JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Nov. 2013. Vol. **7**(Spl. Edn.), p. 765-770

Effects of Chemical Modification on Laccase Stability and Degradation of Indole

Dajun Ren*, Yuanyuan Zhang, Qin Xu, Chunxing Wang, Tao Wang, Su Huang and Shuqin Zhang

College of Resource and Environmental Engineering, Wuhan University of Science and Technology, Wuhan - 430 081, China.

(Received: 27 September 2013; accepted: 04 November 2013)

Since the natural laccase is inclined to be inactivated at industrial application conditions, it is especially important and urgent to improve the stability of laccase. Taking laccase secreted by *Pleurotus ostreatus* as the research object, the laccase is chemically modified with dextran (Dex) and succinic anhydride (SA). The pH stability, thermal stability as well as degradation efficiency toward indole of natural laccase and modified laccase are compared. The enzyme-catalyzed reaction reveals the Michaelis–Menten constant K_m value of modified laccase rises, while the pH stability and thermal stability of modified laccase are superior to those of natural laccase. Moreover, the degradation efficiency toward indole for dextran modified laccase (Dex-Lac) and succinic anhydride modified laccase (SA-Lac) are 37.2% and 35.3% at 50ºC after 3h, respectively, achieving nearly 2 times higher than natural laccase.

Key words: Laccase; Chemical modification; Stability; Degradation efficiency.

The laccase (benzenediol: oxygen oxidoreductase, ECl. 10. 3. 2) is a copper-containing polyphenol oxidase. It can directly oxidize phenols and aromatic compounds using oxygen molecules. Accompanied by the transfer of electrons, the molecular oxygen is reduced to water[1]. The properties of laccase have been extensively studied[2, 3] and it has favorable application prospect in various fields, such as environmental governance, paper industry, biological testing and so on[4, 5, 6].

The key requirement for industrial biocatalyst is higher stability. However, the pH stability and thermal stability of natural laccase are fairly poor and it is prone to be deactivated in application environment in addition to its inferior resistance to organic solvents, which will limit its industrial application[7, 8]. Thus the improvement of the laccase stability has become essential and urgent. The current stability enhancement measures for laccase focus on the development of novel chemical modification methods[9]. Gary[10] and others with modified laccase with chitosan, obtaining improved pH stability and thermal stability for modified laccase, along with decreased kinetic constant K_{cat}.

In this paper, utilizing the methods of side chain group modification and macromolecular modification, succinic anhydride and dextran are selected to modify natural laccase. The degradation efficiency to indole is studied with indole as simulate substrate. Particularly, as a typical nitrogen-containing heterocyclic compound, indole possesses the features of high toxicity, teratogenicity, carcinogenicity and nonbiodegradability and widely exists in wastewater, pharmaceutical waste, dye wastewater as well as other persistent industrial wastewater[11]. The results of this study may also provide

* To whom all correspondence should be addressed.
Tel.: +86 27 68862029; Fax: +86 27 68862029.
E-mail: dj_ren@163.com

significant reference for developing laccase to remove nitrogen-containing heterocyclic compounds.

MATERIALS AND METHODS

Chemicals

Laccase (secreted by *Pleurotus ostreatus*, ≥4.0 U/mg) and 2, 2' - Azino - bis - (3 - ethylbenzothiazoline - 6 - sulfonic acid) diammonium salt (ABTS) are purchased from Sigma-Aldrich Co. Indole (≥99.8 %) is provided by Sinopharm Chemical Reagent Co., Ltd. Parachlorophenol (≥99.0 %) is got from Tianjin Kaitong Chemical Reagent Co. Dextran is provided by Pharmacia Co. Succinic anhydride (≥99.0 %) is provided by Tianjin Baishi Chemical Co. All other reagents are analytical reagents from China. The water was double-distilled.

Enzyme assay

ABTS was employed as substrate. 1 mL diluted enzyme solution was added to 1.5 mL 0.05 M acetate buffer solution with pH 3.5, followed by addition of 0.5 mL 0.5 mM ABTS. The reaction was initiated with UV-2550 spectrophotometer at 25 ºC. The increase of absorbance of the reaction solution at 420 nm was tested immediately in 3 min. With the molar adsorption coefficient of μ= 36000 L/mol·cm, the enzyme activity unit U was defined as converting 1 ¼mol substrate at 25 ºC per minute.

Chemical modification of laccase

Macromolecular modification: 100 mg dextran was oxidized by 100 mg $NaIO_4$ oxidize and dissolved in 1 mL 0.05 M phosphate buffer of pH 7.0 and then was stirred for 12 h in dark. The oxidation product was dialyzed in 0.05 M phosphate buffer of pH 7.0 for 2 h. The 1mL solution after dialysis was linked with 5mg enzyme and was stirred in dark for 12 h. Then 100 mL 0.1mM Tris-HCl buffer solution of pH 8.0 was added and the solution was stirred for 2 h to seal excess aldehyde groups in dextran. After that, 20 mg $NaBH_3CN$ was added and stirring continued for 4 h in dark. At last, the product was dialyzed in 0.05 M phosphate buffer of pH 7.0 for 2 h. The obtained modified enzyme was used for subsequent enzymatic experiments.

Side chain group modification: 5 mg laccase was dissolved in 1 mL 0.05 M phosphate buffer solution of pH 7.0 and dialyzed for 12 h. Then 0.15 mL dimethylsulfoxide (DMSO) solution with 1.2 mg succinic anhydride was added to the dialyzed solution. The total volume was adjusted to 2 mL using 0.05 M phosphate buffer solution of pH 7.0. Afterwards, the solution was stirred in the dark at 4 ºC for 5 h. At last, the product was dialyzed in 0.05 M phosphate buffer solution of pH 7.0 at 4 ºC for 12 h. The obtained modified enzyme was used for subsequent enzymatic experiments.

Assay of enzyme stability

Determination of pH stability: At 25 ºC, the enzyme activities of natural laccase (Lac) and modified laccase (Dex-Lac, SA-Lac) with the same molar concentration were measured in 0.05 mol/L phosphate buffer of different pH values (pH 2.0~3.5) and 0.05 mol/L acetic acid-sodium acetate buffer of different pH values (pH 4.0~6.5), so as to obtain their respective optimum reaction pH values. Lac, Dex-Lac and SA-Lac of same molar concentration were mixed in equal volume with buffers of different pH values, respectively, and preserved in 25 ºC water bath for 1 h. Then the detection method of each laccase activity was the same as Enzyme assay. The activity of the native laccase at pH 3.5 and 25 ºC was taken as 100 % (100 %=3444 U/L), and then the relative enzyme activity was calculated.

Determination of thermal stability: Under the respective optimum pH, the enzyme activity of Lac, Dex-Lac and SA-Lac with the same molar concentration were measured at different temperatures (30 ºC~80 ºC), so as to obtain their respective optimum reaction temperature. Lac, Dex-Lac and SA-Lac of same molar concentration were preserved under respective optimum pH value in water bath of different temperatures for 1h. Then the enzyme activity of each laccase was determined after the solution was cooled in ice bath to 25 ºC. The activity of the native laccase at pH 3.5 and 25 ºC was taken as 100 % (100 %=3444 U/L), and then the relative enzyme activity was calculated.

Determination of kinetic constant of enzyme-catalyzed reaction

The enzyme activity of Lac, Dex-Lac and SA-Lac of same molar concentration were determined at their respective optimum pH and optimum temperature by changing concentration of the substrate ABTS (0.1~0.5 mmol/L) and was denoted as *v*. According to Michaelis-Menten equation, the Michaelis-Menten constant K_m and

catalytic rate constant of enzyme-catalyzed reaction K_{cat} were calculated.

$[S]/v = 1/V_m \times [S] + K_m/V_m$ ....(1)

$K_{cat} = V_m/[E_0]$ ....(2)

Removal efficiency of indole

120 mg indole /L, 0.05 M phosphate buffer of pH 3.5 and 1 U/mL Lac or modified laccase (Dex-Lac or SA-Lac) were mixed at a volume ratio of 1:1:1. The beginning concentration of indole in each system just after mixing was determined with UltiMate-3000 high performance liquid chromatography (HPLC) and was denoted as the initial concentration. Then the mixture was preserved in 50 ºC water bath for reaction. The content of residual indole was measured every 0.5 h. HPLC was conducted with C-18 reverse column and methanol (4:1, v/v) as the mobile phase at 25 ºC, under 1.0 mL/min. The wavelength for indole detection was 270 nm. The indole removal efficiency was calculated using the following formula.

$RE = (A_0-A)/A_0 \times 100\%$ ...(3)

Where A_0 was the initial concentration of indole, A was the remaining concentration after reaction.

RESULTS AND DISCUSSIONS

The pH stability of enzymes

The enzyme-catalyzed reactions were carried out with ABTS as substrate at different pH conditions. With the enzyme activity of natural enzyme at 25 ºC and pH=3.5 as a control, the relative activity of natural laccase and modified laccase were calculated at each pH, as shown in Fig. 1 and Fig. 2.

As presented in Fig. 1, both natural laccase and modified laccase possessed high activity under acidic conditions and achieved the highest activity at pH=3.5 where the maximum activity of Dex-Lac and SA-Lac were 6% and 4% larger than that of Lac. As the pH increased, the activity was gradually decreased. The same pH response of natural laccase and modified laccase indicated that the active center of laccase had not been changed during modification process and the amino acid sequence of laccase was conserved. Taking 70% of the maximum enzyme activity as the boundary to determine suitable pH range for enzyme catalytic, the appropriate pH range for natural laccase was pH 3.5~4, while for Dex-Lac and SA-Lac were pH 3.5~5 and pH 3.5~4.5, respectively. The wider suitable pH range of modified laccase suggested that the pH stability of the two modified laccase was both improved, in favor of the industrial application of modified laccase.

Table 1. Kinetic constants of reactions catalyzed by natural laccase and modified laccase toward ABTS

Material	K_m / mM	k_{cat} / s^{-1}	(k_{cat}/K_m) / $s^{-1}\cdot mmol^{-1}\cdot L$
Lac	0.27	8.89×10^8	32.92×10^8
Dex-Lac	0.59	9.65×10^8	16.36×10^8
SA-Lac	0.57	6.45×10^8	11.31×10^8

Table 2. Kinetic equations and parameters for indole degradation systems

Indole degradation system	Kinetic equation	K_o	Correlation coefficient r^2
Lac	-0.0109t+3.9107	0.0109	0.9854
Dex-Lac	-0.1006t+3.9343	0.1006	0.9494
SA-Lac	-0.0551t+3.8307	0.0551	0.9692

The enzyme activity of natural laccase and modified laccase were reduced to some degree after preservation in water bath for 1 h, as shown in Fig.2. The activity of natural laccase declined most greatly. At pH=3.5, the enzyme activity of Dex-Lac decreased by 28.8%, while the activity of natural laccas ezyme decreased by 54.6% which was close to two times the decay for modified laccase. The above data indicated that the natural laccase was

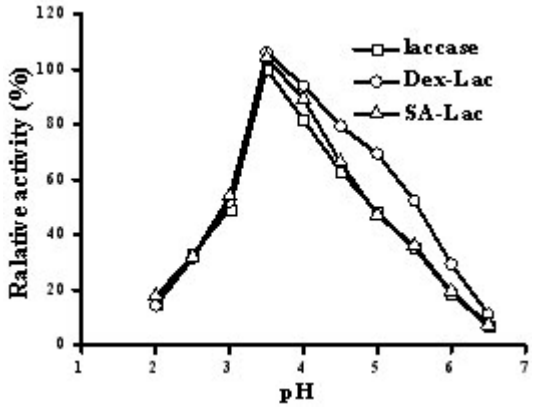

Fig. 1. Catalytic activity of natural laccase and modified laccase at different pH conditions

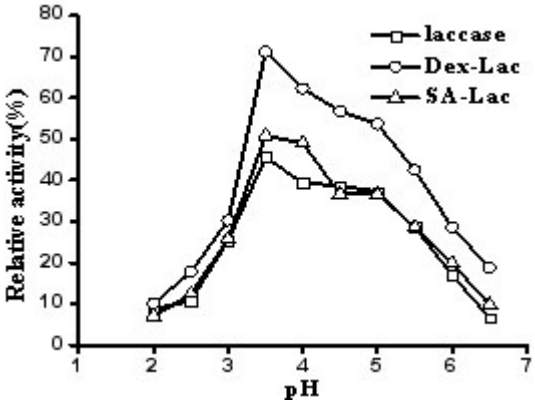

Fig. 2. Stability of natural laccase and modified laccase under different pH

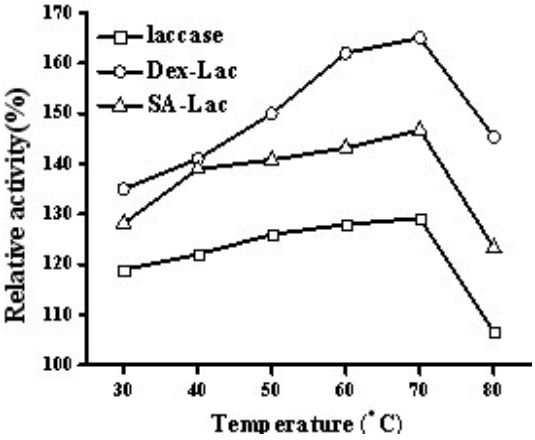

Fig. 3. Catalytic activity of natural laccase and modified laccase at different temperatures

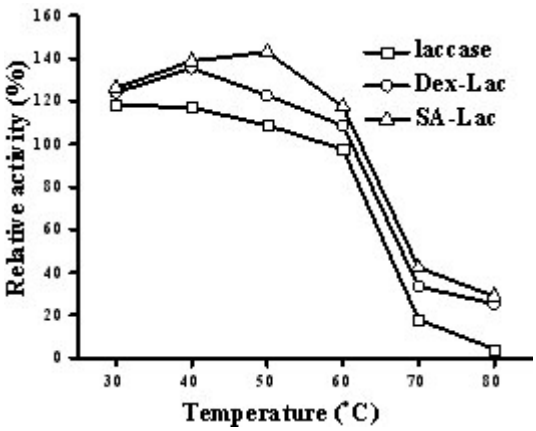

Fig. 4. Stability of natural laccase and modified laccase at different temperatures

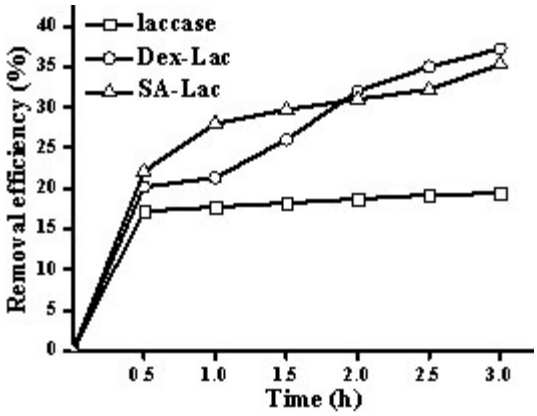

Fig. 5. Degradation rate of natural laccase and modified laccase to indole

easy to lose activity at its optimum pH value, and the stability of Dex-Lac under acidic conditions had been significantly enhanced. The improved stability of modified laccase with dextran may be because that dextran crosslinks with multiple sites on laccase surface and thus the overall stability of the enzyme structure was effectively promoted. Additionally, a number of hydroxyl groups in dextran could efficiently maintain the hydrophilic microenvironment on enzyme surface[12].

The thermal stability of enzymes

The enzyme-catalyzed reactions were conducted with ABTS as substrate at different temperatures. With the enzyme activity of natural laccase at 25 ºC and pH=3.5 as a control, the relative activity of natural laccase and modified laccase at various temperatures were calculated, as exhibited Fig. 3 and Fig. 4.

It could be seen from Fig. 3 that natural laccase and modified laccase both had higher activity at temperatures of 30 ºC-70 ºC and reached greatest activity at 70 ºC followed by significantly reduced enzyme activity at 80 ºC. The change in the spatial structure of enzyme at the high temperature of 80 ºC rendered rapid enzyme inactivation. The optimum reaction temperature for laccase did not alter after modification and the variation in enzyme activity of natural and modified laccase showed the same trend with the change of temperature. Notably, the activity of Dex-Lac and SA-Lac exceeded that of natural laccase, demonstrating that the activity of modified laccase was greatly enhanced at various temperatures. Furthermore, the activity of Dex-Lac was improved more than that of SA-Lac.

As revealed in Fig. 4, the enzyme activity of natural laccase and modified laccase both descended to a certain degree after incubated in water bath of various temperatures after 1 h. The activity loss was less at 30 ºC-60 ºC and remarkable at 60 ºC-70 ºC. Since the thermal energy of reactants had highest value at the optimum temperature of enzyme and thus the reaction rate was fastest, the enzyme activity decreased most rapidly. The spatial structure of natural laccase completely changed at 80 ºC and thus the activity was totally lost. However, the remaining activity of Dex-Lac and SA-Lac at each temperature were higher than that of natural laccase, suggesting the temperature stability of modified laccase was better than that of natural laccase. This may be because the natural conformation of enzyme was provided with some "rigidity" after the modifying agent covalently attached to enzyme molecules, and thus the enzyme was difficult to be stretched and inactivated with reduced thermal vibration of internal groups in enzyme molecules. Consequently, the thermal stability of enzyme was improved[13].

Catalytic reaction kinetics

The kinetic constants of reactions catalyzed by natural laccase and modified laccase toward ABTS were assayed as listed in Table 1.

The K_m value of laccase was increased from 0.27 mM to 0.59 mM and 0.57 mM after modified with dextran and succinic anhydride, respectively. This could be because the more regular and compact structure of modified enzyme rendered that the enzyme molecules lacked the necessary molecular chain flexibility for binding with substrate when the enzyme actived sites combine with substrate, causing limited conformational change of enzyme and thus reduced affinity to substrate[14].

Indole degradation efficiency and reaction kinetics analysis

Indole was degraded by natural and modified laccase in water bath of 50 ºC, respectively. As shown in Fig. 5, within the initial 0.5 h, both natural and modified laccase had apparent degradation effect toward indole with a very quick reaction rate. However, the degradation rate of natural laccase to indole displayed almost no growth after 0.5 h, while the degradation rate of modified laccase rose sustainably. Then the degradation data of different laccases to indole were fitted linearly according to first-order kinetic equation of the linear fit (Table 2). It was stated in Table 2 that reaction rate constant for Dex-Lac and SA-Lac were 10 and 5 times as much as that for natural laccase, respectively. Because the abundant hydroxyl groups in dextran effectively maintained the hydrophilic microenvironment of the surface of enzyme, indole was easier to approach active center of Dex-Lac and thus Dex-Lac possesses higher reaction rate constant to indole than natural laccase. The introduction of side chain groups would affect the charge distribution of enzyme molecules[15], which could coordinate with charge of indole more facilely and therefore the reaction

rate was faster. Additionally, it was further demonstrated that the stability of modified laccase was more excellent than that of natural laccase by comparing the trend of reactions catalyzed by natural and modified laccase after 0.5 h.

CONCLUSIONS

The pH and thermal stability of laccase were improved by side group modification with succinic anhydride and also by macromolecules modification with dextran. Compared with natural laccase, the optimum pH ranges for catalytic reaction were broadened. Obviously, the stability of Dex-Lac with an optimum pH range of pH 3.5~5 preceded that of SA-Lac.

The degradation trended to indole by natural and modified laccase within 0.5~3 h further declare that the stability of modified laccase was superior to that of natural laccase. The first-order kinetic constant of Dex-Lac and SA-Lac were 8 and 4 times higher than that of Lac, respectively. In 3 h of reaction process, the degradation efficiency for Dex-Lac and SA-Lac toward indole was 37.2% and 35.3% respectively, achieving nearly 2-fold more than that for natural laccase.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Grant no. 41201306) and the National Science & Technology Pillar Program of China (No. 2012BAC02B04).

REFERENCES

1. Solomon, E.L., Sundaram, U.M., Machonkin, T.E. Multicopper oxidases and oxygenases. Chemical Reviews, 1996; **96**(7): 2563-2605.
2. Gianfreda, L., Xu, F., Bollag, J. Laccases: a useful group of oxidoreductive enzymes. *Bioremediation Journal*, 1999; **3**(1): 1-26.
3. Bourbonnais, R., Leech, D., Paice, M.G. Electrochemical analysis of the interactions of laccase mediators with lignin model compounds. Biochimica et Biophysica Acta (BBA) - *General Subjects*, 1998; **1379**(3): 381-390.
4. Bulter, T., Alcalde, M., Sieber, V., Meinhold, P., Schlachtbauer, C., Arnold, F.H. Functional expression of a fungal laccase in *Saccharomyces cerevisiae* by directed evolution. *Applied and Environmental Microbiology*, 2003; **69**(2): 987-995.
5. Thurston, T.F. The structure and function of fungal laccases. *Microbiology*, 1994; **140**: 19-26.
6. Zinnai, A., Venturi, F., Sanmartin, C., Quartacci, M.F., Andrich, G. Chemical and Laccase catalysed oxidation of gallic acid: Determination of kinetic parameters. *Research Journal of Biotechnology*, 2013; **8**(7): 62-5.
7. Champagne, P.P., Ramsay, J.A. Reactive blue 19 decolouration by laccase immobilized on silica beads. *Applied Microbiology and Biotechnology*, 2007; **77**(4): 819-823.
8. Svobodova, K., Majcherczyk, A., Novotny, C., Kuees, U. Implication of mycelium-associated laccase from *Irpex lacteus* in the decolorization of synthetic dyes. *Bioresource Technology*, 2008; **99**: 463-471.
9. Liu, J.Z., Song, H.Y., Weng, L.P., Ji, L.N. Increased thermostability and phenol removal efficiency by chemical modified horseradish peroxidase. *Journal of Molecular Catalysis B: Enzymatic*, 2002; **18**(4-6): 225-232.
10. Gary, D., Li, Q., Yu, J. Activity and stability of laccase in conjugation with chitosan. *International Journal of Biological Macromolecules*, 2005; **35**(1-2): 89-95.
11. Chen, Y., Xie, X.G., Ren, C.G., Dai, C.C. Degradation of N-heterocyclic indole by a novel endophytic fungus *Phomopsis liquidambari*. *Bioresource Technology*, 2013; **129**: 568-574.
12. Tinoco, R., Vazquez-Duhalt, R. Chemical modification of cytochrome C improves their catalytic properties of polycyclic aromatic hydrocarbons. *Enzyme and Microbial Technology*, 1998; 22(1): 8-12.
13. Gomez, L., Villalonga, R. Functional stabilization of invertase by covalent modification with pectin. *Biotechnology Letters*, 2000; **22**(14): 1191-5.
14. Polizzi, K.M., Bommarius, A.S., Broering, J.M., Chaparro-Riggers, J.F. Stability of biocatalysts. *Current Opinion in Chemical Biology*, 2007; **11**: 220-5.
15. Vandertol-Vanier, H.A., Vazquez-Duhalt, R., Tinoco, R., Pickard, M.A. Enhanced activity by poly (ethylene glycol) modification of *Coriolopsis gallica* laccase. *Journal of Industrial Microbiology & Biotechnology*, 2002; **29**: 214-220.