

## IMPROVED IMMOBILIZATION OF LACCASE ON A GLASSY CARBON ELECTRODE BY ORIENTED COVALENT ATTACHMENT

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**Abstract** – A laccase from *Thermus thermophilus* HB27 was reported to be potentially useful in the design of a temperature-controlled biofuel cell. For enhancing its application in different thermal conditions, we engineered a laccase-oriented immobilized electrode. A site-directed mutant N323C of the laccase was constructed. A photometric assay was employed in order to compare the catalytic properties of wild-type laccase and mutant. The mutant was attached to a glass carbon electrode by covalent cross-linking. The electrochemical properties of the immobilized laccase were investigated by cyclic voltammetry. This immobilization allowed the active electrode to function at temperatures up to 95°C. The thermal and pH dependence profiles were similar to those of the soluble enzyme investigated by spectrophotometry.

**Key words:** laccase; thermophilic; site directed mutagenesis; immobilization; electrochemistry

### INTRODUCTION

Laccases are multicopper oxidases oxidizing a wide variety of substrates such as lignin, dyes, polyaromatic hydrocarbons, etc. The oxidation takes place with only a concomitant reduction of O<sub>2</sub> to H<sub>2</sub>O (Solomon et al., 1996; Thurston, 1994; Mayer and Staples, 2002). Most laccases are monomeric proteins. While there is low overall sequence similarity, the structure and catalytic mechanism of laccases are conserved (Nakamura and Go, 2005). Most blue copper proteins consist of three cupredoxin domain folds (Sirim et al., 2011). As shown in Fig. 1, the first (N-terminal, domain 1) cupredoxin-like domain contributes to the formation of the binding site of the trinuclear copper center, which is located in the interface between domains 1 and 3. Domain 3 usually contains the mononuclear copper center. Moreover, domain 3 includes the putative substrate binding site, located close to the type I mononuclear copper center. The

overall fold of the second cupredoxin-like domain (domain 2) acts as a bridge between domains 1 and 3. Besides the domain folds, further similarities are more significant in the N- and C-terminal regions corresponding to domains 1 and 3 in laccase structures. The amino acids at the C terminal form a lid-like structure over the substrate-binding site. Many engineered mutations in the C-terminal regions of laccases have been reported to affect various properties of laccases (Koschorreck et al., 2009; Mate et al., 2010; Blevé et al., 2013).

Based on their catalytic characters, laccases are attractive for various applications such as pulp bleaching, biosensors, degradation of polycyclic aromatic hydrocarbon and biofuel cells (Mayer and Staples, 2002). Therefore, laccase immobilization, as a fundamentally important factor in many protein application fields, is an indispensable part of laccase researches to improve the performance of laccases

(Ribeiro et al., 2011). Specifically in biofuel cells, many engineered immobilizations of laccases have been reported. Several researches have reported to immobilize laccases on materials by cross-linking (Franzoi et al., 2009), entrapping (Liu et al., 2011), adsorption (Liu et al., 2012), and terminal covalent binding (Freire et al., 2001; Yinghui et al., 2002; Stanescu et al., 2010). These methods still suffer from low reproducibility, poor deposition or low catalytic ability. This study aimed to improve a thermosensitive laccase-immobilized electrode (Liu et al., 2011). In a previous study, *Thermus thermophilus* HB27 (*Tth*-laccase) was simply fixed on the surface of a glassy carbon electrode (GCE) using a kind of high melting-point agarose gel to describe its thermal-sensitive property. However, the gel could only achieve the immobilization support at a temperature of 70°C and the protein easily leaked out, whereas *Tth*-laccase shows its own best catalytic activity at 92°C (Miyazaki, 2005). In order to obtain a conducting surface matching the thermal property of *Tth*-laccase, oriented covalent protein immobilization was employed at a site that does not affect the enzyme active center. Though specific sites such as the N and C terminal ends were reported to serve for laccase immobilization, it could possibly hinder substrates from getting into the enzyme activity center or affect the functional structure (Stanescu et al., 2010; Bleve et al., 2013). Therefore, in this study, a different orientation of the immobilization is presented and was performed by engineering a third-site covalent coupling. Since laccases have similar domain structures (Nakamura and Go, 2005), the corresponding site attachment strategy could also be considered for the immobilization of many other laccases on different support materials.

#### Experimental procedure

##### Reagents

An expression vector pQE-70 containing cDNA of the *Tth*-laccase was kindly provided by Dr. Bachas' lab (University of Miami). The oligonucleotides used for mutagenesis were synthesized by Invitrogen Biotechnology Co., Ltd (Shanghai, China). Reagents used for site-directed mutation and molecular

weight markers were from New England Biolabs Beijing Ltd (Beijing, China). *E. coli* strain Top10 was used for cloning, and *E. coli* BL21 (DE3) was used for protein expression. The Ni-NTA Superflow resin was acquired from Qiagen China Co., Ltd (Shanghai, China). 2,2-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) was procured from Sigma (Shanghai, China). The cross-linker Sulfo-EMCS and other required specific reagents for cross-linking were from Thermo Fisher Scientific China (Beijing, China). All other chemicals were of reagent grade or better and commercially available. Deionized water was used in the preparation of all solutions.

#### Site-directed mutagenesis

The asparagine 323 to cysteine (N323C) mutation was performed according to the standard protocol. PQE-70 plasmid containing the cDNA of laccase was used as the template for introduction of the site-directed mutation. Primers were designed as follows: 5-T TAC CTC ATC GCC CCC AAG TGC CCC AAG CCC TTA CCC CT, sense; and 5-AG GGG TAA GGG CTT GGG GCA CTT GGG GGC GAT GAG GTA A, antisense. The PCR temperature program was initiated at 95°C for 5 min, followed by 18 cycles at 95°C for 60 s, 55°C for 45 s, 68°C for 90 s, and a final extension at 68°C for 5 min. The desired mutation was confirmed by DNA sequencing and the plasmid was named QE-70-N323C.

#### Expression, purification and copper binding of *Tth*-laccase apoenzyme

The procedure was mainly the same as that described in a previous paper (Liu et al., 2011). *E. coli* BL21 (DE3) harboring pQE-70-N323C was grown in LB medium containing ampicillin and induced to express the mutated *Tth*-laccase. The cells were collected and suspended in 40 ml of lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, 10 mM β-Mercaptoethanol, pH 8.0) for subsequent purification.

The cells were disrupted by sonication on ice. The protein was partially purified by heating and

chilling the lysate to precipitate the thermally unstable proteins. After centrifugation, the supernatant, containing recombinant laccase, was loaded onto a Ni-NTA column equilibrated with lysis buffer. Then the column was washed with wash buffer (50 mM  $\text{NaH}_2\text{PO}_4$ , 300 mM NaCl, 60 mM imidazole, 10 mM  $\beta$ -Mercaptoethanol, pH 8.0). Proteins were eluted under gradient elution by increasing the concentration of imidazole up to 300 mM in the wash buffer. Protein samples were collected and analyzed by 10% SDS-polyacrylamide gel electrophoresis. The protein bands were visualized by Coomassie brilliant blue R250 staining.

After purification, the eluted protein was dialyzed twice against 1 L of 20 mM acetate buffer containing 0.1 mM  $\text{CuSO}_4$  (pH 6.0) at 4°C for 48 h. Then the protein solution was dialyzed three times against 1 L of 20 mM acetate buffer (pH 4.5) without copper ion at 4°C for another 48 h. The precipitate was removed by centrifugation at  $18\,000\times g$ , 4°C, for 10 min. The supernatant was collected for further tests, and the protein concentration was determined using the Bradford method (Bradford, 1976).

#### *Laccase activity assay*

The oxidase activity of laccase was measured spectrophotometrically in air-saturated 20 mM sodium acetate buffer with 100 mM  $\text{CuSO}_4$ , pH 4.5 (Li et al., 2007). The method was based on the oxidation of the substrate 2,2-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS), which results in a colored product that absorbs at 420 nm with an absorbance coefficient value  $\epsilon=36,000\text{ M}^{-1}\text{ cm}^{-1}$ . The reaction was carried out for 5 min at pH 4.5, the referenced optimum pH of recombinant *Tth*-laccase. The specific activity was expressed as micromole of ABTS oxidized per minute per milligram of protein. The concentration of the purified protein in the assay solution was 10  $\mu\text{g/mL}$ . Buffer without protein was used as control. By using the enzyme activity assay, the temperature dependence and pH dependence of free enzymes were investigated. Reactions were performed in triplicate at different temperatures using a water-jacketed cuvette. 50 mM citric acid- $\text{Na}_2\text{HPO}_4$

buffer was used as the reaction buffer for varying the pH from 2.5 to 7.0.

#### *Site-directed immobilization*

The working electrode was a glassy carbon electrode (GCE, diameter, 3.0 mm) (Bioanalytical Systems, BAS MF-2012) modified with laccase on its working surface adapted from the cross-linking product instructions. The electrode surface was polished for 5 min with a polishing cloth and alumina-water slurry; then it was sonicated for 5 min to remove any adsorbed alumina and was rinsed with deionized water. After that, the working surface was immersed in a 2% solution of 3-aminopropyltriethoxysilane in acetone for 30 s, rinsed with dry acetone and allowed to air-dry. A solution of 2 mg cross-linker Sulfo-EMCS in 1 ml Coupling Buffer (50 mM Phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2) was prepared before use. The dried silylated surface was incubated in Sulfo-EMCS solution for 1 h at room temperature. Then the modified surface was rinsed with coupling buffer. The maleimide-activated surface material was covered with the laccase solution that was dialyzed in coupling buffer to be 20  $\mu\text{g/mL}$  for 4 h at room temperature. Lastly, the surface was thoroughly rinsed with coupling buffer to ensure that only covalently attached *Tth*-laccase molecules remained. The electrode was ready to use or could be dried and stored at 4°C overnight.

#### *Electrochemical assay*

A PARSTAT 2273 advanced electrochemical system (AMETEK Princeton Applied Research, Oak Ridge, TN) was used to obtain cyclic voltammograms of ABTS, with a saturated Ag/AgCl electrode as the reference electrode, platinum foil as the counter electrode, and the modified GCE as the working electrode. Measurements were performed in assay buffer (20 mM sodium acetate buffer, 1 mM ABTS, pH 4.5). Before the experiment, the working electrode was placed in the assay buffer. The buffer was saturated with air before starting the experiments and kept saturated by bubbling air during the experiments. Control experiments were performed using the same

**Table 1.** Kinetic properties of recombinant wide-type *Tth*-laccase and the N323C mutant in solution (data are averages  $\pm$  standard deviation,  $n=3$ ).

Tth-laccase types	$K_M$ (mM)			Specific activity ( $\mu\text{mol min}^{-1}\text{mg}^{-1}$ )			$k_{\text{cat}}/K_M$ ( $\text{min}^{-1}\text{mM}^{-1}$ )		
	25 ( $^{\circ}\text{C}$ )	65 ( $^{\circ}\text{C}$ )	90 ( $^{\circ}\text{C}$ )	25 ( $^{\circ}\text{C}$ )	65 ( $^{\circ}\text{C}$ )	90 ( $^{\circ}\text{C}$ )	25 ( $^{\circ}\text{C}$ )	65 ( $^{\circ}\text{C}$ )	90 ( $^{\circ}\text{C}$ )
wild type	0.329 $\pm$ 0.121	0.161 $\pm$ 0.013	0.128 $\pm$ 0.003	0.019 $\pm$ 0.002	0.046 $\pm$ 0.005	0.106 $\pm$ 0.004	3.195 $\pm$ 0.723	15.079 $\pm$ 1.285	42.995 $\pm$ 2.593
N323C	0.26 $\pm$ 0.012	0.156 $\pm$ 0.005	0.131 $\pm$ 0.007	0.017 $\pm$ 0.001	0.046 $\pm$ 0.002	0.096 $\pm$ 0.007	3.32 $\pm$ 0.183	15.398 $\pm$ 0.744	38.269 $\pm$ 2.779

reaction mixtures with the electrode being modified with wild-type *Tth*-laccase instead. Using cyclic voltammetry, the  $K_M$  and  $k_{\text{cat}}$  values of the immobilized laccase were calculated. The reaction rate was determined using five substrate (ABTS) concentrations in the range 0.25 to 1.5 M. The pH and temperature dependence of immobilized N323C *Tth*-laccase were also investigated by cyclic voltammetry method.

## RESULTS AND DISCUSSION

### *Mutant preparation*

Our work started with the design of the site-directed mutagenesis of *Tth*-laccase for oriented covalent protein immobilization. In order to minimize the effect of the mutation on the properties of the enzyme, we chose asparagine 323 as the mutation site to be changed to cysteine. As shown in Fig. 1, 323N is at the bottom point of the cupredoxin structure of laccase domain 2 (residues 164–332; Fig. 1), a surface location away from the enzyme active site and the copper binding sites. Therefore, this is considered an ideal location away from the enzyme active site and other effect sites. The enzyme molecules can be oriented on the support surface so that their active sites uniformly face the solution phase. It is conceivable that enzymes immobilized in this oriented manner will have a higher specific activity than randomly immobilized enzymes. *Tth*-laccase has a cysteine near its T1 copper. Considering its essential role for enzyme activity and its entrapped location in the enzyme structure, it was assumed that the original cysteine should be retained and that it would not influence the cysteine 323 immobilization much.

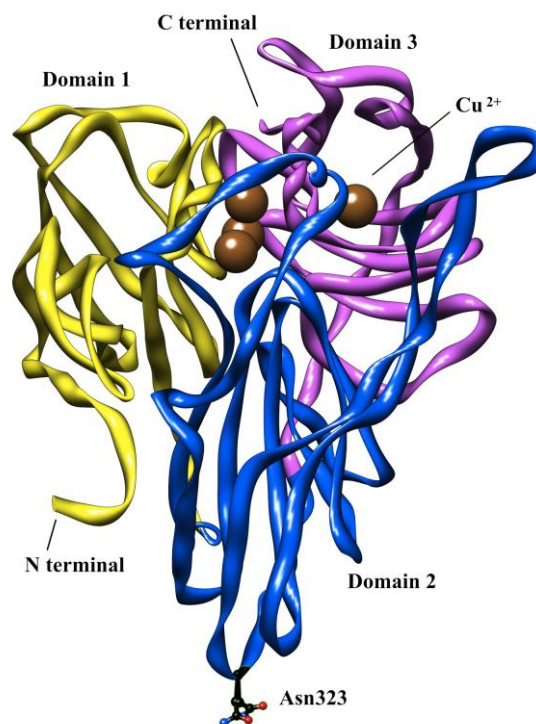


Fig. 1. Overall structure of *Tth*-laccase. The domains, terminals and  $\text{Cu}^{2+}$  cations are shown. N 323 is highlighted with stick mode. The structure was obtained from the RCSB protein data bank. The copper centers are shown with circles. The three related domains are colored separately.

The purification of the mutant was mainly performed following the protocol for wide-type *Tth*-laccase in reference (Liu et al., 2011). Cysteine 323 on the surface of the mutant *Tth*-laccase tends to form an intermolecular disulfide linkage, thus  $\beta$ -mercaptoethanol was added into buffers in the purification steps to reduce the *Tth*-laccase dimer. Approximately 2 mg of the 53 kDa blue-colored mature

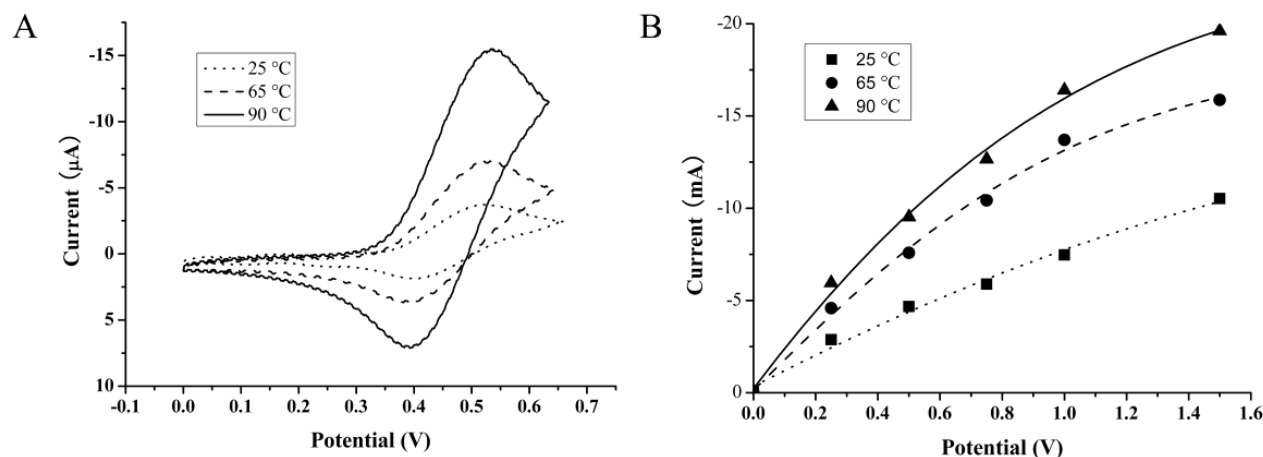


Fig. 2. Cyclic voltammograms for the electrochemical behavior of 1 mM ABTS with N453C mutant on a glassy carbon electrode (scan rate= 40 mV/s). A. Electrochemical properties of electrode-immobilized *Tth*-laccase mutant at three different temperatures (solid line 90°C, broken line 65°C, dotted line 25°C). B. The effect of ABTS concentration on current at three different temperatures (triangle square 90°C, circle 65°C, square 25°C).

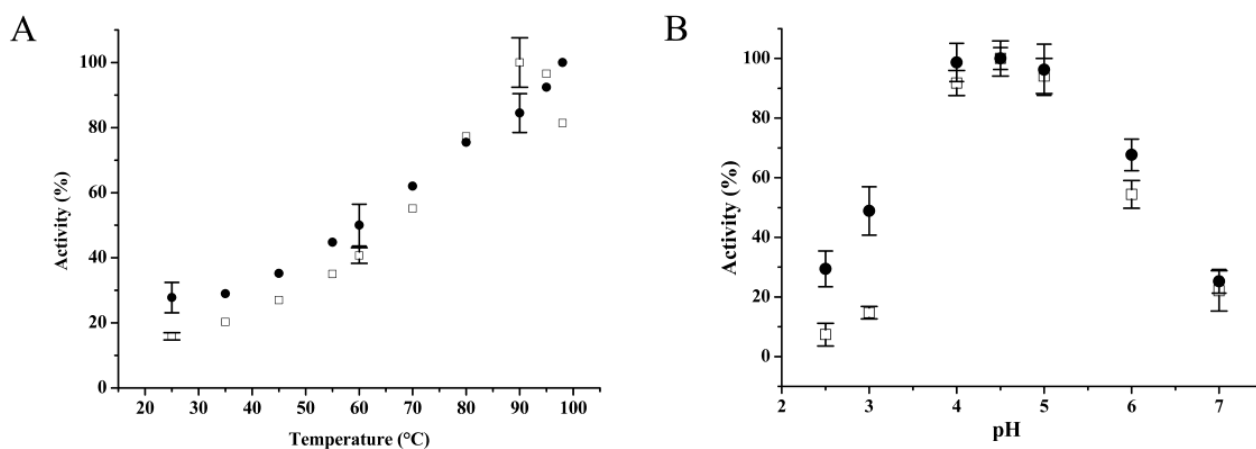


Fig. 3. Catalytic properties of free N453C and immobilized mutant. A. Temperature dependence of activity (open square free enzyme, closed circle immobilized enzyme). B. pH dependence of activity (open square free enzyme, closed circle immobilized enzyme).

monomer per liter of culture was yielded for further experiments.

#### *Catalytic properties of free N323C mutant*

In order to investigate the catalytic kinetic behavior of free *Tth*-laccase mutant N323C, the effect of the substrate concentration on the rate of ABTS oxidation was studied at low and high temperatures.

Lineweaver-Burk analysis was used to obtain the specific activity and  $K_M$  values, and the kinetic parameters of the mutant *Tth*-laccase were compared to that of the wild-type enzyme (Table 1). Previous studies reported that wild-type *Tth*-laccase has an increase of activity from 25°C to 92°C (Miyazaki, 2005). This behavior was similarly observed with the free mutant N323C (Table 1). At three low and high temperatures, the activity and  $K_M$  value of the free



N323C mutant were observed to be similar to that of wild-type *Tth*-laccase. This indicated that the N323C site mutation did not affect much of the activity in enzyme center, the affinity for the substrate, or enzyme thermophilic properties.

#### *Catalytic properties of immobilized N323C mutant*

Following the precedent set in previous works, the electrochemical properties of our immobilized laccase were investigated. Fig. 2a shows cyclic voltammograms of ABTS with laccase immobilized on a GCE. The current change is temperature-dependent. By comparing the anodic (ABTS oxidation) peaks at 25, 65, and 90°C, it is evident that at 90°C the enzyme is more active than at 65°C and 25°C by ~100% and ~300%, respectively. These data correlate well with those obtained by optical detection. The result indicates that the cross-linker was long enough to couple with the side chain of cysteine 323 to engineer a *Tth*-laccase active surface of GCE. Moreover, it is suggested that this laccase oriented-immobilized method is more fitting than the previous agarose cross-linking method for the design of a high temperature activated biofuel cell.

The  $K_{M\text{ ABTS, app}}$  of the immobilized laccase was also calculated by using cyclic voltammetry, given that the peak currents are proportional to the rate of the enzymatic reactions (Liu et al., 2011; Farneth et al., 2005) (Fig. 2b). The  $K_{M\text{ ABTS, app}}$  was found to be 1.9 mM by this method in an oxygen-saturated environment at room temperature, or 1.5 mM at 65°C, and 1.2 mM at 90°C. These apparent  $K_M$  values are larger than those of the enzyme in solution (Table 1), a common occurrence among immobilized enzymes. This is possibly because, when using an immobilized enzyme, due to the crowded amino acid branches, the concentration of substrate in solution is higher than the corresponding concentration in the microenvironment of the immobilized enzyme (Nednoor et al., 2004).

The temperature-dependent increase of activity was observed with the immobilized N323C mutant by cyclic voltammograms broadly from 25°C to

100°C (Fig. 3a). Compared with the behavior of the free mutant, which was investigated spectrophotometrically, the relative activity of the immobilized mutant was systematically higher than the free one at room temperature. Along with the temperature increase, the ascending activity of the immobilized mutant appeared to decrease gradually. While the free N323C showed its highest activity at about 90°C, the immobilized enzyme showed an increasing activity even when the temperature was up to 100°C.

pH-dependent properties were also studied in free as well as immobilized forms of the N323C mutant (Fig 3b). The activity of the immobilized mutated *Tth*-laccase decreased in slightly a different manner when varying the pH from 2.5 to 7.0 in 50 mM citric acid- $\text{Na}_2\text{HPO}_4$  buffer. At pH below 4.0, the activity loss of the free laccase was more rapid than that of the immobilized one, and the immobilized enzyme exhibited a slight shift of the maximum activity towards pH 4.0, whereas the free laccase had the highest activity at pH 4.5.

It is well known that an enzyme, when immobilized, sometimes turns out to be more resistant to heat and chemicals than the native enzyme (Martinek et al., 1977). The protein content on the electrode working surface could have effected protein folding when the temperature increased, which in some cases is an important determinant of the thermal properties of thermophilic enzymes (Sirim et al., 2011). The difference of pH dependence profiles between the free and the immobilized N323C mutant could be explained by the influence of the carrier microenvironment on the reaction conditions (King, 2005), for example, the redundant amine ( $-\text{NH}_2$ ) on the glassy carbon electrode surface after reacting with the cross-linker. Broad-ended pH profiles can also be seen if diffusion on the substrate to the enzyme is hindered upon immobilization (Hermanson, 2008).

## CONCLUSIONS

The N323C mutation of *Tth*-laccase and the enzyme immobilization did not exhibit large changes in catalytic properties. This immobilization provides

an opportunity for an improved application of thermal laccase as a catalyst for temperature-controlled biofuel cells. For better effects, different immobilized protein contents and longer spacers should be investigated further.

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