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Specificity enhancement towards phenolic substrate by immobilization of laccase on surface plasmon resonance sensor chip

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ABSTRACT

In the modern biomedical and environmental technology, development of high performance sensing methods for phenolic compound is a critical issue because of its potential toxicity for human and environment. Laccases are polyphenol oxidases that exhibit broad substrate specificity; they act on both phenolic and non-phenolic compounds. Constructing a selective, sensitive and fast phenolic detecting system is a challenge for modern technologies. In the present study a laccase from *Bacillus* sp. HR03 was immobilized on the carboxymethyl dextran chip that altered its substrate specificity toward phenolic substrate. Since the opposite side of active site was enrich of Lys, oriented attachment via amine coupling was expected. Atomic force microscopy revealed a uniform distribution of the enzyme over the sensor surface. Surface plasmon resonance demonstrated no interactions toward non phenolic substrate (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) and HPLC analysis of the reaction products revealed no peak. Significant conformational changes of the free enzyme toward non-phenolic substrate were detected using fluorescence spectroscopy. Therefore, the inactivation of immobilized enzyme toward non phenolic substrate was due to rigidification. The results of the current study could lead to the utility of laccase in developing a sensitive and specific catalytic detection system of phenolic compound based on surface plasmon resonance.

1. Introduction

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) belong to the group of blue oxidases and represent the largest subgroup of multicopper oxidases. These enzymes are able to oxidize a broad range of phenolic and non-phenolic substrates, and they can be considered as generalists [1]. In general, laccases oxidize phenolic and non-phenolic compounds such as anilines, aryl diamines, hydroxyindols, benzenethiols, bilirubin, ascorbate and lignin related molecules. Among them, phenol compounds are byproducts of large-scale production such as drugs, dyes, antioxidants, paper pulp and pesticides that cause ecologically undesirable effects [2]. Nonetheless, most phenolic compounds are distinguished by their toxic, noxious, mutagenic and carcinogenic activity [3,4]. Therefore, detection and elimination of hazardous phenolic compounds using laccases has gained attention during recent decades. To date, various biosensors for both environmentally important pollutants and clinically relevant metabolites have been developed [5–7]. Although the determination of phenol and its derivative compounds is of environmental greatness [8,9], but in clinical detection system preparation, specific detection of a single substrate in complex mixtures is more critical and requires the use of enzymes with high substrate specificity, which means that laccase with broad range specificity [10], could not be a highly suitable candidate for detection. In fact, in all clinical laccase based phenolic detection system, such as detection of dopamine or catechol, it is necessary to study significant interference like ascorbic acid, as a non-phenolic laccase substrate in real mixture samples [11]. In order to reduce and completely omit the necessity of considering interference in the mixture samples, the enhancement of laccase specificity and activity towards the target metabolites could be the fundamental step in its detection system construction. Recently, to create practical catalysts, few researches reported the narrowing of laccase substrate specificity toward non-phenolic substrate, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), by using mutagenesis [1,10]. In the current study, we have achieved a novel specific laccase towards phenolic compounds by immobilization on the surface plasmon resonance (SPR). Different methodologies have been used for laccase immobilization such as, polypropylene membrane [12], sol–gel matrix of

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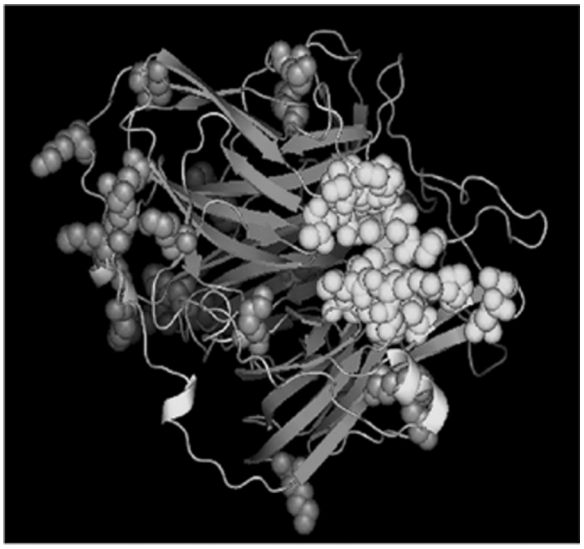

Fig. 1. Side view of the three-dimensional model of laccase from *Bacillus* HR03 was built using Swiss model utilizing CotA (1GSK) as the template. The model shows that lysine residues (gray sphere) are located far away from the active site (white sphere).

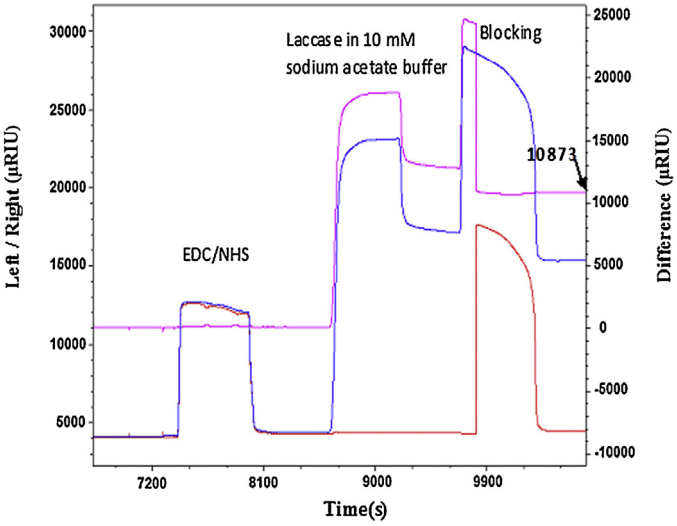

Fig. 2. Sensogram for covalent immobilization of laccase onto SPR-CMD surface. Carrier solution: 20 mM phosphate buffer (pH 7), flow speed 25 μl/min, flow duration: 10 min.

diglycerysilane and Ca and Cu–alginate beads [13,14], to construct laccase biosensors for phenolic compounds [15], but they did not report any innovation about laccase specificity and activity towards its substrate during immobilization. In this study, we investigated, for the first time the immobilization of laccase (CotA) from *Bacillus* sp. HR03 [16] on the SPR carboxymethyldextran (CMD) chip and employed surface plasmon resonance (SPR) system as a powerful and sensitive technique to monitor the activity and specificity of the immobilized enzyme. The activity of immobilized laccase was promoted towards its phenolic substrate, syringaldazine (SGZ). Further consideration using fluorescence and HPLC techniques have proved the specificity enhancement of laccase by immobilization. Our results represent a simple and new strategy to construct more effective and specific clinical catalytic detection system of phenolic compounds.

2. Materials and methods

2.1. Materials

All the reagents were prepared with chemicals of analytical grade. All chemicals were purchased from Sigma–Aldrich Chemical (USA). The carboxymethyldextran (CMD 200 M), sensor chip and the amine-coupling kit containing *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(3-diethylaminopropyl) carbodiimide (EDC), and ethanolamine hydrochloride were obtained from Xantec Bioanalytics (Germany). Laccase (CotA) from *Bacillus* sp. HR03 was expressed in *Escherichia coli* BL21 (DE3) cells in our laboratory [16].

2.2. Methods

2.2.1. Immobilization of laccase onto CMD chip surface

The enzyme was immobilized via its primary amine groups (lysine residues) through EDC/NHS esters [17,18]. The immobilization protocol was designed to obtain an increased binding capacity (R_{max}) of the sensor chip (i.e., with a large amount of covalently bound protein). For this purpose, the activation of the surface carboxyl groups was performed by injecting a 10 min pulse of EDC/NHS. For immobilization, 250 μl of laccase (0.2 mg/ml) in 10 mM acetate buffer (pH 4.5) was injected over the activated chip. The remaining active sites of the SPR sensor were blocked by 1 M ethanolamine (pH 8.5). All of the mentioned operations were followed by rinsing with a phosphate buffer solution. The SPR SR7500DC instrument detects changes in refractive index and measures these changes in microrefractive-index units (μRIU). A change in refractive index is proportional to the quantity (mass) of analyte interacting with the surface. Over amine coupling process, the signal up to 10,873 microrefractive-index units (μRIU) was achieved by laccase immobilization on the surface.

2.2.2. SPR measurements

SPR measurements were performed on an SPR SR7500DC instrument (XanTec bioanalytics GmbH, Germany) equipped with an automatic flow injection system. The interaction between substrates and immobilized laccase was studied by flowing substrate over the sensor surface. After optimizing the pH condition for good binding interaction, 20 μM of ABTS in 100 mM phosphate buffer (pH 4) was injected over the laccase immobilized CMD surface for 10 min with a flow rate of 25 μl/min at 25 °C [16,19]. Subsequently, the flow-through was collected for the HPLC analysis. 10 μM of SGZ (100 mM phosphate buffer, pH 7) was also injected for 5 min with a flow rate of 50 μl/min at 25 °C. The collected sample was analyzed by HPLC. A blank control run was performed by injecting the above mentioned buffers. Reference sensorgrams were subtracted from binding sensorgrams using the Scrubber analysis program (Biologic Software Pty. Ltd., Canberra, Australia).

2.2.3. Laccase activity assay

Laccase activity was measured as previously described [16]. Briefly, the oxidation of ABTS (2 mM) in 100 mM phosphate buffer (pH 4) was measured by the increase in absorbance at 420 nm (ϵ = 36,000 M^{-1} cm^{-1}) [20]. Oxidation of SGZ in 100 mM phosphate buffer (pH 7) was monitored at 525 nm (ϵ = 65,000 M^{-1} cm^{-1}) [21]. One activity unit (*U*) was defined as the amount of enzyme that oxidized 1 μmol of substrate per minute at 25 °C.

2.2.4. High performance liquid chromatography (HPLC) assay

Chromatographic analysis was performed on a reverse-phase high-performance liquid chromatography (HPLC) system (Knauer, Berlin, Germany) using an analytical Inertsil ODS-3 column (In Ertsil, Eindhoven, Netherlands). Samples were filtered through a 0.2-μm syringe filter prior to HPLC analysis. A mobile phase composed of 50% methanol and 50% water was used at a low rate of 0.5 ml min^{-1}. The injection of the samples was performed on an