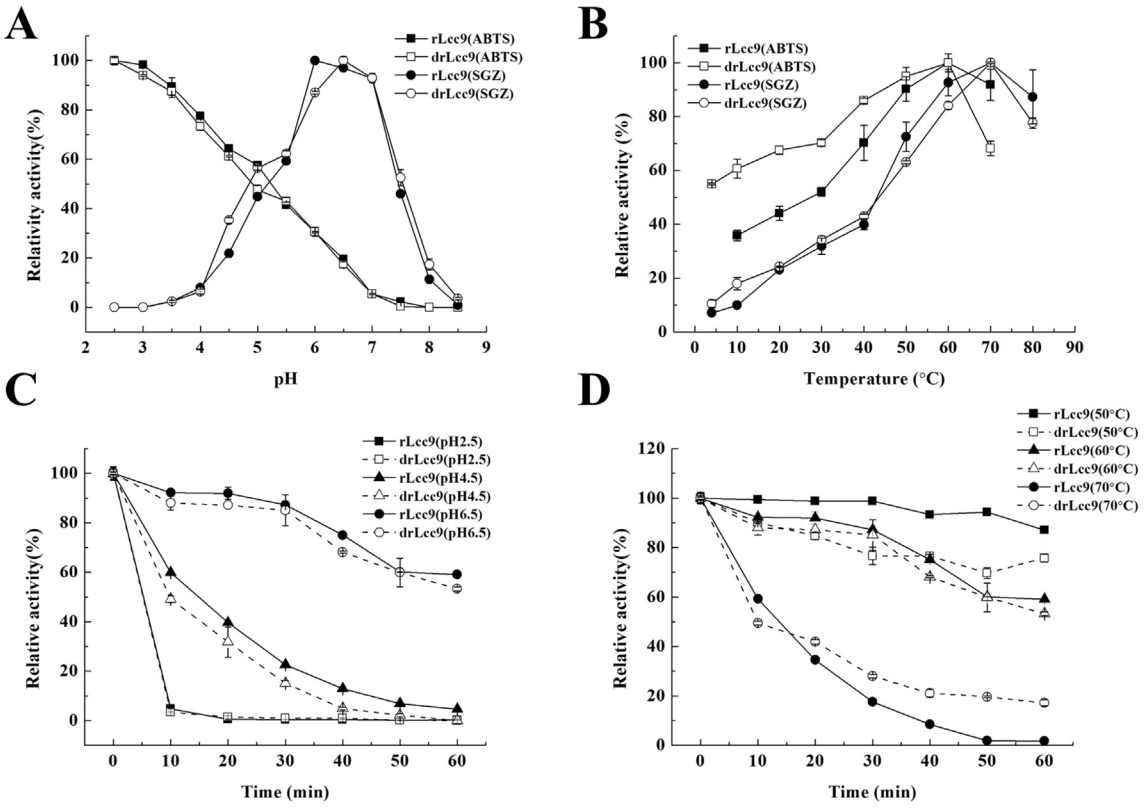

FIG. 2. Effects of pH (A, C) and temperature (B, D) on the activity and stability of rLcc9 and drLcc9. The data presented are the average values from triplicate measurements.

than that of rLcc9. dnLcc9 possessed a specific activity of 79.7 U/mg, in comparison to that of 92.9 U/mg of nLcc9.

The optimum temperature and pH of the deglycosylated proteins were basically consistent with rLcc9 and nLcc9 (Figs. 2A,B and 3A,B). rLcc9 and drLcc9 also showed comparable pH and thermal stabilities (Fig. 2C and D). For instance, after incubation at pH 6.5 and 50°C for 1 h, rLcc9 and drLcc9 retained 87.10 ± 0.22% and 75.81 ± 1.58%, respectively, of the original activities (Fig. 2D). However, nLcc9 and dnLcc9 showed apparent differences in terms of pH and thermal stabilities (Fig. 3C and D). nLcc9 was more stable than dnLcc9 at the temperature range of 50–70°C. For example, the half-lives of nLcc9 and dnLcc9 were 40 min and 10 min, respectively, at pH 6.5 and 70°C (Fig. 3D).

When tested using ABTS as a substrate, deglycosylation did not apparently change the K_m value of rLcc9. In comparison, the turnover number (k_{cat}) of drLcc9 decreased by 6.34-fold compared to that of rLcc9. As a result, the catalytic efficiency of drLcc9 was about a quarter of that of rLcc9 (Table 2). On the other hand, the K_m of dnLcc9 increased by 1.76-fold compared to that of nLcc9, and the turnover number (k_{cat}) of dnLcc9 decreased by about 50% compared to that of nLcc9. Correspondingly, the catalytic efficiency of dnLcc9 was about 17% of that of nLcc9 (Table 2). In total, our results

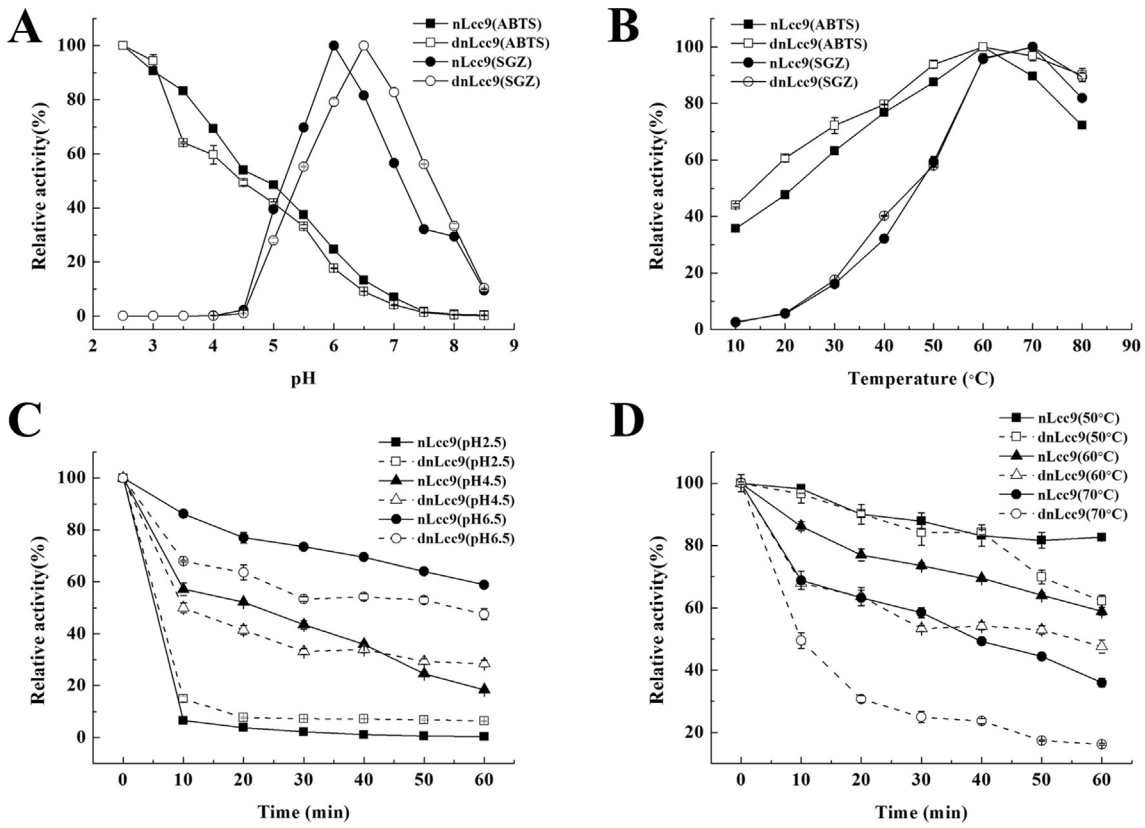

FIG. 3. Effects of pH (A, C) and temperature (B, D) on the activity and stability of nLcc9 and dnLcc9. The data presented are the average values from triplicate measurements.

evidenced that glycosylation affects the specific activity, as well as kinetic parameters of rLcc9 and nLcc9.

Contribution of each *N*-glycan on the enzymatic characteristics of rLcc9 To evaluate the effect of glycosylation at each site on Lcc9 properties, the potential glycosylation sites were predicted in Lcc9 amino acid sequence by using the NetNGlyc 1.0 Server. Three potential glycosylation sites were predicted in Lcc9 at residues N293, N313, and N454 based on the consensus sequence of Asn-X-Thr/Ser. Comparison of the Lcc9 amino acid sequence with other characterized fungal laccase sequences showed that glycosylation site N454 was highly conserved in fungal laccases, whereas N293 and N313 have no counterpart (Table 3).

Three asparagine residues in potential glycosylation sites were independently converted to glutamine residues to determine the effect of each *N*-glycan on the enzymatic characteristics. Three mutant laccases, including N293Q, N313Q, and N454Q were expressed as extracellular secreted proteins under methanol induction for 8 days. They were successfully purified from the culture supernatants and showed different molecular weights on SDS-PAGE (Figs. 4A and S3). Further analysis showed that the molecular weights of rLcc9 and three mutant proteins (N293Q, N313Q, and N454Q) were 60.19 kDa, 59.99 kDa, 57.24 kDa, and 59.34 kDa, respectively, as determined by using MALDI-TOF/MS (Table 4 and Fig. S4). These results suggested that N293 might not have been glycosylated, while the other two glycosylation sites (N313, N454) were decorated with high-mannose-type oligosaccharides. Based on the molecular weights, the glycosylation patterns of N313 and N454 were postulated to be $Man_{14\text{-}16}(GlcNAc)_2$ and $Man_{2\text{-}4}(GlcNAc)_2$ (Table 4) (16).

Native-PAGE analysis showed that the rLcc9 and N293Q laccases exhibited activity to substrate ABTS within 9 min, while the activities of the N313Q and N454Q mutants appeared at around 20 min (Fig. 4B). The specific activities of purified N293Q, N313Q, and N454Q to ABTS were 318.6, 57.2, and 64.8 U/mg, refer to 101%, 28% and 25% of specific activity relative to rLcc9 (100%), respectively (Table 2), suggesting that loss of *N*-glycosylation chain at the two sites N313 or N454 caused significant changes in Lcc9 specific activity. Results from the double point mutant protein N313Q/N454Q further supported this conclusion, e.g., the specific activity of purified N313Q/N454Q was 82.5 U/mg (Table 2).

TABLE 2. Kinetic properties of proteins.

Enzyme	Specific activity (U/mg)	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	Reference
nLcc9	92.9	$(1.10\pm0.05)\times10^{-5}$	213.83 ± 0.74	1.95×10^{7}	15
dnLcc9	79.7	$(3.01\pm0.08)\times10^{-5}$	100.82 ± 2.86	3.35×10^{6}	This study
rLcc9	315.3	$(2.24\pm0.02)\times10^{-5}$	604.22 ± 3.09	2.70×10^{7}	15
drLcc9	119.9	$(1.44\pm0.10)\times10^{-5}$	95.33 ± 1.67	6.62×10^{6}	This study
N293Q	318.6	$(4.52\pm0.46)\times10^{-5}$	744.28 ± 65.56	1.65×10^{7}	This study
N313Q	57.2	$(7.67\pm0.25)\times10^{-5}$	286.73 ± 13.03	3.74×10^{6}	This study
N454Q	64.8	$(2.55\pm0.46)\times10^{-5}$	56.20 ± 9.09	2.21×10^{6}	This study
N313Q/N454Q	82.5	$(1.96\pm0.19)\times10^{-5}$	77.00 ± 0.18	3.93×10^{6}	This study

TABLE 3. The sequence alignments of *Coprinopsis cinerea* Lcc9 with other Basidiomycete laccases.

Species	Glycosylation sites		
	Asn^{293}	Asn^{313}	Asn^{454}
Lcc9	287 GSNNLPNFSSGGINS	305 RYAGAPNANPTSTPV	452 SDNVTIRFTTDNPGP
Lentinus sp.	287 GNVGFT ….NGINS	301 RYDGAAVAEPATAIP	456 GDNVTIRFVTDNPGP
T. hirsuta	266 GNVGFD ….GGINS	280 RYDGAPAVEPTTNQT	434 GDNVTIRFDTNNPGP
C. maxima	266 GNVGFN ….GGINS	280 RYDGAPAVEPTTNQT	434 GDNVTIRFLTNNPGP
T. versicolor	266 GNVGFT ….GGINS	280 RYDGAAAVEPTTTQT	434 GDNVTIRFRTDNPGP
T. ochracea	266 GNVGFT ….NGINS	280 RYSGAAATQPTTSQT	434 NDNVTIRFKTNNPGP
C. gallica	265 GTVNFA ….GGTNS	279 RYDGAAPVEPTTSQT	431 GDNVTIRFRTDNPGP
T. trogii	265 GTRNFD ….GGVNS	279 RYDGAAPVEPTTSQT	431 GDNVTIRFRTDNPGP
L. tigrinus	266 GTTGFA ….DGVNS	280 RYDDADPVEPVTNQT	433 GDNVTIRFQTDNPGP

The predicted *N*-glycosylation sites on Lcc9 are underlined. *Lentinus* sp. (PDB #3X1B), *Trametes hirsute* (PDB #3FPX), *Cerrena maxima* (PDB #3DIV), *Trametes versicolor* (PDB #1KYA), *Trametes ochracea* (PDB #2HZH), *Coriolopsis gallica* (PDB #4A2E), *Trametes trogii* (PDB #2HRG), and *Lentinus tigrinus* (PDB #2QT6).

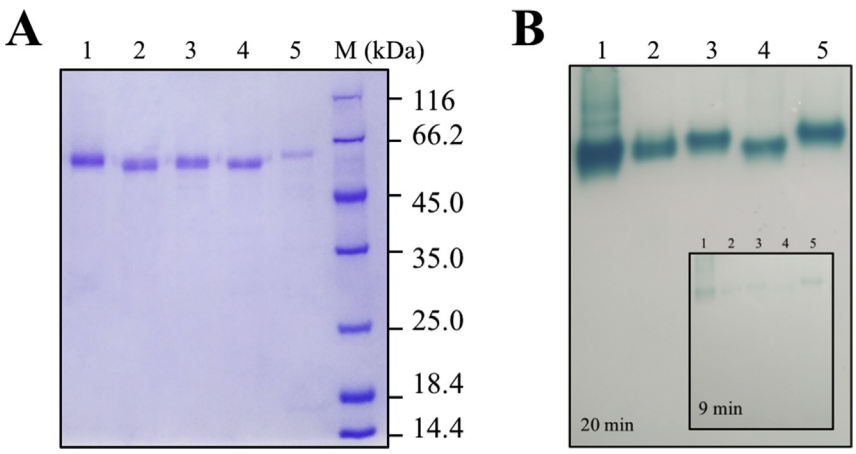

FIG. 4. Expression profile of recombinant wild-type and mutant forms of Lcc9 in the cultural media of *P. pastoris*. (A) SDS-PAGE of WT and mutant Lcc9 proteins. Lane 1, N293Q; lane 2, N313Q; lane 3, N454Q; lane 4, N313Q/N454Q; lane 5, rLcc9; M, protein marker. (B) Native-PAGE of WT and mutant Lcc9 proteins. Lane 1, N293Q; lane 2, N313Q; lane 3, N454Q; lane 4, N313Q/N454Q; lane 5, rLcc9.

TABLE 4. Molecular mass of rLcc9 and its mutants expressed in *P. pastoris* and the presumed glycan structures at different asparagine residue.

	Relative molecular mass (kDa)[a]	Presumable *N*-glycan structure[b]
rLcc9	60.19 ± 0.1	–
N293Q	59.99 ± 0.1	–
N313Q	57.24 ± 0.1	$Man_{14\text{-}16}\ (GlcNAc)_2$
N454Q	59.34 ± 0.1	$Man_{2\text{-}4}\ (GlcNAc)_2$

[a] The MALDI-TOF mass spectrometry is shown in Fig. S2.
[b] The *N*-glycan structure at each site was presumed based on reports of the *N*-glycan structure pattern.

rLcc9 and its four *N*-glycosylation mutants (N293Q, N313Q, N454Q, and N313Q/N454Q) showed the same optimal temperatures and optimal pHs (data not shown). However, N454Q and N313Q/N454Q were apparently less stable than the rLcc9 when incubated at pH 6.5 and 60°C (Fig. 5). The two mutants retained 75% and 66% of their original activities, respectively, after 20 min incubation at pH 6.5 and 60°C. In comparison, rLcc9 retained 94% of the original activity under the same condition (Fig. 5A).

The catalytic efficiency of nLcc9, rLcc9 and N293Q were 1.95×10^7, 2.70×10^7 and 1.65×10^7, respectively. Therefore, we concluded that there was not much difference refers to the catalytic efficiency of nLcc9, rLcc9, and N293Q. However, a decrease of 7.22, 12.22 and 6.87-fold in k_{cat}/K_m was noted for N313Q, N454Q, and N313Q/N454Q compared to rLcc9 (Table 2). Among them, the K_m of N313Q showed a 3.42-fold increment compared to rLcc9, while the turnover number (k_{cat}) of N454Q and N313Q/N454Q were decreased 10.75 and 7.85-fold, respectively, when compared to rLcc9 (Table 2). These results showed that de-glycosylation at N313 affects the affinity of the enzyme toward the substrate (ABTS), and de-glycosylation at N454 affects the catalytic rate.

Structural modeling confirms the critical role of *N*-glycan Sequence blast result showed that laccase Lcc4 from Basidiomycete *Lentinus* sp. shared 53.4% sequence identity with Lcc9 (8). As a result, a three-dimensional structure of the *C. cinerea* nLcc9 was modeled using the crystallographic structure of Lcc4 as a template. The model structure of nLcc9 comprises 31 β-strands, 4 α-helices, and 9 3/10-helices, which fold into three sequentially-arranged cupredoxin-like domains: domain 1 (D1, residues 22–149), domain 2 (D2, residues 167–306), and domain 3 (D3, residues 347–517) (Fig. 6A).

The model structure clearly showed that N293 and the other two *N*-glycosylation sites were located in two loops (D1-D2, D2-D3). Particularly, N293 was located in a flexible loop and had no counterpart in *Lentinus* sp. Lcc4. On the other hand, structural alignment clearly showed that N454 of nLcc9 corresponded to N458 of Lcc4 (Fig. 6A and B), which was confirmed as a glycosylation site (8). The third predicted glycosylation site of nLcc9, N313, corresponded to an E in other laccases (Table 3), which is not a glycosylation site, suggesting that N313 was a unique glycosylation site in nLcc9.

DISCUSSION

Till now, about 40 fungal laccases have been heterologously expressed in *P. pastoris* (17,18). It is well known that *P. pastoris* can *N*-glycosylate proteins through mannose oligosaccharide (12). Due

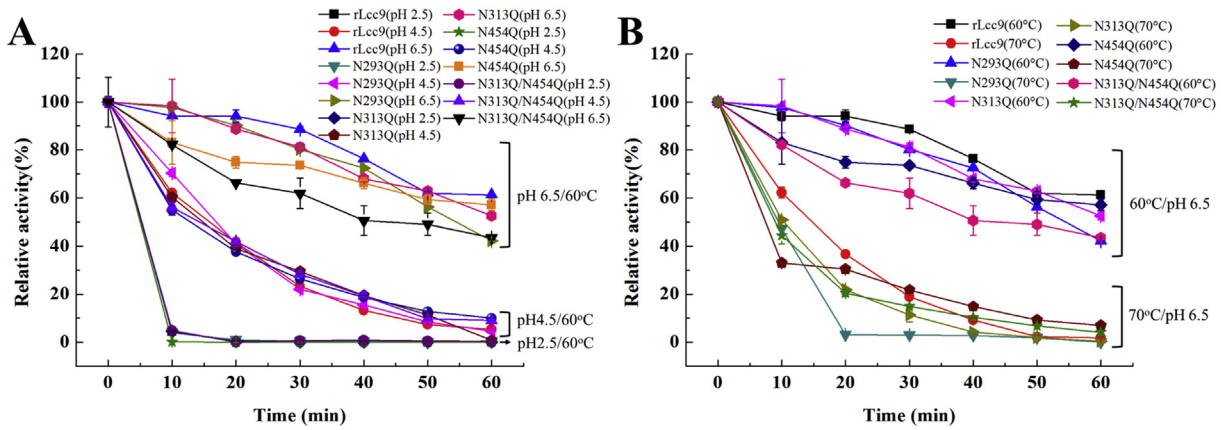

FIG. 5. The changes of the stabilities of rLcc9 and mutant Lcc9 proteins. (A) pH stabilities; (B) temperature stabilities. The data presented are the average values from triplicate measurements.

to the different glycosylation patterns between the host and wild-type strain, more than 10 heterologous expressed fungal laccases exhibit different biochemical characteristics compared to the native fungal laccases. For example, Garg et al. (19) reported that the changed pattern of glycosylation increased thermal stability and salt tolerance of the laccase from *Cyathus bulleri* after expressed in *P. pastoris*. In another case, the hyper-glycosylation caused the decreased substrate affinity of laccase from *Trametes versicolor* because the addition of a large glycan attached to the protein backbone can alter the structure and consequently the function of the protein local architecture (20). However, the effect of glycosylation on the specific activity of fungal laccases is rarely reported. Previously, we found that the glycosylation may affect the specific activity of Lcc9 of *C. cinerea*. Thus, in this study, we did a systematic analysis to get deep insight into the effect of glycosylation on promoting Lcc9 specific activity.

Fungal laccases are glycosylated proteins that usually contain 3–10 potential glycosylation sites in the amino acid sequences (4). Consistent to most fungal laccases, our data showed that rLcc9 and nLcc9 were glycosylated laccases as suggested by Endo H hydrolyzation experiments (Fig. 1). Three glycosylation sites (N293, N313, and N454) were predicted in Lcc9 amino acid sequence (Table 3). However, based on the mutation experiments and MALDI-TOF/MS analysis, results showed that only N313 and N454 were occupied by glycans (Table 4). The fact that not all the predicted glycosylation sites are occupied by glycans was also reported in other cases (8,21). For example, four potential *N*-glycosylation sites (N75, N162, N238, and N458) were predicted in nLcc4 of *Lentinus* sp. However, N162 was actually not glycosylated in the protein (8). Three potential *N*-glycosylation sites (N432, N468, and N474) were predicted in DLac of *Cerrena* sp. RSD1, but only two *N*-glycosylation sites (N432 and N468) were observed in the crystal structure (21). In total, our results demonstrated that rLcc9 was a glycosylated fungal laccase with two sites occupied by glycans.

drLcc9 and dnLcc9 showed apparently lower specific activity than that of rLcc9 and nLcc9, respectively (Fig. 1 and Table 2). In particular, compared to rLcc9, the single point mutation (N313Q or N454Q) and double point mutation (N313Q/N454Q) caused significant loss of laccase specific activities (4–5 folds) (Table 2). All of these results support our previous hypothesis that glycosylation affects the specific activity of Lcc9. However, enzymatic cleavage of the *N*-glycans presented in the laccase from *Pycnoporus sanguineus* with endoglycosidase F1 resulted in slight changes in the kinetic parameters of the enzyme (20). Furthermore, when compared to the specific activities of drLcc9, which remained only one GlcNAc residue at each *N*-glycosylation site, mutant proteins including N313Q, N454Q, and N313Q/N454Q showed even lower specific activities, indicating that GlcNAc played some role in Lcc9 specific activity. Different from our results, comparison of the biochemical properties of laccase nLcc4 from *Lentinus* sp. with its Endo H

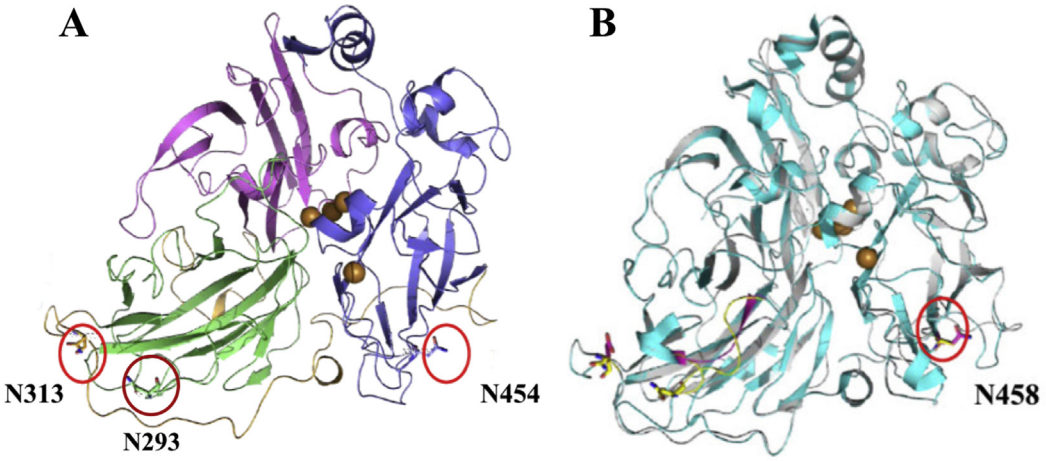

FIG. 6. The substructure of the three glycosylation sites in Lcc9. (A) The substructure of Lcc9 predicted by SWISS-MODEL. Three domains were highlighted in different colors, D1 (red), D2 (green) and D3 (blue) respectively; (B) comparison of glycosylation sites between Lcc9 of *C. cinerea* and Lcc4 of *Lentinus* sp.

deglycosylated form indicated that the GlcNAc residue remained at each of the three *N*-glycosylation sites play a critical role in keeping the kinetic efficiency of the enzyme (16).

Mutation experiments were conducted using rLcc9 to get a deep insight into how the glycosylation affects Lcc9 specific activity. Our results suggested that glycosylation at N454 may have little effect on substrate affinity, but have significant effect on the catalytic rate of the enzyme toward ABTS. N454 was identical to the N458 in nLcc4 of *Lentinus* sp. (Fig. 6) (8). According to the theory of Rivera-Hoyos, it is speculated that the removal of the glycan on N454 resulted in the increased motion of substrate binding pocket loops I, II, and IV. The D2-D3 loop also underwent a profound conformational change, resulting in the change in the catalytic rate (8). In comparison, laccase from *Cerrena* sp. RSD1 also showed no significant difference on k_{cat}/K_m after treated with Endo H. However, its catalytic efficiency decreased by 41% when the laccase was treated with PNGase F (21).

On the other hand, our results showed that glycosylation at N313 influenced the protein's affinity toward the substrate. N313 have no counterpart when compared to other fungal laccases (Table 3). From the assimilated Lcc9 structure, N313 was located in a flexible loop between Domain 2 and Domain 3 on the surface of the protein and showed no apparent contact with the local amino acids (Fig. 6). As a result, we proposed that this effect should not be due to a global change in the enzyme structure. It is reasonable that the large glycan chain will cause the redistribution of charges at the local place. In addition, recently, two small molecule transport channels from the surface of laccase to TNC catalytic center were assimilated, they input oxygen molecules and release product water molecules, respectively (Fig. S5). N313 was located near these two channels (Fig. S5). As a result, we proposed glycosylation at N313 may cause the local variations in the structure that probably at the periphery of the active site of the protein.

In addition, drLcc9 and dnLcc9 showed relatively lower pH and thermal stabilities than that of rLcc9 and nLcc9, respectively (Figs. 2 and 3). This is consistent with the observations that Vite-Vallejo and coworkers made on dLcc from *P. sanguineus*, which was less stable at temperatures below 50°C and decrease the activity at 20°C (22). Furthermore, we noticed that deglycosylation caused apparently different changes in stabilities and substrate affinity between nLcc9 (dnLcc9) and rLcc9 (drLcc9) (Figs. 2 and 3). Because nLcc9 showed a relatively lower molecular weight than the rLcc9, and the fact that *C. cinerea* and *P. pastoris* possess different glycosylation patterns, we proposed that the different chain lengths and/or the glycosylation on different sites between rLcc9 and nLcc9 may cause the different properties among nLcc9, dnLcc9, rLcc9, and drLcc9. However, we failed to remove the glycans from rLcc9 and dLcc9 by using PNGase F due to the steric hindrance of the proteins. As a result, further experiments such as overexpression of these mutant proteins in *C. cinerea* are necessary to elucidate this hypothesis.

In conclusion, rLcc9 was *N*-glycosylated when expressed in *P. pastoris*. The *N*-glycans of rLcc9 on the different sites had different functions for the enzymatic properties of the laccase Lcc9. Our report may also provide theoretical support for the improvement of laccase activity and stability based on the *N*-linked glycosylation modification to meet the future needs of the biotechnological industry.

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