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Research Article 研究报告

Semi-rational engineering of microbial laccase Lac15 for enhanced activity

XIE Yanan[1,2,3], WANG Rui[1,2,3], LI Zhi[1,2,3], LI Jie[1,2,3], FANG Zemin[1,2,3], FANG Wei[1,2,3], ZHANG Xuecheng[1,2,3*], XIAO Yazhong[1,2,3*]

1 School of Life Sciences, Anhui University, Hefei 230601, Anhui, China
2 Anhui Provincial Engineering Technology Research Center of Microorganisms and Biocatalysis, Hefei 230601, Anhui, China
3 Anhui Provincial Key Laboratory of Modern Biomanufacturing, Hefei 230601, Anhui, China

Abstract: [Objective] Laccases can oxidize a wide range of compounds and have great application value in diverse industries. Lac15, a microbial laccase with considerable application potential, can be modified by protein engineering to expand the application range. **[Methods]** By structure-based analysis, we chose some residues supposed to contribute directly or indirectly to electron/proton transfer or substrate interaction for site-directed mutagenesis, and measured the activities of the mutants for various substrates and the enzymatic properties. **[Results]** Some of the mutants showed significantly enhanced activity towards particular substrates. To be specific, the catalysis efficiency of mutant D216N toward 2,2′-azino-bis(3-ethylbenzthiazoline-6-suphonic acid) (ABTS) was 1.0 fold higher, and that of mutants R178V and K433T toward dopamine was 1.2 and 11.1 folds, respectively, higher than that of Lac15. Moreover, the mutants maintained the advantages of Lac15 in application, such as the neutral optimum pH and salt activation. **[Conclusion]** By structure-based semi-rational engineering, the specific activities of Lac15 toward some substrates can be improved. A conserved Asp in laccases, corresponding to D216 in Lac15, can be a common engineering target for enhancing the activity of the enzymes with ABTS as substrate or mediator.

Keywords: microbial laccase; Lac15; semi-rational engineering; activity; specificity

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***Corresponding authors.** Tel: +86-551-63861063; Fax: +86-551-63861861; E-mail: ZHANG Xuecheng, turenzh@ahu.edu.cn, XIAO Yazhong, yzxiao@ahu.edu.cn

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半理性改造提升细菌漆酶 Lac15 的催化活性

谢雅楠[1,2,3], 王瑞[1,2,3], 李治[1,2,3], 李杰[1,2,3], 方泽民[1,2,3], 房伟[1,2,3], 张学成[1,2,3*], 肖亚中[1,2,3*]

1 安徽大学生命科学学院，安徽 合肥 230601
2 安徽省微生物与生物催化工程技术研究中心，安徽 合肥 230601
3 安徽省现代生物制造重点实验室，安徽 合肥 230601

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摘　要：【目的】漆酶可氧化各种底物，在多个工业领域有很好的潜在应用价值。Lac15 是一种微生物漆酶，已表现出可观的应用潜能，可望通过蛋白质工程改造提升和拓展其应用。【方法】通过基于结构分析的半理性改造策略，选取推测与电子/质子转移或底物结合直接或间接相关的位点进行定点突变，并测定突变酶对各种底物的活性及酶学性质。【结果】部分突变体对某些底物的活性显著提升，其中 D216N 对 ABTS 的催化效率提高了 1.0 倍，R178V 和 K433T 对多巴胺的催化效率分别增加了 1.2 和 11.1 倍。同时，这些突变体保留了野生型酶在应用中的优点如中性最适 pH 和盐促活等性质。【结论】利用基于结构的半理性改造方法，可改善漆酶 Lac15 对某些底物的活性。漆酶中的一个保守 Asp，对应于 Lac15 中的 D216，可作为提升以 ABTS 为底物或介体时漆酶活性的一个共同改造目标。

关键词：细菌漆酶；Lac15；半理性改造；活性；特异性

Laccases (benzenediol: oxygen oxidoreductases, EC1.10.3.2) are multicopper oxidases (MCOs) with a superior efficiency for oxidation of organic compounds. In fungi and plants, laccases and laccase-like multi-copper oxidases (LMCOs) are thought to play important roles in both the synthesis and degradation of lignin[1], while in bacteria they are related with pigment synthesis, protection from stress and putatively ligninocellulose degradation etc[2–3]. The substrates of laccases and LCMOs, varying from one to another in a wide range, include lignin-like phenolic molecules such as polyphenols, methoxy substituted phenols, non-phenolic compounds such as aromatic diamines, and even inorganic compounds[4–5]. Compared with polyphenols substrates, monophenols such as 2,6-dimethoxy phenol and guaiacol are better substrates for laccase[1,6]. Among them, syringaldazine (SGZ) is often used to identify if an enzyme is laccase. Complex biopolymer substrates such as lignin, due to large size or high redox potential, cannot be directly oxidized by laccases. In these cases, some small low redox potential chemical compounds may be used as a mediator, whose oxidized radical is then able to oxidize large high redox potential substrate targets[2,7]. 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) is one of the most widely used mediators in the application of laccases[8]. Despite displaying various substrate specificity, all laccases share the same structural fold pattern, whose activity center

consists of one type 1 copper (T1), one type 2 copper (T2) and two type 3 copper (T3). The T1 extracts electrons from the substrate and transfers them to T2/T3, where oxygen is reduced and seize proton to transform to water. All laccases have similar substrate binding pocket profiles, delimited by several loops[9], and relatively conserved proton donor for oxygen, a Glu/Asp in a loop nearby T2/T3[10–11]. In the substrate binding pocket, hydrophobic residues aligned along the wall are thought important for recognition of the substrate[12–14], and a conserved Asp/Glu located at the bottom is believed to play an important role in deprotonation of the substrate and electron transfer[14–17]. Due to wide range of substrates, lack of requirement for exogenous co-factors, and with water as the only by-product, laccases as a green tool have great application potential in diverse industrial fields including environmental protection, medicine, organic synthesis, biodegradation and biofuels production etc[3,7–8,18–19].

Compared with the laccases from fungi and plants, those from bacteria have several applicational advantages such as neutral to alkaline optimum pH, salt tolerance and increased thermal and chemical stability, thus are more compatible with some industrial processes such as paper pulp bleaching and dye decolorization[2–3,20]. Recent screening of metagenomic libraries of diverse natural ecosystems has identified lots of new bacterial laccases with diverse and novel enzymatic properties[3]. Among them, Lac15 is a laccase previously identified from marine microbial metagenome, showing outstanding salt tolerance and application potential in dye decolorization[21]. Some studies showed that the substrate binding and electron transfer of laccases are dependent on the features of the substrate binding pocket, and mutations of the residues located around the pocket may have influence on the enzyme activity[14,22–24]. Rational design on the substrate pocket has been demonstrated to be able to guide the engineering of laccases, e.g. CotA from *Bacillus subtilis* and *Bacillus pumilus*, and PM1L from basidiomycetes PM1, better suited for target applications[3,14,22–26]. However, laccases with high structural similarity may have quite different residues delimiting the substrate binding pocket, making rational design on it differ from case to case. In present study, to improve and broaden the application potential of Lac15, we engineered it to enhance the activities towards specific substrates by semi-rational design. Based on the crystal structure of Lac15 (PDB: 4f7k)[27], some sites supposed to be related to activities towards specific substrates were chosen for site-directed mutagenesis, and the mutants were biophysically and biochemically characterized. The results showed that some mutants exhibited significantly enhanced activity towards some substrates, especially the D216 mutants, K433T and R178V displayed higher activity towards ABTS or catechol derivatives. Our results would help improve the application potential of Lac15 and understand the specificity of laccase activity.

1 Materials and methods

1.1 Reagents and strains

Laccases' substrates including 2,2′-azino-bis(3-ethylbenzthiazoline-6-suphonic acid) (ABTS), 2,6-dimethoxyphenol (2,6-DMP), 2-methoxyphenol (guaiacol), dopamine salt, catechol, and syringaldazine (SGZ) were purchased from Aladdin® (Shanghai). Other analytical grade chemicals and solvents were purchased from Sangon Biotech (Shanghai). *Escherichia coli* BL21(DE3) and pET-22b vector were purchased from TransGen Biotech (Beijing). PrimeSTAR HS DNA Polymerase, T4 DNA ligase, restriction enzyme *Nde* I and *Xho* I were purchased from TaKaRa (Dalian). *Escherichia coli* BL21(DE3) cells carrying pET22b-*lac15* was constructed previously in our laboratary[21].

1.2 Structure and sequence analysis

The structures of the laccases were downloaded from Protein Data Bank (PDB) and analyzed using UCSF Chimera[28].

1.3 Gene cloning, protein expression and purification

Single amino acid substitutions were created using through whole plasmid polymerase chain reaction (PCR), using a pair of complementary primers containing the substitution. For PCR, plasmid pET22b-*lac15* (containing wild-type Lac15 DNA sequence) was used as template. The PCR products were transformed into *E. coli* BL21(DE3) competent cells, and the clones were tested by sequencing to screen correct construct.

For expression, *E. coli* BL21(DE3) containing pET22b-*lac15* or mutant clone was cultivated in 400 mL LB medium containing 100 μg/mL ampicillin at 37 °C with shaking at 180 r/min to OD_{600}=0.6–0.8, and then induced with 0.2 mmol/L isopropyl β-D-thiogalactoside at 16 °C. After an additional culture of 20 h at 16 °C, the liquid growth media was centrifuged at 5 000×*g* for 20 min to collect cells, which were then resuspended and disrupted by sonication. The precipitate was removed by centrifugation at 12 000×*g* for 30 min, and the supernatant was applied to HiTrap™ Capto™ Q affinity chromatography (GE Healthcare) to purify the target protein. The protein was eluted from the column with a linear gradient of NaCl (0.1–1.0 mol/L) in 20 mmol/L Tris/HCl buffer (pH 8.0) at a flow rate of 1 mL/min. The eluted protein was dialyzed against a 50 mmol/L Na_2HPO_4-KH_2PO_4 buffer (pH 7.5).

1.4 Intrinsic fluorescence spectroscopy

The intrinsic fluorescence emission spectra of Lac15 and mutants D216N, K433T and R178V were record on an F-4500 FL spectrophotometer (Hitachi Limited, Japan), with excitation wavelength of 293 nm and emission wavelength range of 300–400 nm. All measurements were performed at 20 °C. The spectra of the buffers were subtracted from those of the samples to obtain the spectra of the protein. The samples contained 0.5 mg/mL purified protein in 50 mmol/L Na_2HPO4-KH_2PO_4 buffer (pH 7.5), with 100 μmol/L $CuSO_4$.

1.5 Enzyme assay

Laccase activity was quantified using SGZ, ABTS, catechol, dopamine, guaiacol, and 2,6-DMP as substrates. The assay mixture consisted of 10 μL of appropriately diluted laccase protein and 990 μL buffer containing 100 μmol/L $CuSO_4$, and 100 μmol/L SGZ, or 500 μmol/L ABTS, 2 mmol/L catechol, 2 mmol/L dopamine, 1 mmol/L guaiacol or 1 mmol/L 2,6-DMP. The buffers used were 50 mmol/L Na_2HPO_4-KH_2PO_4 (pH 7.5, 6.0 and 7.0) for SGZ, catechol, and dopamine, 50 mmol/L NaAC-HOAc (pH 6.0) for ABTS, and 50 mmol/L Tris-HCl (pH 8.0) for guaiacol and 2,6-DMP. After incubation at 45 °C for 5 min, the mixture was transferred into an ice-water bath for 30 second to stop the reaction. The absorbance was measured at 525 nm for SGZ's product [ε=65 000 L/(mol·cm)], 420 nm for ABTS's [ε=36 000 L/(mol·cm)], 388 nm for catechol's [ε=1 300 L/(mol·cm)], 475 nm for dopamine's [ε=2 834.7 L/(mol·cm)], 465 nm for guaiacol's [ε=1 200 L/(mol·cm)], 468 nm for 2,6-DMP's [ε=49 600 L/(mol·cm)]. One activity unit (U) was defined as the amount of laccase required to oxidize the substrate to produce 1 μmol product per minute. Reactions with heat-inactivated laccase were used as controls.

For optimal pH measurements, 50 mmol/L NaAc-HOAc buffer was used for pH 4.5–6.0, 50 mmol/L Na_2HPO_4-KH_2PO_4 for 6.0–8.0, and 50 mmol/L Tris-HCl for 8.0–9.0.

The enzyme kinetic analyses were carried out in reaction mixtures containing appropriate amounts of the purified enzymes under the optimal conditions at 45 °C. Initial rates of the reactions were tested at various concentrations of substrate in the range of 0.01–10.00 mmol/L for ABTS, 0.1–50.0 mmol/L for guaiacol, 0.1–10.0 mmol/L for dopamine, and 0.1–30.0 mmol/L for catechol. Non-linear regression analysis and Michaelis-Menten curve fitting were performed using Origin 8.5 to calculate the kinetic parameters.

2 Results and analysis

2.1 Design and preparation of Lac15 mutants

Lac15 is a laccase obtained from the South China Sea microbial metagenome by sequence screening, and it shows outstanding salt tolerance and application potential in dye decolorization[21]. To improve and broaden the application potential of Lac15, in this study we engineered it to enhance the activities towards specific substrates by semi-rational design. The structure of Lac15 has been resolved by X-ray crystallography previously (PDB: 4f7k)[27]. However, the T1 copper and three fragments comprising residues 172–181, 320–345 and 431–432, are missing in the crystal structure (Figure 1), likely due to their structural flexibility. Notably, by aligning Lac15's structure with other laccases', all these fragments correspond to be located within the loops involved in substrate binding (172–181 and 320–345) or T1 coordinating (431–432). This makes it difficult to rationally design the enzyme to improve its activity towards specific substrate based on the structure of the binding pocket. Therefore, we randomly chose some sites within these fragments and several other specific sites, which were supposed related to the activity by contributing to substrate binding or electron/proton transfer according to references[14–17,29–32] (Figure 1), and performed site-directed mutagenesis on them to investigate their roles in the enzyme activity. The mutants that are successfully recombinantly expressed are listed in Table 1, with the supposed roles of the mutated sites included. Among the chosen sites, D216 is the counterpart of the Asp/Glu that assists deprotonation of phenol and amine substrates in fungal laccases[15–17]; M432 is the counterpart of axial ligand of the T1 copper, related to the redox

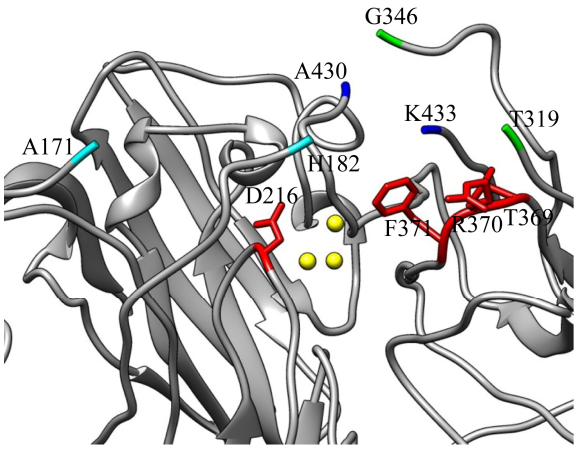

Figure 1 Diagram of the sites chosen for mutation in Lac15. The residues immediately flanking the fragments 172–181, 320–345, and 431–432, which are missing in the X-ray crystallography structure, are colored in cyan, green, and blue, respectively. In addition, other residues nearby the substrate pocket and the T1 center and chosen for mutation are colored in red. The image was generated using UCSF Chimera[28], with the coppers shown in yellow.

Table 1 List of Lac15 mutants successfully constructed and recombinantly expressed

Putative role of mutated site	Location	Mutants
Substrate binding related	A171-H182 loop	H182D, A181D, D179K, D179A, D179R, D179K, R178D, R178V, R178A, E175D, E175F
	T369-F371 loop	F371G, F371T, R370D, T369D, T369A
	T319-G346 loop	S345A, Q344E, G343A, N342D, M330S, M329K, R328K, R328D, R327D, S326L, S326K, M325D, E321K, M320A, M320K, M320S, T319K, M329S/M330S, M329K/M330K, M329F/M330F
	Q428-K432 loop	Q428E, Q428N, Q428A, Q428D, G431E
Proton transfer related	K433	K433E, K433D, K433A, K433T, K433R, K433F
Redox potential related	Axial ligand of the T1 copper	M432F, M432L
Substrate binding and deprotonation related	D216	D216A, D216T, D216N, D216E, D216K

potential[29–32]; others are supposed to directly or indirectly interact with the substrates. In addition, in the loop nearby T2/T3 of Lac15, there is no acidic residue counterpart to the conserved proton donor Glu/Asp in other laccases e.g. E498 in *Bacillus subtilis* CotA[10–11]. We mutated K433 in Lac15, the structural counterpart of E498 in *B. subtilis* CotA, as putative proton transfer related residues, to test if the mutations could enhance proton transfer thus improve the enzyme activity to all the substrates. 18 mutant proteins, expressed solubly with considerable amount and of more than 95% purity assessed by SDS-PAGE after purification (Figure 2), were further biophysically and biochemically characterized.

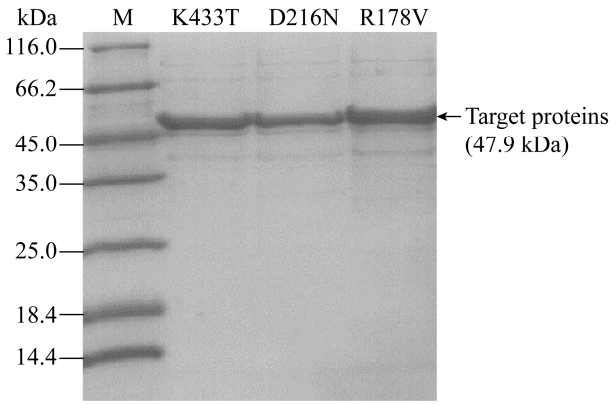

Figure 2 SDS-PAGE result for part of purified recombinant Lac15 mutants.

2.2 Characterization of Lac15 mutants

Most of the tested mutants showed decreased activity compared with wide type Lac15, except for a few displaying enhanced activities towards particular substrates (Table 2). Mutation of K433E was expected to improve the enzyme activities of Lac15 towards all the substrates by enhancing proton transfer. However, the activities of this mutant were instead decreased in various extents. Moreover, other mutants of this site displayed decreased or increased activities. These results indicated that there are other sites playing

Table 2 Relative activities (unit: %) of Lac15 mutants towards different substrates compared with the wide type enzyme

Enzymes	SGZ	ABTS	Catechol	Dopamine	Guaiacol	2,6-DMP
Lac15	100.00	100.00	100.00	100.00	100.00	100.00
T369D	38.80	24.68	8.70	76.21	6.69	13.04
M432F	2.16	/	5.63	/	/	2.90
M432L	17.23	/	57.88	/	/	37.69
M320F	61.04	113.79	57.95	89.74	56.12	77.41
M320A	326.90	145.60	25.92	36.11	16.80	33.42
M320S	43.63	58.21	29.06	87.76	14.11	17.89
M320K	4.78	10.94	13.35	13.33	2.44	3.67
D216A	32.82	**173.82**	72.02	30.47	102.19	59.94
D216T	55.62	**167.25**	111.22	39.81	68.00	61.36
D216N	20.49	**228.34**	57.18	30.49	48.86	35.98
D216E	38.77	**134.96**	109.17	49.77	59.42	76.99
D216K	51.84	**209.21**	94.46	34.58	130.05	60.10
K433F	115.45	10.31	131.62	82.58	67.32	62.43
K433E	71.69	32.48	58.86	86.63	43.47	65.01
K433R	81.48	90.48	115.98	116.19	80.07	102.01
K433T	85.25	18.85	**136.82**	**136.23**	51.56	60.29
R178V	102.68	44.81	**145.66**	**249.75**	87.34	74.09
Q428E	60.22	10.10	86.51	51.97	25.19	46.64

Values in bold are those significantly higher than the wild type enzyme.

role as proton donor while K433 contributes to the activity through other ways than donating proton. Notably, mutation of the sites nearby K433 to Glu did not enhance the enzymatic activity (data not shown), indicating in Lac15 there might be other types of proton donor than acidic residues.

Among the mutants, those of T369 uniquely displayed significantly decreased activity towards all the substrates, implying this site might play critical role in Lac15's activity. However, in the structure of Lac15, there is not any interaction observed between T369 and the residues involved in the activity center, maybe because the T1 copper nearby is missing in the structure. While, in the structure of an extensively studied bacterial laccase CotA (PDB: 3zdw), T415, the counterpart of T369 in Lac15, contributes to the T1 center constitution by its side chain hydrogen bonding to that of H419, which is a coordinating residue of the T1 copper. Putatively, in the complete form of Lac15 in which T1 is present, T369 plays the same role as T415 in CotA. So mutation of the T369 would remove the hydrogen bond, therefore destabilize T1 center and impair electron transfer.

The axial ligand of T1 copper in the laccasses from fungi and plants is always a Phe or Leu, resulting in a relatively higher redox potential[29–32]. In contrast to expectation, mutation of the axial ligand of the T1 copper in Lac15, M432, to Phe or Leu reduced rather than enhanced the enzymatic activity towards the substrates with high redox potential. This result, along with situations observed in other laccases, evidenced the complexity of factors affecting laccases' redox potential[30,33].

Mutation of M320 to polar residues decreased the enzymatic activities towards all the tested substrates, while mutation to hydrophobic residues resulted in varying effects, with mutant M320A specifically displaying higher activity towards SGZ and ABTS. In the structure of CotA complex with ABTS (PDB: 3zdw), Q378, the counterpart of M320 in Lac15, is located close to one of the two symmetrical units of ABTS, indicating this site may contribute to docking the bulky substrate. SGZ, like ABTS, is also composed of two symmetrical parts. Thus the mutation of M320 to A, decreasing the side chain size and reducing steric hindrance, may facilitate binding of these bulky substrates.

Another site whose mutations showed positive effects on the activity of Lac15 towards ABTS is D216, with mutant D216N displaying the highest activity. However, the mutations at the same time led to negative effect on the activities towards SGZ and most of other substrates. This implied D216 is unfavorable to interact with ABTS, probably due to its negative charge that would repulse the same charged SO_3^- of the substrate. Notably, in fungal laccases, the residue corresponding to D216 in Lac15 is conserved Asp/Glu, which is responsible for their acidic optimum pH[3,17,32,34]; while in bacterial laccases such as CotA and CueO, there is a Thr at the same site. In the structure of *Trametes versicolor* laccase complex with xylidine (PDB: 1KYA), D206, the counterpart of D216 in Lac15, interacts with the substrate through the terminal oxygen of its side chain hydrogen bonding to xylidine and facilitates deprotonation of the substrate[15]. While, in a structure of CotA complex with ABTS (PDB: 1OF0), T260, the counterpart of D216 in Lac15, faces to the substrate with its hydrophobic methyl group instead of polar hydroxyl group. Therefore the mutations at this site in Lac15 would reduce the activities towards phenol and arylamine substrates, by impairing substrate binding and deprotonation, while enhance the activity towards ABTS by facilitating substrate binding[17].

Mutations at K433 enhanced the activity towards some of the tested substrates; especially the mutations to Arg and Thr enhanced the activity towards the catechol derivatives. As in the loop nearby T2/T3 in Lac15 there is no Glu/Asp that could play a role as proton donor, K433, the structural counterpart of the proton donor in other laccase, a conserved Glu/Asp[10–11], may have effect on proton transfer or substrate binding. Mutant R178V also displayed enhanced

activity towards the catechol derivatives, probably due to higher contribution of Val to hydrophobic interaction with the aromatic ring of the phenol substrates.

To exclude the contribution of structural change to the activity variation caused by the mutations, tertiary structure of the mutants was measured by recording their intrinsically fluorescence spectra. As shown in Figure 3, the maximum emission wavelengths of the tested mutants were all identical with that of wild-type Lac15, indicating the overall tertiary structure was not disturbed by the mutations. Therefore, the activity change might be caused by other effects than structural one derived from the mutation.

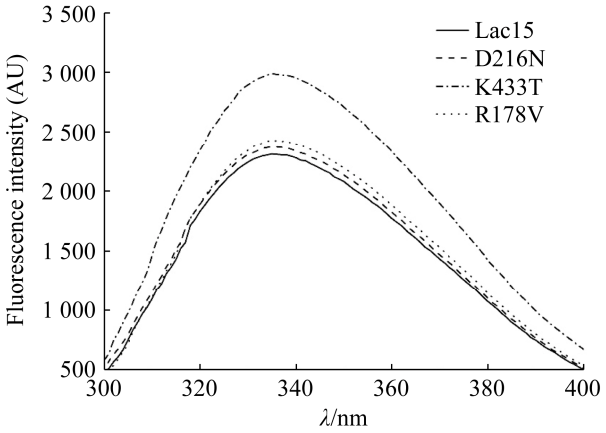

Figure 3 Intrinsic fluorescence emission spectra of Lac15 and its mutants, with the excitation wavelength of 280 nm.

2.3 Activities of Lac15 mutants D216N, K433T and R178V towards ABTS and catechol derivatives

Among the mutants, D216N, K433T and R178V showed the highest activity towards ABTS and the catechol derivatives respectively. We then measured the optimum conditions and kinetic parameters for the activity of the mutants to assess their relevant application potentials. As shown in Figure 4, optimum pHs for the activity

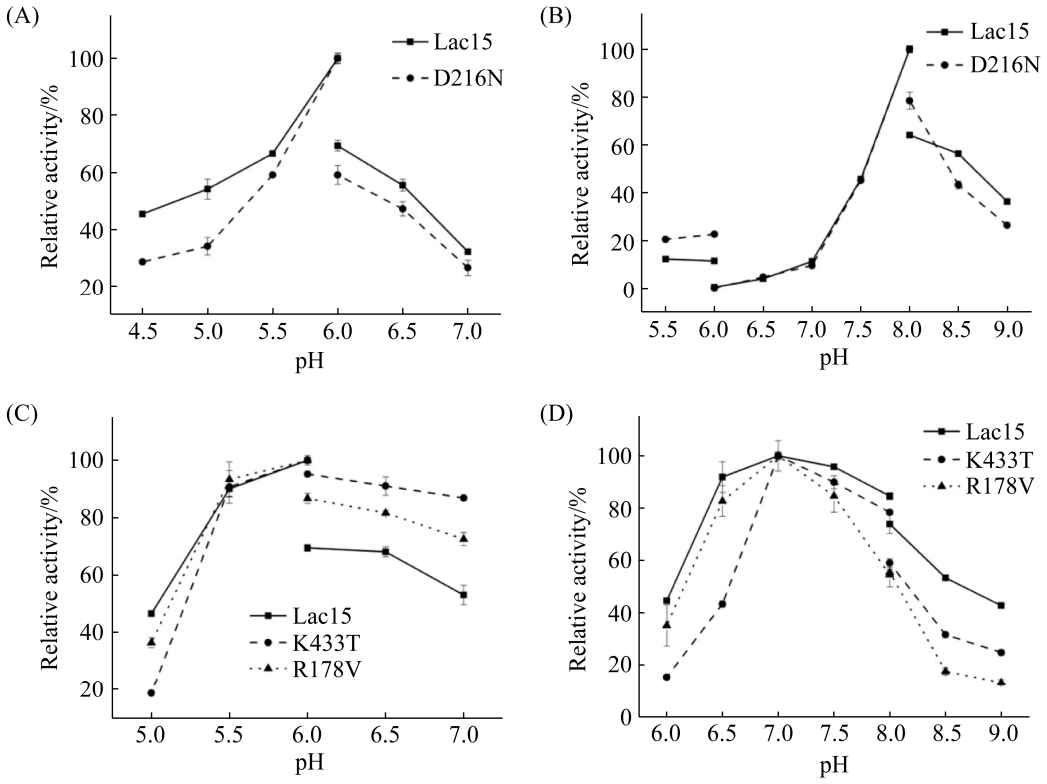

Figure 4 Activity-pH profiles of Lac15 and mutants D216N, K433T and R178V towards ABTS (A), guaiacol (B), catecol (C) and dopamine (D).

of Lac15 towards all the tested substrates are higher than 6, which is an advantage of bacterial laccases in application. Notably, in comparison with fungal laccases, which present conserved Asp/Glu at the site corresponding to D216 in Lac15 and display acidic optimum pHs[17], Lac15 displayed much higher optimum pHs. Besides, D216N mutation in Lac15 had no influence on the optimum pHs (Figure 4A and 4B), while mutations of the counterpart Asp in a laccase from fungus *Trametes versicolor* to Asn and Ala shift its optimum pHs to higher values[14]. In addition to the fact that various mutation at D216 in Lac15 exerted either enhancement or reduction effect on the enzyme activities towards different substrates (Table 2), these indicated this residue plays distinctive roles from its counterparts in fungal laccases. In addition to near neutral optimum pH, salt tolerance and activation are another advantage of bacterial laccases in application. As shown in Figure 5, Lac15 exhibited salt tolerance and activation towards all the tested substrates, and mutant D216N maintained the property. As for mutants R178V and K433T, the neutral optimum pHs were maintained while the salt activations were eliminated compared with the wide type enzyme. Since both R178 and K433 are positively charged, putatively they are the sites where Cl^- could bind to activate the enzyme activity.

To reveal the details of effects of the mutations on the enzymatic activity, kinetic parameters were measured for Lac15 and mutants D216N, R178V and K433T (Table 3). For ABTS as substrate, the substitution of D216 with Asn led to an about 1-fold decrease in the K_m value,

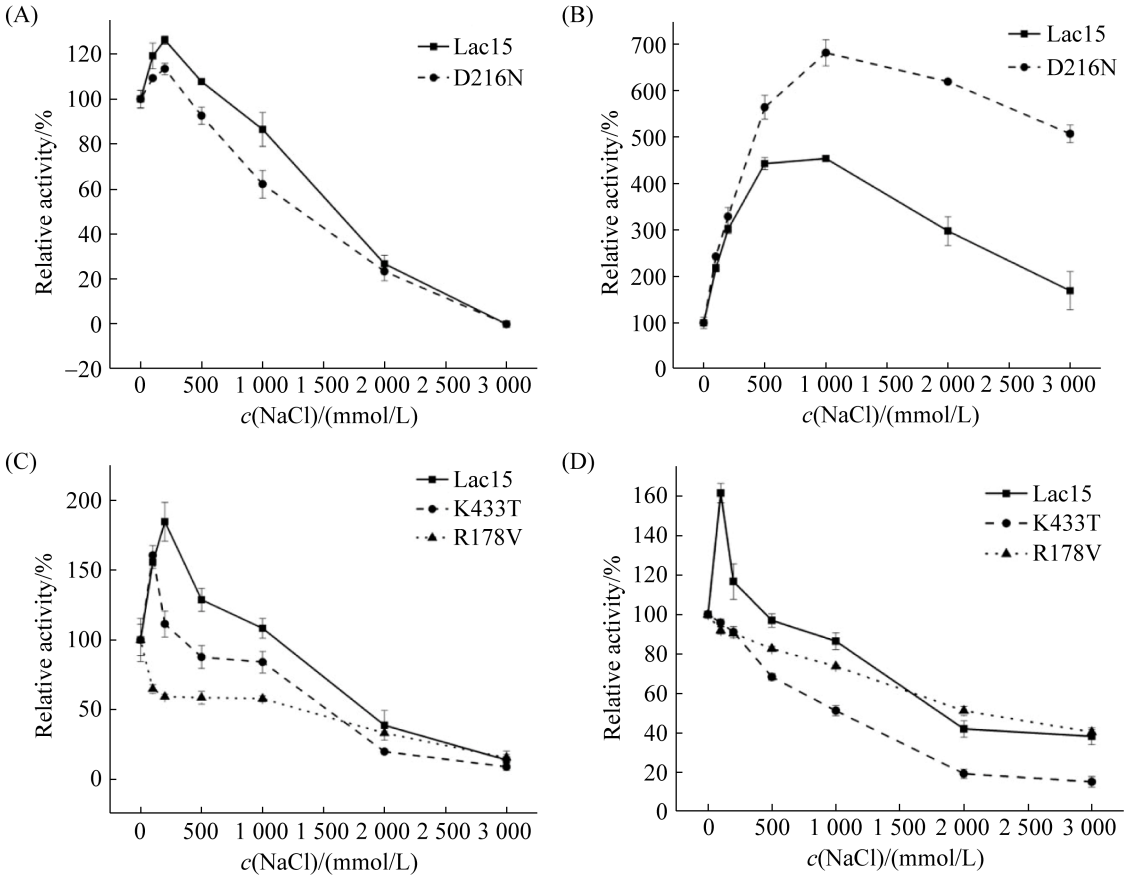

Figure 5 Dependence of the enzyme activity on NaCl for Lac15 and mutants D216N, K433T and R178V towards ABTS (A), guaiacol (B), catecol (C) and dopamine (D).

Table 3 Kinetic parameters of Lac15 and mutants D216N, R178V, and K433T towards ABTS and phenolics

Substrates	Variant	K_m/(μmol/L)	k_{cat}/s^{-1}	k_{cat}/K_m/[L/(μmol·s)]
ABTS	Lac15	951.781±161.670	0.297±0.007 7	3.124E–04±7.69E–05
	D216N	511.80±42.42	0.324±7.59E-03	6.33E–04±1.79E–04
Guaiacol	Lac15	11 714.60±2 302.99	1.527±0.220	1.303 5E–04±9.73E–05
	D216N	5 210.48±658.81	0.411±0.029 1	7.887 9E–05±4.41E–05
Dopamine	Lac15	1 548.90±131.71	0.955±0.026 9	6.165 6E–04±2.04E–04
	R178V	613.79±50.87	0.845±0.016 1	1.376 69E–03±3.17E–04
	K433T	211.81±16.86	1.582±0.020	7.468 96E–03±1.18E–03
Catechol	Lac15	2 449.27±305.34	7.321±0.384	2.989E–03±1.26E–03
	R178V	1 942.64±234.31	8.048±0.374	4.143E–03±1.59E–03
	K433T	5 186.5±994.8	23.429±2.679	4.517E–03±2.69E–03

denoting an increase in the affinity for the substrate. The mutation at the same time resulted in a slight increase in k_{cat}, denoting a little enhancement of the catalysis rate. As a comparison, mutation of the counterpart Asp in the laccase of *Trametes versicolor* to Asn resulted in more than two folds decrease in K_m and one fold increase in k_{cat} for ABTS[17]. This is consistent with the deduced negative role of the Asp in binding to the same charged ABTS. With guaiacol as substrate, D216N showed decreased K_m and k_{cat}, denoting enhanced substrate binding while reduced catalytic rate. This is consistent with the positive roles of its counterpart Asp/Glu in fungal laccases in binding and deprotonation of phenol and arylamine substrates[15–17]. For the mutant R178V, a significant enhancement of K_m was found towards both the catechol derivatives compared with wild-type Lac15, while the changes of k_{cat} were much slighter. This indicated R178 may be involved in only substrate binding but catalysis process; substitution of the polar Arg with hydrophobic Val may facilitate binding of hydrophobic phenyl ring of the catechol derivatives. Compared with wild-type Lac15, mutant K433T showed significant decrease in K_m and increase in k_{cat}, implying both substrate binding and catalysis process were influenced by the mutation. As the structural counterpart of the proton donor Glu in other laccase, K433 may influence the proton transfer in Lac15, therefore its mutation would change catalytic rate. In sum, compared with wild-type Lac15, the three mutants exhibited higher catalysis efficiency, with the k_{cat}/K_m of D216N to ABTS, R178V and K433T to dopamine were enhanced by 1.0 fold, 1.2 folds, and 11.1 folds, respectively.

3 Discussion and conclusion

In this study, some sites in Lac15 supposed to be involved in substrate binding and electron/proton transfer between the substrates and the laccase were chosen to be mutated, and the mutants were biophysically and biochemically characterized and compared with the wild type enzyme. The results identified some sites especially D216, R178 and K433 whose mutations significantly changed the enzyme activity towards particular substrates, and provide hints for the roles of these sites in the enzyme activity, with R178 related to substrate binding while D216 and K433 referred to both substrate binding and proton transfer. Our results may contribute to improving application of Lac15 and better understanding of activity mechanisms especially substrate specificity of laccases. Notably, mutations at D216 in Lac15, as those at its conserved counterpart Asp/Glu in fungal laccases, would lead to higher catalytic efficiency to ABTS and maintained optimum conditions. As reported in the reference [17], at most a 3-fold increase was obtained for k_{cat} between the wild-type *Trametes versicolor* laccase and the most efficient mutant of Asp206 (counterpart to D216 of Lac15) with ABTS

as a substrate, similar to the result obtained in this study. These suggest this site may be chosen as a common target for engineering to enhance laccase activity when using ABTS as substrate or mediator. However, compared with *T. versicolor* laccase and its D206 mutants, Lac15 and its D216 mutants have higher optimum pH, supporting application in a wider range.

References

[1] Arora DS, Sharma RK. Ligninolytic fungal laccases and their biotechnological applications. *Applied Biochemistry and Biotechnology*, 2010, 160(6): 1760–1788.

[2] Chauhan PS, Goradia B, Saxena A. Bacterial laccase: recent update on production, properties and industrial applications. *3 Biotech*, 2017, 7(5): 323.

[3] Martins LO, Durão P, Brissos V, Lindley PF. Laccases of prokaryotic origin: enzymes at the interface of protein science and protein technology. *Cellular and Molecular Life Sciences*, 2015, 72(5): 911–922.

[4] Janusz G, Pawlik A, Świderska-Burek U, Polak J, Sulej J, Jarosz-Wilkołazka A, Paszczyński A. Laccase properties, physiological functions, and evolution. *International Journal of Molecular Sciences*, 2020, 21(3): 966.

[5] Kudanga T, Burton S, Nyanhongo GS, Guebitz GM. Versatility of oxidoreductases in the remediation of environmental pollutants. *Frontiers in Bioscience: Elite Edition*, 2012, 4: 1127–1149.

[6] Baldrian P. Purification and characterization of laccase from the white-rot fungus *Daedalea quercina* and decolorization of synthetic dyes by the enzyme. *Applied Microbiology and Biotechnology*, 2004, 63(5): 560–563.

[7] Riva S. Laccases: blue enzymes for green chemistry. *Trends in Biotechnology*, 2006, 24(5): 219–226.

[8] Kudanga T, Roes-Hill M. Laccase applications in biofuels production: current status and future prospects. *Applied Microbiology and Biotechnology*, 2014, 98(15): 6525–6542.

[9] Hakulinen N, Rouvinen J. Three-dimensional structures of laccases. *Cellular and Molecular Life Sciences*, 2015, 72(5): 857–868.

[10] Bento I, Silva CS, Chen Z, Martins LO, Lindley PF, Soares CM. Mechanisms underlying dioxygen reduction in laccases. Structural and modelling studies focusing on proton transfer. *BMC Structural Biology*, 2010, 10: 28.

[11] Chen ZJ, Durão P, Silva CS, Pereira MM, Todorovic S, Hildebrandt P, Bento I, Lindley PF, Martins LO. The role of Glu498 in the dioxygen reactivity of CotA-laccase from *Bacillus subtilis*. *Dalton Transactions*: *Cambridge*, *England*, 2010, 39(11): 2875–2882.

[12] Cambria MT, Di Marino D, Falconi M, Garavaglia S, Cambria A. Docking simulation and competitive experiments validate the interaction between the 2,5-xylidine inhibitor and *Rigidoporus Lignosus* laccase. *Journal of Biomolecular Structure and Dynamics*, 2010, 27(4): 501–510.

[13] Galli C, Gentili P, Jolivalt C, Madzak C, Vadalà R. How is the reactivity of laccase affected by single-point mutations? Engineering laccase for improved activity towards sterically demanding substrates. *Applied Microbiology and Biotechnology*, 2011, 91(1): 123–131.

[14] Pardo I, Camarero S. Laccase engineering by rational and evolutionary design. *Cellular and Molecular Life Sciences*, 2015, 72(5): 897–910.

[15] Bertrand T, Jolivalt C, Briozzo P, Caminade E, Joly N, Madzak C, Mougin C. Crystal structure of a four-copper laccase complexed with an arylamine: insights into substrate recognition and correlation with kinetics. *Biochemistry*, 2002, 41(23): 7325–7333.

[16] Kallio JP, Gasparetti C, Andberg M, Boer H, Koivula A, Kruus K, Rouvinen J, Hakulinen N. Crystal structure of an ascomycete fungal laccase from *Thielavia arenaria*-common structural features of asco-laccases. *The FEBS Journal*, 2011, 278(13): 2283–2295.

[17] Madzak C, Mimmi MC, Caminade E, Brault A, Baumberger S, Briozzo P, Mougin C, Jolivalt C. Shifting the optimal pH of activity for a laccase from the fungus *Trametes versicolor* by structure-based mutagenesis. *Protein Engineering, Design and Selection*, 2006, 19(2): 77–84.

[18] Giardina P, Faraco V, Pezzella C, Piscitelli A, Vanhulle S, Sannia G. Laccases: a never-ending story. *Cellular and Molecular Life Sciences*, 2010, 67(3): 369–385.

[19] Pezzella C, Guarino L, Piscitelli A. How to enjoy laccases. *Cellular and Molecular Life Sciences*, 2015, 72(5): 923–940.

[20] Singh G, Arya SK. Utility of laccase in pulp and paper industry: a progressive step towards the green technology. *International Journal of Biological*

Macromolecules, 2019, 134: 1070–1084.

[21] Fang ZM, Li TL, Wang Q, Zhang XC, Peng H, Fang W, Hong YZ, Ge HH, Xiao YZ. A bacterial laccase from marine microbial metagenome exhibiting chloride tolerance and dye decolorization ability. *Applied Microbiology and Biotechnology*, 2011, 89(4): 1103–1110.

[22] Chen Y, Luo Q, Zhou W, Xie Z, Cai YJ, Liao XR, Guan ZB. Improving the catalytic efficiency of *Bacillus pumilus* CotA-laccase by site-directed mutagenesis. *Applied Microbiology and Biotechnology*, 2017, 101(5): 1935–1944.

[23] Giardina P, Sannia G. Laccases: old enzymes with a promising future. *Cellular and Molecular Life Sciences*, 2015, 72(5): 855–856.

[24] Khodakarami A, Goodarzi N, Hoseinzadehdehkordi M, Amani F, Khodaverdian S, Khajeh K, Ghazi F, Ranjbar B, Amanlou M, Dabirmanesh B. Rational design toward developing a more efficient laccase: catalytic efficiency and selectivity. *International Journal of Biological Macromolecules*, 2018, 112: 775–779.

[25] Arregui L, Ayala M, Gómez-Gil X, Gutiérrez-Soto G, Hernández-Luna CE, Herrera de Los Santos M, Levin L, Rojo-Domínguez A, Romero-Martínez D, Saparrat MCN, Trujillo-Roldán MA, Valdez-Cruz NA. Laccases: structure, function, and potential application in water bioremediation. *Microbial Cell Factories*, 2019, 18(1): 200.

[26] Stanzione I, Pezzella C, Giardina P, Sannia G, Piscitelli A. Beyond natural laccases: extension of their potential applications by protein engineering. *Applied Microbiology and Biotechnology*, 2020, 104(3): 915–924.

[27] Ge HH, Xu PS, Xu Y, Fang ZM, Xiao YZ. Purification, crystallization and preliminary crystallographic analysis of recombinant Lac15 from a marine microbial metagenome. *Acta Crystallographica Section F Structural Biology and Crystallization Communications*, 2011, 67(8): 956–958.

[28] Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. UCSF Chimera-a visualization system for exploratory research and analysis. *Journal of Computional Chemistry*, 2004, 25(13): 1605–1612.

[29] Durão P, Bento I, Fernandes AT, Melo EP, Lindley PF, Martins LO. Perturbations of the T1 copper site in the CotA laccase from *Bacillus subtilis*: structural, biochemical, enzymatic and stability studies. *Journal of Biological Inorganic Chemistry*, 2006, 11(4): 514–526.

[30] Mot AC, Silaghi-Dumitrescu R. Laccases: complex architectures for one-electron oxidations. *Biochemistry: Moscow*, 2012, 77(12): 1395–1407.

[31] Xu F, Berka RM, Wahleithner JA, Nelson BA, Shuster JR, Brown SH, Palmer AE, Solomon EI. Site-directed mutations in fungal laccase: effect on redox potential, activity and pH profile. *Biochemical Journal*, 1998, 334: 63–70.

[32] Xu F, Palmer AE, Yaver DS, Berka RM, Gambetta GA, Brown SH, Solomon EI. Targeted mutations in a *Trametes villosa* laccase: axial perturbations of the T1 copper. *Journal of Biological Chemistry*, 1999, 274(18): 12372–12375.

[33] Cambria MT, Gullotto D, Garavaglia S, Cambria A. *In silico* study of structural determinants modulating the redox potential of *Rigidoporus lignosus* and other fungal laccases. *Journal of Biomolecular Structure and Dynamics*, 2012, 30(1): 89–101.

[34] Xu F. Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases. *Journal of Biological Chemistry*, 1997, 272(2): 924–928.

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