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Construction and direct electrochemistry of orientation controlled laccase electrode

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ABSTRACT

A laccase has multiple redox centres. Chemisorption of laccases on a gold electrode through a polypeptide tag introduced at the protein surface provides an isotropic orientation of laccases on the Au surface, which allows the orientation dependent study of the direct electrochemistry of laccase. In this paper, using genetic engineering technology, two forms of recombinant laccase which has Cys-6×His tag at the N or C terminus were generated. Via the Au-S linkage, the recombinant laccase was assembled orientationally on gold electrode. A direct electron transfer and a bioelectrocatalytic activity toward oxygen reduction were observed on the two orientation controlled laccase electrodes, but their electrochemical behaviors were found to be quite different. The orientation of laccase on the gold electrode affects both the electron transfer pathway and the electron transfer efficiency of O_2 reduction. The present study is helpful not only to the in-depth understanding of the direct electrochemistry of laccase, but also to the development of laccase-based biofuel cells.

1. Introduction

Laccase is a copper-containing oxidase [1,2]. Each laccase protein contains four copper ions, which are divided into T1 (one), T2 (one) and T3 (two) categories according to their spectral characteristics. The T1 site is for aromatic substrate oxidation and T2 and T3 copper ions form a trinuclear copper cluster active site for oxygen reduction. During the catalytic oxidation of substrates, one electron from the substrate is transferred to the T1 site, and subsequently it passes through the intramolecular His-Cys-His bridge to the T2/T3 cluster where molecular oxygen is reduced to water instead of the intermediate H_2O_2. Due to these unique properties, laccase has been tried to construct biofuel cells [3–5]. However, the direct electron transfer (DET) of laccase on conventional electrodes is found to be difficult. It depends upon the electrode materials and laccase immobilization strategies [3,6–10]. It has been found that the electrocatalytic reduction of oxygen at non-oriented laccase electrode is accompanied by the production of the intermediate H_2O_2, which decreases the efficiency of the O_2 reduction [11]. It follows that the oriented immobilization of laccase is a prerequisite for understanding the above phenomena and also it is the difficult point for studying the DET of laccase.

The site-directed biological modification of functional proteins is an ideal approach to achieving an oriented assembly of the proteins [12–17]. The basic strategy is to use genetic engineering technology to modify functional proteins with linkers which provide oriented attachment of the proteins on a specific support. The Au–S bond is usually used to fabricate an assembled film on Au electrode. Therefore, the introduction of cysteine at the N or C-terminus of laccase peptide chain facilitates the oriented immobilization of laccase. To obtain a recombinant laccase with Cysteine-6×Histidine (Cys-6×His) tag at the N or C terminus (named rLacA-N and rLacA-C, respectively), a gene sequence encoding the Cys-6×His tag was fused in the present study to the 3′/5′ end of the *lacA* gene from *Trametes* sp. AH 28-2. The obtained recombinant laccase was assembled orientationally on a gold electrode via the Au-S bond. The orientation dependent direct electron transfer of laccase and its electrocatalysis toward oxygen reduction were then investigated and compared. The present study is helpful to the in-depth understanding of the direct electrochemistry of laccase and to the development of laccase-based biofuel cells.

2. Materials and methods

The construction of the expression vector of rLacA with Cys-6×His tagged at the N or C terminus (named pPlacA-$N_{Cys\text{-}His}$ and

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pPlacA-$C_{Cys\text{-}His}$, respectively) and its heterologous expression in *Pichia pastoris* strain GS115 (Invitrogen, Carlsbad, CA) were performed according to the Xiao's method [18]. The PCR with designed primers that contained the sequence of the tag (the underlined) was carried on the plasmid pPlacA (kindly provided by Dr. Xiao et al. [19]) by standard procedures. For the construction of pPlacA-$N_{Cys\text{-}His}$, the upstream and downstream primers were 5′-CCGgaattcTGCCATCATCACCATCATCACGCCATTGGGCCCACCGCTGACCTCA-3′ and 5′-AATAgcggccgcCTGGTCGTTGACATCGAGCGCG-3′, respectively. For the construction of pPlacA-$C_{Cys\text{-}His}$, the two primers were 5′-CCGgaattcGCCATTGGGCCCACCGCTGACCTCA-3′ and 5′-AATAgcggccgcGCAGTGATGATGGTGATGATGCTGGTCGTTGACATCGAGCGCG-3′, respectively. Sequence analyses of the target expression vectors (pPlacA-$N_{Cys\text{-}His}$ and pPlacA-$C_{Cys\text{-}His}$) were performed.

The laccase producing transformants were cultured under the conditions described in the manual of the *Pichia* Expression Kit (Invitrogen). To harvest the expressed rLacA, the cells were first removed by centrifugation and the supernatant was then concentrated with a 10 kDa cut-off centrifugal filtering device (Amicon Ultra-15, Millipore Corp. MA). The resulting rLacA was purified with a His-Trap FF column (GE Healthcare) at 4 °C. The purification was monitored by SDS–PAGE. The activity of purified laccases (both free and immobilized) were determined spectrophotometrically [18] and one activity unit (U) was defined as the amount of the enzyme required to oxidize 1 μmol 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) per minute at pH 4.0 at 30 °C.

The rLacA modified electrode was prepared by immersing a pretreated gold disc electrode [20] (3 mm in diameter) into the enzyme solution at 4 °C for 24 h, followed by rinsing with phosphate buffer (100 mM, pH 6.0). The immobilized laccase was determined using quartz crystal microbalance (ANT Q300 affinity sensor, Taiwan). Cyclic voltammetry was performed using a CHI 630 Electrochemical Analyzer (Shanghai). A bare or laccase modified gold electrode was used as a working electrode, a platinum wire and a saturated calomel electrode (SCE) were used as counter electrode and reference electrode, respectively. Unless otherwise specified, the electrolyte phosphate buffer (100 mM, pH 4.6) was thoroughly deoxygenated with pure N_2 for ca. 30 min prior to experiments and the N_2 atmosphere was kept during the experiments. The CVs were recorded over a broad potential range and with different scan rates. The content of H_2O_2 formed during the electrocatalytic reduction of O_2 was measured according to the method described elsewhere [11].

3. Results and discussion

Sequence analysis indicated that the cDNA fragment of *lacA* with Cys-6×His tag in N or C-terminus was correctly inserted into pPIC9K, resulting in two 15.4 kb plasmids without gene mutation. The gene was expressed in *P. pastoris* with an expression level of 35.9 mg L^{-1} (3.04×10^4 U L^{-1}) for rLacA-N and 19.9 mg L^{-1} (0.83×10^4 U L^{-1}) for rLacA-C, indicating that the heterologous expression of the rLacA was successful.

SDS–PAGE experiments indicated that the separated rLacA-N and rLacA-C were both pure with molecular weight estimated to be both 64 kDa, which is very close to that of the native LacA (nLacA) from *Trametes* sp. AH28-2 (62 kDa) [19]. The apparent kinetic parameters of K_m and k_{cat}, determined with ABTS as substrate, are 16.7 μM and 68.6 s^{-1} for rLacA-N, and 17.2 μM and 65.3 s^{-1} for rLacA-C, respectively. These data suggest that there is little difference between rLacA-N and rLacA-C in the catalytic performance and the insertion of Cys-6×His tag has no notable effect on the conformation of laccase.

The structure of LacA from *Trametes* sp. AH 28-2 is not reported, but that of LacB from the same strain has been released (Protein Data Bank code 3KW7) [21]. The structure of rLacA was built via homology modelling using the SWISS-MODEL function in Swiss Pdb viewer version 3.7 [22]. Its structure and probable orientation on gold surface (the rLacA was immobilized via the Au–S bond) is schematically represented in Fig. 1.

In rLacA-N, the T1 site is away from the Au electrode surface, while the T2/T3 site is near the electrode surface; in rLacA-C, however, the distance between the T1 and the electrode surface is nearly the same as that between the T2/T3 and the electrode surface. It follows that the difference in site for tag modification results in different orientations of laccase on the gold surface.

The amount of immobilized laccase on the gold surface shows that the unmodified laccase is not readily adsorbed on the gold surface, while the modified laccase is easily immobilized on the gold surface due to the thiol from the Cys-6×His tag. The surface coverages of the two modified laccases are $(5.41 \pm 0.28) \times 10^{-12}$ mol cm^{-2} for rLacA-N and $(4.99 \pm 0.19) \times 10^{-12}$ mol cm^{-2} for rLacA-C, respectively. These data are close to the theoretical value of 4.65×10^{-12} mol cm^{-2} calculated based on the monolayer assembly of laccase [7]. The slight difference in the coverage may be due to differences in orientation, transverse sectional area and steric hindrance, etc.

The measurements of the activity of the immobilized laccase shows that after chemisorption of rLacA on the Au surface, the

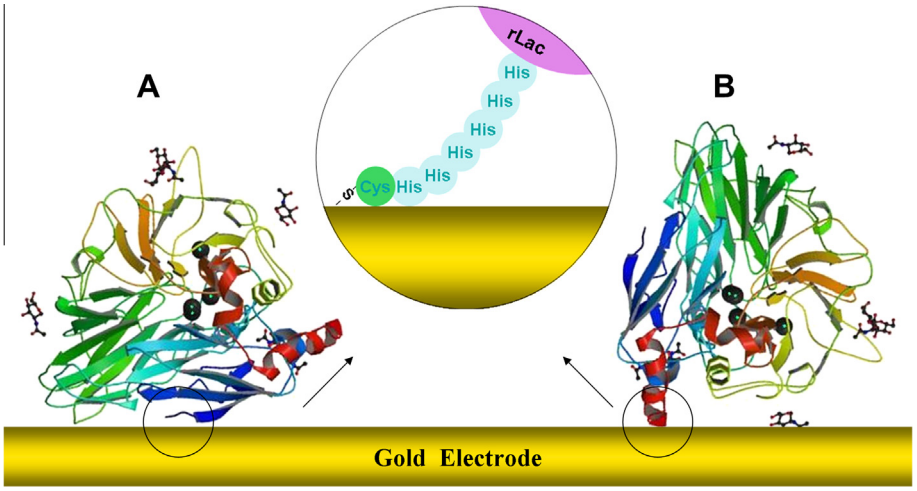

Fig. 1. Schematic representation of rLacA immobilized orientationally on bare gold surface with Cys-6×His tag at its N-terminus (A) or C-terminus (B).