JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Purification and Characterization of a Temperature- and pH-Stable Laccase from the Spores of *Bacillus vallismortis* fmb-103 and Its Application in the Degradation of Malachite Green

Chong Zhang, Shuang Zhang, Hanwen Diao, Haizhen Zhao, Xiaoyu Zhu, Fengxia Lu, and Zhaoxin Lu*

Laboratory of Enzyme Engineering, College of Food Science and Technology, Nanjing Agriculture University, Nanjing 210095, People's Republic of China

ABSTRACT: Malachite green residue can affect aquaculture food safety. Bioremediation of contaminated water by enzyme treatment is an environmentally friendly and economical way to remove contaminating substances. In the present study, a temperature- and pH-stable laccase was purified from the spores of *Bacillus ballismortis* fmb-103 and was used to degrade malachite green. The laccase from fmb-103 (fmb-L103) was purified 15.2-fold to homogeneity (389.9 mU/mg protein with respect to ABTS as a substrate) by precipitation with 30−80% $(NH_4)_2SO_4$, DEAE-Sephadex A-50 ion exchange chromatography, and Sephadex G-100 chromatography. fmb-L103 is a nonblue laccase with a molecular weight of 55.0 kDa and Cu content of 2.5 (mol:mol). fmb-L103 retained more than 50% activity after 10 h at 70 °C and demonstrated broad pH stability in both acidic and alkaline conditions. The effects of inhibitors and metal ions on fmb-L103 activity were also examined. A kinetic study revealed that ABTS was a suitable substrate with a K_m of 22.7 μmol and a V_{max} of 3.32 μmol/mL/min. fmb-L103 can efficiently degrade malachite green after a 48 h treatment period in combination with a mediator, without the appearance of leucomalachite green.

KEYWORDS: *Bacillus vallismortis fmb-103, laccase, purification and characterization, malachite green degradation*

INTRODUCTION

Malachite green (MG) is a type of triphenylmethane dye, which can be used as an effective therapy for the treatment of ectoparasitic and fungal infections in aquaculture.[1] However, the use of MG is limited because of its metabolism to unwanted toxic metabolites. It is well known that MG can be readily absorbed by fish and metabolically reduced to the lipophilic leucomalachite green (LMG). Residues of MG and LMG in water or in fish can result in serious pollution of the ecological environment and also affect the safety of food products and consequently threaten human health. As a result, the use of MG in aquaculture has been prohibited in many countries since the 1990s.[1] However, MG is still illegally used in the fish-farming industry because of its low cost, easy availability, and high efficacy against fungi, bacteria, and parasites.[2]

To date, many researchers have investigated the distribution of MG in aquatic foodstuffs using a variety of detection technologies.[3−5] In addition, it has been important to consider appropriate means of degrading MG to maintain pollution-free aquatic food products. Various combinations of physical, chemical, and biological technologies useful for restoring farming water have been investigated. Compared with physical and chemical methods, enzyme-catalyzed processes are environmentally friendly and economical for purposes of remediation.[6]

Laccase is an enzyme involved in the catalytic decomposition of dyes, and it has been investigated for its biotechnological applications in bioremediation of contaminated soil or wastewater.[6] Laccase (EC 1.10.3.2) is a blue multicopper oxidase that catalyzes the oxidation of a variety of aromatic substrates coupled with the reduction of molecular oxygen to water.[6] These enzymes were first detected in the varnish tree *Rhus vernicifera* and subsequently in many other plants, insects, Archae, and bacteria.[7] Many fungal laccases have been purified and characterized[8−10] and have been used in the removal of dyes. However, fungal laccases usually lose their activity rapidly at high temperature and pH.[11] Compared with fungal laccases, bacterial laccases are much more thermotolerant and pH stable, two characteristics that are advantageous for the biodegradation of industrial textile dyes.[12] However, to date, only a few spore laccases have been purified and characterized.[13−15]

In our previous work, we described a *Bacillus vallismortis* strain fmb-103 that produces a spore laccase.[16] It was quite stable at high temperature and demonstrated broad pH stability in both acidic and alkaline conditions. It could efficiently degrade triphenylmethane dyes in combination with a mediator. However, spores containing polysaccharides, protein, nucleic acids, or some other substance may influence the catalytic process of laccase. In the present study, laccase from the spores of *B. vallismortis* strain fmb-103 was purified, characterized, and tested in the MG degradation process.

MATERIALS AND METHODS

Bacterial Strain. *B. vallismortis* strain fmb-103 producing a spore laccase was isolated as described elsewhere.[16] The fmb-103 strain was cultured in LB liquid medium at 37 °C, 180 rpm for 12 to 14 h.

Purification of Laccase from *Bacillus vallismortis* fmb-103. Preparation of the spores of this novel dye degradation microorganism was carried out as earlier reported.[17] The collected spores were ground for 5 min, and then the isolation of the proteins from the spores was performed as described earlier.[15]

Received: March 7, 2013
Revised: May 22, 2013
Accepted: May 24, 2013
Published: May 24, 2013

Table 1. Purification of the Laccase from Spores of Strain fmb-103

purification	total activity (mU)	total protein (mg)	specific activity (mU/mg)	recovery (%)	purification (fold)
crude mixture (isolation from the spores)	12 454.8	487.2	25.6	100	1
$(NH_4)_2SO_4$ (30−80%)	9335.5	173.1	53.9	74.9	2.1
DEAE-Sephadex A-50	6607.2	41.1	160.8	53.4	6.3
Sephadex G-100	3665.9	9.4	389.9	29.4	15.2

The proteins isolated from the spores were precipitated overnight with 80% ammonium sulfate by slowly stirring at 4 °C for 2 h. The precipitate was then dissolved in 0.05 M potassium phosphate buffer (pH 5.0) and dialyzed against distilled water using a cellulose membrane. The highest activity fraction was loaded onto a DEAE-Sephacel column equilibrated in 0.05 M potassium phosphate buffer (pH 5.0), and bound protein was eluted with a liner gradient formed from three buffer solutions containing 0.1, 0.3, and 0.5 M NaCl at a flow rate of 0.5 mL/min. Fractions with laccase activity were collected and loaded onto a Sephadex G-100 column equilibrated with 0.5 M potassium phosphate buffer (pH 5.0). Elution was carried out with the same buffer at a flow rate of 0.3 mL/min. The active fractions were pooled and concentrated.

The protein concentration was determined by the method of Bradford using bovine serum albumin as the standard. The purity of the laccase was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with a 5% stacking gel and 10% separating gel. Proteins were stained with Coomassie Brilliant Blue R-250. PAGE under nondenaturing conditions was performed using 8% (w/v) polyacrylamide gels,[9] and activity was detected in the gels using the guaiacol assay in citrate-phosphate buffer (pH 5.0).

Enzyme Assay. Laccase activity was assayed at 45 °C using ABTS and syringaldazine as the substrate. The oxidation of ABTS (1 mM) was measured at 420 nm (ε_{420} = 36 000 M^{-1} cm^{-1}) in 0.1 M citrate-phosphate buffer.[14] The oxidation of syringaldazine (SGZ) (0.1 mM) was detected at 525 nm (ε_{525} = 65 000 M^{-1} cm^{-1}) in 0.1 M citrate-phosphate buffer.[14] The oxidation of 2,6-dimethoxyphenol (2,6-DMP) (0.1 mM) was detected at 465 nm (ε_{465} = 49 600 M^{-1} cm^{-1}) in 0.1 M citrate-phosphate buffer.[18] The oxidation of guaiacol (0.1 mM) was detected at 465 nm (ε_{465} = 49 600 M^{-1} cm^{-1}) in 0.1 M citrate-phosphate buffer.

Spectrum and Copper Content. A sample of 1.5 mg/L of the purified laccase was subjected to a wavelength scan in the range 200−800 nm using a UV−visible spectrophotometer. The Cu content of the purified laccase was determined by a colorimetric assay using the chromogenic ligand 1,10-phenanthroline monohydrate as described elsewhere.[19]

Biochemical Characterization of the Purified Laccase. *Effect of Temperature and pH on Laccase Activity and Stability.* The optimum temperature for activity was determined using ABTS as the substrate at different temperatures (25−90 °C). For determination of temperature stability, the laccase solution was incubated for 10 h at different temperatures (70, 80, and 90 °C); then the residual activity was measured. The enzyme storage stability was determined at 20 °C for the selected times.

The effect of pH on the activity of the laccase was determined at 45 °C in 0.1 M citrate-phosphate buffer (pH 2.2−7.0) or 0.1 M Tris-HCl buffer (pH 7.0−9.0). The pH stability of the laccase was assayed by incubating the laccase at 4 °C at pH 3.0, 7.0, and 8.0 and then measuring the residual activity.

The activity at the optimum temperature or optimum pH was used as the control to determine the effect of the temperature or the pH on the laccase, respectively. For the temperature or pH stability experiments, the test system without manipulations at these conditions was used as the control.

Effect of Inhibitors on Laccase Activity. The effects of inhibitors on spore activity at different concentrations were determined by the enzyme assay described above using ABTS as the substrate. The purified laccase was preincubated with various inhibitors for 10 min at 37 °C. Residual activity was expressed as the percentage of the activity observed in the absence of any compound.

Effect of Metal Ions on Laccase Activity. The effects of metal ions on the activity were investigated in the presence of K^+, Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, and Fe^{2+}. The purified enzyme was preincubated with various metal ions at a concentration of 5 mM for 10 min at 37 °C, and the residual activities were determined. The activity of the enzyme in the absence of metal ions was taken as 100%.

Kinetic Constants of the fmb-L103. The substrate specificity of the enzyme was determined at the wavelength of maximum absorption of each substrate including ABTS, 2-DMP, SGZ, and guaiacol. Different concentration ranges of the four substrates were used for the kinetic studies.

Degradation Tests. Degradation of MG was tested in the presence or absence (0.1 mol/L) of ABTS, acetosyringone, and syringaldehyde as the mediator. The enzymatic treatment was performed in 6 mL of 0.1 M citrate-phosphate buffer (pH 4.0), containing fmb-L103 (400 mU/mL) and MG (50 mg/L). The reaction mixtures were incubated at 37 °C for 24 h. The degradation of MG was determined spectrophotometrically as the relative decrease of absorbance at the maximal absorbance wavelength of 617 nm.

Decolorization activity was calculated according to Parshetti et al. (2006).

$$\%\text{Decolorization} = [(I - F)/I] \times 100$$

where I = initial absorbance and F = absorbance of decolorized medium.

Decolorization was monitored by UV−vis spectroscopic analysis. Spectral analysis was carried out using a UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan), where changes in its absorption spectrum (200−800 nm) were recorded.

The LMG Elisa kit (CFC019D) was purchased from Suzhou ARD Biological Co. Ltd. (China) and was used to detect LMG.

All assays were carried out in triplicate.

RESULTS AND DISCUSSION

Purification of the Laccase from Strain fmb-103 Spores. The laccase from *B. vallismortis* fmb-103 was purified using a combination of chromatographic steps listed in Table 1. Before performing the purification steps, the spores with laccase activity (6 U/g) were disaggregated as described in the Materials and Methods section. After isolation, the crude mixture from the spores was subjected to ammonium sulfate precipitation and DEAE-Sephadex A-50 and Sephadex G-100 column chromatography to purify the enzyme to homogeneity. The collected fraction showed a single band of approximately 55 kDa by SDS-PAGE (Figure 1a), and the laccase active band was visualized by soaking the gel in guaiacol solution (Figure 1b). This active protein was named fmb-L103. The enzyme was purified 15.2-fold, with a final yield of 29.4%. The specific activity of the final enzyme preparation was estimated to be 389.9 mU/mg protein using ABTS as the substrate.

The UV−vis spectra of purified fmb-L103 (data not shown) did not contain an absorption peak around 600 nm, which is expected for the type 1 copper site found in most laccases. The atomic absorption spectroscopy results suggested that 2.5 mol/mol of copper was present in the purified fmb-L103, indicating that fmb-L103 belonged to the nonblue laccase family. This unusual laccase has been previously purified from other microorganisms including *Bacillus* sp. ADR,[20] *Pleurotus*

dx.doi.org/10.1021/jf4010498 | J. Agric. Food Chem. 2013, 61, 5468−5473

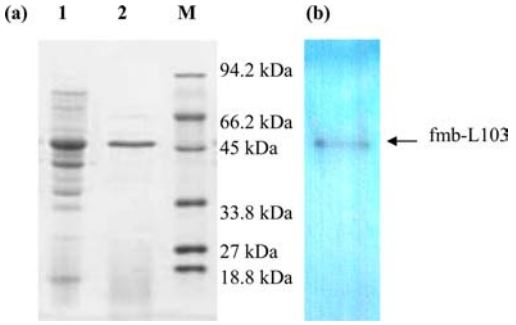

Figure 1. (a) SDS-PAGE of the crude mixture and the purified fmb-L103: 1, crude mixture; 2, purified fmb-L103; M, molecular mass marker. (b) Activity staining with guaiacol after PAGE under nondenaturing conditions.

ostreatus,[21] and *Trametes hirsute*.[22] Compared with blue laccases, there are some differences in the catalytic reaction of nonblue laccases,[22] although further studies are needed to define the mechanism.

Effect of Temperature on fmb-L103 Activity and Stability. The effects of temperature on the purified fmb-L103 are shown in Figure 2a. The fmb-L103 was active in the

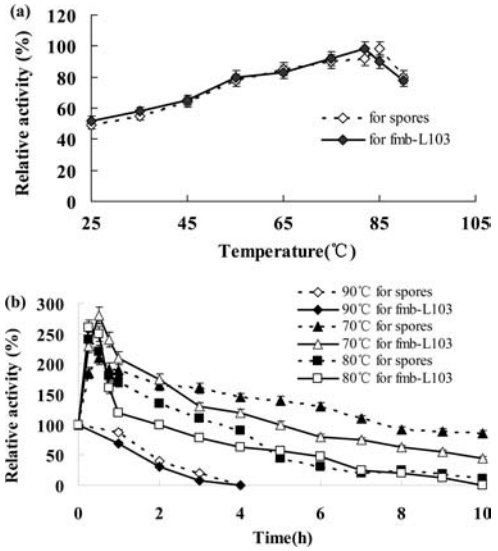

Figure 2. Effect of temperature on activity (a) and stability (b) of fmb-L103 and the spore laccase with ABTS as the substrate at pH 4.4. (a) Laccase activity was measured at different temperatures (25−90 °C); (b) residual activity was measured after incubation for 0−10 h.

temperature range 25−90 °C, with a maximum activity at 82 °C. The residual activities of fmb-L103 incubated for 0−10 h at different temperatures (70, 80, 90 °C) are shown in Figure 2b. The half-life of fmb-L103 was about 10, 4, and 1.2 h at 70, 80, and 90 °C, respectively. These results showed that fmb-L103 had greater thermal stability compared with most fungal laccases such as *Paraconiothyrium variabile*[9] and *Cladosporium cladosporioides*.[10] The thermal stability of the fmb-L103 laccase is even better than the laccase from other *Bacillus* sp. strains such as *B. subtilis*,[14] *Bacillus* sp. ADR,[20] and *Bacillus licheniformis* LS04.[17] Like the spore laccase, fmb-L103 activity was also accelerated after preincubation at high temperature compared with the nonpreincubated samples (Figure 2b). The activity of fmb-L103 increased above 280% after 30 min incubation at 70 °C and increased by nearly 260% after 10 min at 80 °C.

Compared with the spore laccase, the optimum temperature of fmb-L103 decreased at 3 °C (Figure 3a), and the thermal

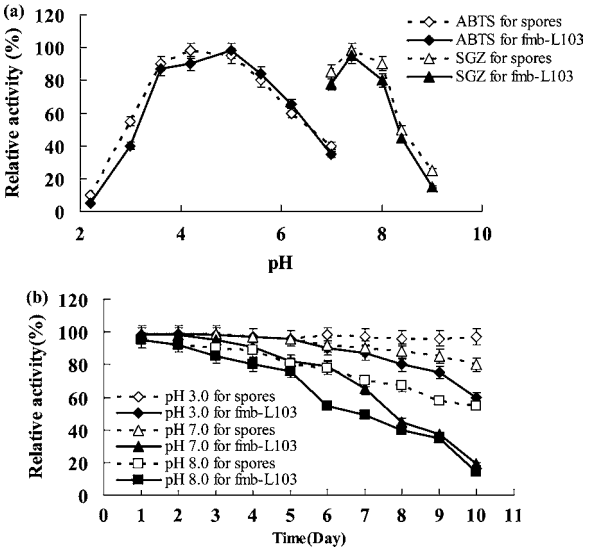

Figure 3. Effect of pH on activity (A) and stability (B) of fmb-L103 and the spore laccase at 45 °C. (A) ABTS, pH 2.2−7.0; syringaldazine, pH 7.0−9.0; (B) ABTS, pH 3.0; syringaldazine, pH 7.0 and 8.0.

stability also decreased (Figure 3b). This may be the result of the spore lacking some protective substance, such as protein and/or polysaccharide, which may have been lost during the purification, although this hypothesis needs to be confirmed.

Effect of pH on fmb-L103 Activity and Stability (Figure 3). The fmb-L103 can oxidize ABTS in acid conditions from pH 2.2 to 7.0 with an optimum pH of 4.8, similar to most fungal (pH2.0−5.0)[11] and *Bacillus* sp. (pH 4.2)[23] laccase (Figure 3a). For the oxidation of syringaldazine, similar to other bacterial laccases,[24] the catalysis pH range of fmb-L103 was 7.0 to 9.0, and the optimum pH was 7.4. Thus, similar to fungal[9,10] and bacterial laccases,[25] fmb-L103 was stable at both acidic and alkaline pH conditions (Figure 3b). The relative activity was 40%, 80%, and 85% at pH 3, pH 7, and pH 8 after 10 days, respectively.

Compared with the spore laccase, the optimum pH for catalysis of ABTS shifted to alkaline conditions and the pH stability also decreased by a small amount. The absence of protective substances, removed during purification, may also account for these results as well as the variations in the thermal stability.

Effects of Inhibitors and Metal Ions on fmb-L103 Activity. The effects of several laccase inhibitors were determined with ABTS as a substrate (Table 2). Similar to the spore laccase, the activity of fmb-L103 was absolutely inhibited by NaN_3, L-cysteine, and dithiothreitol. SDS slightly inactivated the enzyme (>40% inhibition) at 1 mM, while 5 mM EDTA resulted in greater inhibition (>85%). NaCl decreased fmb-L103 activity to 50.7% and 20.5% of its original activity in the presence of 500 and 1000 mM NaCl, respectively.

The effects of several metal ions on fmb-L103 activity at a final concentration of 5 mM are shown in Table 3. It is noteworthy that the addition of Cu^{2+} into the reaction mixture stimulated fmb-L103 activity, increasing it by approximately

dx.doi.org/10.1021/jf4010498 | J. Agric. Food Chem. 2013, 61, 5468−5473

Table 2. Effects of Inhibitors on fmb-L103 Activity

inhibitor	concentration (mM)	relative activity (%)[a]
none		100.00
SDS	0.1	89.3 ± 2.07
	0.5	70.2 ± 1.98
	1	58.3 ± 3.23
L-cysteine	0.1	0
	0.5	0
dithiothreitol	0.1	0
	0.5	0
NaN_3	0.1	0
EDTA	0.5	85.4 ± 3.32
	2.5	47.5 ± 3.42
	5	13.2 ± 1.06
NaCl	100	90.3 ± 2.12
	500	50.7 ± 2.04
	1000	20.5 ± 1.20

[a]Values represent the means ± SD (n = 3) relative to the untreated control samples.

Table 3. Effects of Metal Ions on fmb-L103 Activity

metal ions	relative activity (%)[a] (for fmb-L103)
control	100
Cu^{2+}	158.6 ± 5.4
Ca^{2+}	115.6 ± 3.1
Mn^{2+}	108.7 ± 2.6
Mg^{2+}	106.8 ± 2.7
Fe^{2+}	98.5 ± 1.7
K^+	97.4 ± 1.6
Zn^{2+}	10.9 ± 0.7

[a]Values represent the means ± SD (n = 3) relative to the untreated control samples.

60% compared with the control. Similar results were reported by Chakroun,[8] likely as a result of filling the type-2 copper binding sites with copper ions.[26] The presence of Ca^{2+}, Mg^{2+}, and Mn^{2+} in the reaction mixture appeared to slightly enhance the enzyme activity. Partial inhibition (2−3% inhibition) was observed in the presence of Fe^{2+} and K^+, and the strongest inhibitory effect was obtained for Zn^{2+}.

Kinetic Constants for fmb-L103. Several conventional substrates of laccase such as ABTS, 2-DMP, SGZ, and guaiacol were oxidized by fmb-L103. The K_m's were 22.7, 476.8, 357.9, and 1062.4 μM for ABTS, 2-DMP, SGZ, and guaiacol, respectively. ABTS was the most suitable substrate for fmb-L103, with the lowest K_m and a V_{max} of 3.32 μmol/mL/min. Interestingly, laccase purified from *Bacillus* sp. ADR cannot oxidize ABTS.[20] Compared with fungal laccase, such as those from *Lentinula edodes*[26] and *Paraconiothyrium* variabile,[9] the K_m value of fmb-L103 with respect to the substrate ABTS is lower and similar to that of *Trametes* sp. A282[27] and *Cladosporium cladosporioides*.[10] These results indicate some variations in substrate specificities among the laccases from different species and will only be clarified upon further investigations into the catalysis mechanisms of fmb-L103.

Degradation of MG by fmb-L103. The application of laccases for dye degradation has been reported previously,[12] but there were no reports on its use for the degradation of MG. In this study, three mediators, ABTS, acetosyringone, and syringaldehyde, were used to degrade MG as suggested previously.[25] The degradation of MG with laccase is shown in Figure 4. When only laccase was present, the degradation efficiency was not more than 10% (Figure 4A). In the presence

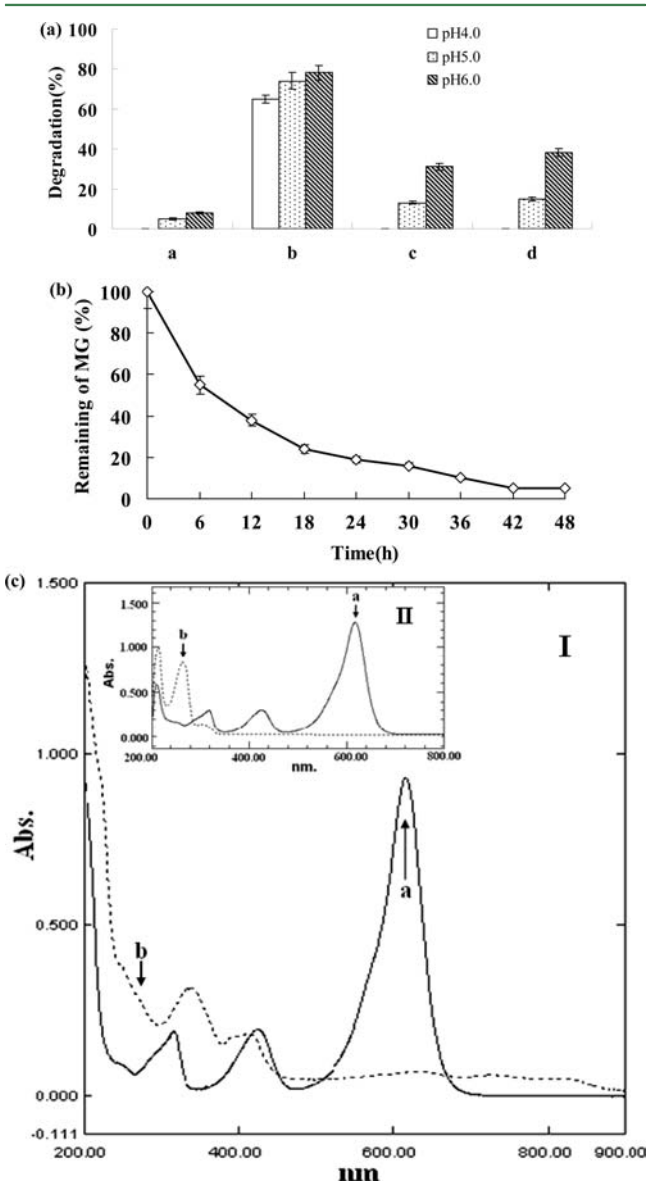

Figure 4. (A) Effects of mediators and pH on the decolorization of MG: (a) laccase + dye; (b) laccase + ABTS + dye; (c) laccase + syringaldehyde + dye; (d) laccase + acetosyringone + dye. (B) Kinetics of MG degradation by fmb-L103. (C) (I) Wavelength scan of MG before and after decolorizing; dotted line for the MG untreated by fmb-L103, solid line for MG catalyzed by fmb-L103; (II) characteristic peak of MG (a) and LMG (b); dotted line for the MG, solid line for LMG.

of ABTS, acetosyringone, or syringaldehyde, the degradation was obviously improved, with the efficiency reaching 79.6% when combined with ABTS.

The effect of pH on the degradation efficiency was also tested, and the degradation efficiency improved as the pH increased from approximately 4.0 to 6.0 (Figure 4A). The highest degradation efficiency was 79.6% with ABTS at pH 6.

The kinetics of the MG degradation process are shown in Figure 4B. More than 70% of the added MG disappeared within 24 h, and almost no MG could be detected in the mixture at the 48 h time point.

dx.doi.org/10.1021/jf4010498 | J. Agric. Food Chem. 2013, 61, 5468−5473

Full wavelength scans of the reaction products from the MG degradation catalyzed for 24 h by fmb-L103 were performed and compared with the control samples obtained in the absence of enzyme catalysis. The relative maximum absorption of the enzymatically treated samples indicated that at least one peak had disappeared (Figure 4C). These results illustrated that MG was catalytically degraded.

As shown in Figure 4C, although the values from 200−400 nm increased, no obvious characteristic peak of LMG appeared, and the Elisa results showed that there was no LMG detected during the MG degradation process. We conferred that the increased values from 200−400 nm were affected by the added laccase and ABTS and/or other catalyzed products. Some strains have been reported to degrade MG, and triphenylmethane reductase has been shown to be responsible for the degradation of MG to LMG in many strains.[28] In this study, the absence of the appearance of LMG implied that fmb-L103 catalyzed a novel degradation pathway. Further studies are needed to identify the decomposed substances and clarify the catalytic mechanism of fmb-L103.

In conclusion, in this study, the laccase fmb-L103 was purified from the spores of the *B. vallismortis* fmb-103 strain. fmb-L103 is temperature and pH stable and belongs to the nonblue laccase family. The ability of fmb-L103 to degrade MG without producing LMG makes it a candidate for potential applications in the bioremediation of wastewater in the aquaculture industry.

AUTHOR INFORMATION

Corresponding Author

*(Z. Lu) E-mail: fmb@njau.edu.cn. Tel: +86-25-84396583. Fax: +86-25-84396583.

Funding

This work was funded by the National Natural Science Foundation of China (31201423; 31071605), Doctoral Fund of the Ministry of Education of China (20120097120004), a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), and The Youth Science and Technology Innovation Fund of Nanjing Agriculture University (KJ2011016).

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Srivastava, S.; Sinha, R.; Roy, D. Toxicological effects of malachite green. *Aquat. Toxicol.* **2004**, *66*, 319−329.

(2) Cha, C.-J.; Doerge, D. R.; Cerniglia, C. E. Biotransformation of malachite green by the fungus *Cunninghamella elegans*. *Appl. Environ. Microbiol.* **2001**, *67*, 4358−4360.

(3) Xing, W.; He, L.; Yang, H.; Sun, C.; Li, D.; Yang, X.; Li, Y.; Deng, A. Development of a sensitive and group-specific polyclonal antibody-based enzyme-linked immunosorbent assay (ELISA) for detection of malachite green and leuco-malachite green in water and fish. *J. Agric. Food Chem.* **2009**, *89*, 2165−2173.

(4) Yang, M. C.; Fang, J. M.; Kuo, T. F.; Wang, D. M.; Huang, Y. L.; Liu, L. Y.; Chen, P. H.; Chang, T. H. Production of antibodies for selective detection of malachite green and the related triphenylmethane dyes in fish and fishpond water. *J. Agric. Food Chem.* **2007**, *55*, 8851−8856.

(5) Andersen, W. C.; Turnipseed, S. B.; Roybal, J. E. Quantitative and confirmatory analyses of malachite green and leucomalachite green residues in fish and shrimp. *J. Agric. Food Chem.* **2006**, *54*, 4517−4523.

(6) Giardina, P.; Faraco, V.; Pezzella, C.; Piscitelli, A.; Vanhulle, S.; Sannia, G. Laccases: a never-ending story. *Cell. Mol. Life Sci.* **2010**, *67*, 369−385.

(7) Claus, H. Laccases and their occurrence in prokaryotes. *Arch. Microbiol.* **2003**, *179*, 145−150.

(8) Chakroun, H.; Mechichi, T.; Martinez, J. M.; Dhouib, A.; Sayadi, S. Purification and characterization of a novel laccase from the ascomycete *Trichoderma atroviride*: Application on bioremediation of phenolic compounds. *Process Biochem.* **2010**, *45*, 507−513.

(9) Forootanfar, H.; Faramarzi, M. A.; Shahverdi, A. R.; Yazdi, M. T. Purification and biochemical characterization of extracellular laccase from the ascomycete *Paraconiothyrium variabile*. *Bioresour. Technol.* **2011**, *102*, 1808−1804.

(10) Halaburgia, V. M.; Sharma, S.; Sinha, M.; Singh, T. P.; Karegoudara, T. B. Purification and characterization of a thermostable laccase from the ascomycetes *Cladosporium cladosporioides* and its applications. *Process Biochem.* **2011**, *46*, 1146−1152.

(11) Baldrian, P. Fungal laccases-occurrence and properties. *FEMS Microbiol. Rev.* **2006**, *30*, 215−242.

(12) Singh, G.; Bhalla, A.; Kaur, P.; Capalash, N.; Sharma, P. Laccase from prokaryote: a new source for an old enzyme. *Rev. Environ. Sci. Biotechnol.* **2011**, *10*, 309−326.

(13) Martins, L. O.; Soares, C. M.; Pereira, M. M.; Teixeira, M.; Costa, T.; Jones, G. H.; Henriques, A. O. Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. *J. Biol. Chem.* **2002**, *277*, 18849−18859.

(14) Held, C.; Kandelbauer, A.; Schroeder, M.; Cavaco-Paulo, A.; Guebitz, G. M. Biotransformation of phenolics with laccase containing bacterial spores. *Environ. Chem. Lett.* **2005**, *3*, 74−77.

(15) Miyazaki, K. A hyperthermophilic laccase from *Thermus thermophilus* HB27. *Extremophiles* **2005**, *9*, 415−425.

(16) Zhang, C.; Diao, H. W.; Lu, F. X.; Bie, X. M.; Wang, Y. F.; Lu, Z. X. Degradation of triphenylmethane dyes using using temperature and pH stable laccase from a strain of *Bacillus vallismortis*. *Bioresour. Technol.* **2012 (a)**, *126*, 80−86.

(17) Lu, L.; Zhao, M.; Wang, T. N.; Zhao, L. Y.; Du, M. H.; Li, T. L.; Li, D. B. Characterization and dye degradation ability of an alkaline resistant and organic solvents tolerant laccase from *Bacillus licheniformis* LS04. *Bioresour. Technol.* **2012**, 11535−11540.

(18) Campos, R.; Kandelbauer, A.; Robra, K. H.; Cavaco-Paulo, A.; Gübitz, G. M. Indigo degradation with purified laccase from *Trametes hirsuta* and *Sclerotium rolfsii*. *J. Biotechnol.* **2001**, *89*, 131−139.

(19) Zhang, C.; Tao, T. T.; Ying, Q.; Zhang, D. L.; Lu, F. X.; Bie, X. M.; Lu, Z. X. Extracellular production of lipoxygenase from *Anabaena sp.* PCC 7120 in *Bacillus subtilis* and its effect on wheat protein. *Appl. Microbiol. Biotechnol.* **2012 (b)**, *94*, 949−958.

(20) Telke, A. A.; Ghodake, G. S.; Kalyani, D. C.; Dhanve, R. S.; Govindwar, S. P. Biochemical characteristics of a textile dye degrading extracellular laccase from a *Bacillus* sp. ADR. *Bioresour. Technol.* **2011**, *102*, 1752−1756.

(21) Palmieri, G.; Giardina, P.; Bianco, C.; Scaloni, A.; Capasso, A.; Sannia, G. A novel white laccase from *Pleurotus ostreatus*. *J. Biol. Chem.* **1997**, *272*, 31301−31307.

(22) Haibo, Z.; Yinglong, Z.; Feng, H.; Peiji, G.; Jiachuan, C. Purification and characterization of a thermostable laccase with unique oxidative characteristics from *Trametes hirsuta*. *Biotechnol. Lett.* **2009**, *31*, 837−843.

(23) Koschorreck, K.; Richter, S. M.; Ene, A. B.; Roduner, E.; Schmid, R. D.; Urlacher, V. B. Cloning and characterization of a new laccase from *Bacillus licheniformis* catalyzing dimerization of phenolic acids. *Appl. Microbiol. Biotechnol.* **2008**, *79*, 217−224.

(24) Wu, J.; Kim, K. S.; Lee, J. H.; Lee, Y. C. Cloning, expression in *Escherichia coli*, and enzymatic properties of laccase from *Aeromonas hydrophila* WL-11. *J. Environ. Sci.* **2010**, *22*, 635−640.

(25) Singh, G.; Capalash, N.; Goel, R.; Sharma, P. A pH-stable laccase from alkalitolerant γ-proteobacterium JB: purification, characterization and indigo carmine degradation. *Enzyme Microb. Technol.* **2007**, *41*, 794−799.

(26) Nagai, M.; Sato, T.; Watanabe, H.; Saito, K.; Kawata, M.; Enei, H. Purification and characterization of an extracellular laccase from the edible mushroom *Lentinula endodes*, and degradation of chemically different dyes. *Appl. Microbiol. Biotechnol.* **2002**, *60*, 327−335.
(27) Zhang, H.; Hong, Y. Z.; Xiao, Y. Z.; Yuan, J.; Tu, X. M.; Zhang, X. Q. Efficient production of laccases by Trametes sp. AH28-2 in cocultivation with a Trichoderma strain. *Appl. Microbiol. Biotechnol.* **2006**, *73*, 89−94.
(28) Jadhav, J. P.; Govindwar, S. P. Biotransformation of malachite green by *Saccharomyces cerevisiae* MTCC 463. *Yeast* **2006**, *23*, 315−323.