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Interaction of small molecules with fungal laccase: A Surface Plasmon Resonance based study

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ABSTRACT

Laccases have a great potential for use in industrial and biotechnological applications. It has affinity towards phenolics and finds major applications in the field of bioremediation. Here, Surface Plasmon Resonance (SPR) as a biosensor with immobilized laccase on chip surface has been studied. Laccase was immobilized by thiol coupling method and compounds containing increasing number of hydroxyl groups were analyzed for their binding affinity at various concentrations in millimolar range. The small molecules like phloroglucinol (1.532×10^{-8} M), crocin (3.204×10^{-3} M), ascorbic acid (8.331×10^{-8} M), kojic acid (6.411×10^{-7} M) and saffron (3.466×10^{-7} M) were studied and respective K_D values are obtained. The results were also confirmed by inhibition assay and IC_{50} values were calculated. All these molecules showed different affinity towards laccase in terms of K_D values. This method may be useful for preliminary screening and characterization of small molecules as laccase substrates, inhibitors or modulators of activity. This method will be useful for rapid screening of phenolics in waste water because of high sensitivity.

1. Introduction

Laccase is belonging to the polyphenol oxidase family found in plant, fungi and bacteria with copper atoms in the catalytic center. Phenolic substrates such as ortho-and para-diphenol, aromatic compounds with hydroxyl and amine groups are [1] oxidized by laccase. It catalyzes the oxidation of organic substrates of broad range such as phenols concomitantly reducing molecular oxygen to water. Because of various functions and broad substrate specificity, laccase can be utilized in industrial processes like, textile dye bleaching, paper and pulp industry, phenolics removal, effluent detoxification, and many other processes [2,3]. In addition, it is used in cosmetics as deodorants, other products such as toothpaste, mouthwash, detergent, soap, and diapers [4–11] also, in food industry for wine stabilization [12], stabilization of fruit juices [13–15], in dough and/or baked products to increase strength of gluten structures [13,16,17], in pharmaceutical industry in anesthetics, anti-inflammatory drugs, also in antibiotics and sedatives [18–21],in nanobiotechnology, as nanoparticles based biosensors and in biosensors for determination of glucose, aromatic amines and phenolic compounds [22–28].

Many reports are available on immobilization of laccase as it can be easily reused and have longer stability compared to free enzyme. Moreover, immobilization of the biocatalyst can reduce the enzyme loss and facilitate their use in continuous processes, such as bioreactors [29–32]. Successful immobilization of laccases were carried out on support materials such as Eupergit® C [33], mesoporous silica spheres which are magnetically separable [34], gold nanoporous particles [35], mesostructured cellular foams [36], fumed silica nanoparticles [37], platinum surface [38–40] etc. Laccase, due to its broad substrate range has wide applications in biosensor technology for the detection of phenolics [41]. Immobilized laccase enzyme was used as biosensor for determination of phenolic acids in human plasma [42], phenols such as catechols detection in tea [43], phenolic compounds in wine, lignin and phenolic residues in wastewaters [44], detection, oxidation and removal of phenolic compounds from wastewater [45,46,33], determination of catecholamine from plasma and urine samples [47]. Reported conventional methods are available for successful immobilization of laccase using electrode surfaces. To avoid these labor-intensive methods Surface Plasmon Resonance with immobilized laccase is studied.

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SPR with different coupling chemistries for immobilization of enzyme facilitates interaction of small molecules and their binding affinity. SPR is optical technique analyzes label-free, real-time detection of binding of biomolecules such as coupling of ligand and receptor, interactions between antigen-antibody and DNA-protein [48]. As a biosensor, SPR has been used in various studies, for detection of bacteria causing water born diseases [49], interaction of nitric oxide synthase I and calmodulin to the CaM-binding site [50] etc. In present study, affinity of ascorbic acid, phloroglucinol, kojic acid, saffron and crocin towards fungal laccase was detected by SPR using surface-immobilized laccase. Change in refractive index indicates binding of analyte to the immobilized ligand at different concentrations [48]. The corresponding binding constants were generated after affinity screening analysis. These results were also confirmed by inhibition assay. This is the first report evaluating the binding and affinity studies of immobilized laccase using SPR towards various compounds. This approach might be helpful for the detection and affinity of various bioactive phenolics from waste generated from beer, wine and fruit juice industrial waste.

2. Material and methods

2.1. Chemicals

Sensor Chips CM5, *N*-ethyl-*N*-(dimethylaminopropyl)-carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), ethanolamine HCl, vials, and caps were obtained from GE Healthcare Life Sciences, Uppsala, Sweden. Saffron, crocin, *Trametes versicolor* laccase and ABTS, were purchased from Sigma (St. Louis, MO, USA) and ascorbic acid, phloroglucinol and kojic acid were obtained from Himedia, Mumbai, India. All other chemicals used in study were of highest purity and analytical grade. Milli Q (Milipore) water was used for preparing buffers and reagents.

2.2. Enzyme activity

2.2.1. Laccase activity assay

Laccase activity of *T. versicolor* was determined by oxidation of 2,2′-azino-bis(3 ethylbenzthiazoline-6-sulfonic acid) (ABTS) as a substrate. The reaction mixture containing 10% ABTS and 100 mM sodium acetate buffer (pH 4.8) [51]. The reaction was started with the addition of enzyme solution. The oxidation of substrate was monitored at 420 nm (ε420 = 36,000 M^{-1} cm^{-1}). One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 μM of ABTS per min. The reaction was carried out at 30 °C using Shimadzu UV visible spectrophotometer.

2.2.2. Inhibition of laccase activity

Inhibitory activity assay was carried out as same as for standard laccase assay. Ascorbic acid, kojic acid, phloroglucinol, saffron and crocin were first dissolved in deionised water and incubated with 0.2 ml of fungal laccase (1 mg/ml) in 100 mM sodium acetate buffer, pH 4.8. The reaction was incubated for 90 s. Then, absorbance of each mixture was determined by the optical density at respective wavelength using Shimadzu UV visible spectrophotometer. ABTS is a positive control. The inhibitory percentage of laccase was calculated as follows:

$$\%\text{inhibition} = \frac{\text{Abs. of test compounds [Final} - \text{Initial]}}{\text{Abs. of ABTS[Final} - \text{Initial]}} \times 100$$

The extent of inhibition by the addition of test compound was expressed as percentage necessary for 50% inhibition (IC_{50}).

Table 1
Binding affinity (K_D) and IC_{50} values of compounds from SPR and spectrophotometric method.

Compound name	K_D values	IC_{50} values
Ascorbic acid	8.331×10^{-8} M	34.66 ± 4.16 μM
Kojic acid	6.411×10^{-7} M	10.33 ± 3.5 μM
Phloroglucinol	1.532×10^{-8} M	0.266 ± 0.06 μM
Saffron	3.466×10^{-7} M	0.19 ± 0.05 μM
Crocin	3.204×10^{-3} M	0.17 ± 0.05 μM

Data is presented as mean of three replicates with standard deviation (±).

2.3. Surface Plasmon Resonance (SPR) studies

SPR interaction studies were performed using a Biacore T200 optical biosensor (GE Healthcare Life Sciences, Bangalore, India). The phosphate buffer saline (PBS) was used to carry out surface sensitive SPR measurements. Working solutions were diluted from analytes stock solutions, in PBS buffer before use on the sensor surface. Data were collected with the Biacore control software. Changes in the refractive index as a function of time were monitored under constant flow conditions [52]. The relative amount of analytes bound to the laccase was determined by measuring the net increase of refractive index over time compared with that of running buffer alone. There is an inline subtraction of reference surface during the run which is reported in response units (RU). The surface, between each increasing concentration of analytes was washed with PBS (running buffer).

Fungal laccase dissolved (1 mg/ml) in 10 mM sodium acetate buffer pH 5.0 was immobilized on CM5 chip by thiol coupling. The surface of flow cell was activated for 7 min using a 1:1 mixture of 100 mM *N*-ethyl-*N*-(dimethylaminopropyl)-carbodiimide (EDC) and 100 mM *N*-hydroxysuccinimide (NHS) (both dissolved in water) with a flow rate of 10 μl/min and subsequently laccase was injected for 7 min, and the surface holding residual activated carboxy methyl groups were blocked by a 7 min injection of 1 M ethanolamine, pH 8.5. A total of 326 (RU) of laccase were immobilized. For this study, flow cell was blank immobilized (without protein) for using as a reference.

To analyze interactions of saffron, ascorbic acid, phloroglucinol, kojic acid and crocin with immobilized laccase, compounds were dissolved in 10 mM PBS pH 7.4 except crocin pH 8.5 containing 0.005% P20 and were injected. Experiments were performed in single cycle kinetics mode with higher concentrations in millimolar range of ascorbic acid (5.7, 2.85, 1.42, 0.712, 0.356), phloroglucinol (8, 4, 2, 1, 0.5), kojic acid (3.5, 1.75, 0.87, 0.437, 0.218), saffron (60, 30, 15, 7.5, 3.75), and crocin (1, 0.5, 0, 25, 0.125, 0.0625). The same buffer was used as the running buffer (Tables 1 and 2). Flow rate was maintained constant throughout the kinetics experiment (10 μl/min), contact time and dissociation time was kept at 180 s

Table 2
Interaction kinetics of *T. versicolor* laccase of analytes with two state fit model.

Analytes	k_a1 (1/Ms)	K_d1 (1/s)	K_a2 (1/s)	k_d2 (1/s)	K_D (M)
Phloroglucinol	7885 ± 8.6E+2	0.07135 ± 0.0053	0.001841 ± 9.0E − 5	3.122×10^{-6} ± 1.8E − 5	1.532×10^{-8} M
Kojic acid	3.145×10^{4} ± 4.0E+4	0.02356 ± 0.018	7.451×10^{-4} ± 0.0016	0.004417 ± 0.0039	6.411×10^{-7} M
Saffron	4.532×10^{4} ± 6.5E+4	0.09320 ± 0.012	8.655×10^{-5} ± 2.2E − 5	1.755×10^{-5} ± 1.8E − 4	3.466×10^{-7} M
Ascorbic acid	1.881×10^{5} ± 1.3E+5	0.01813 ± 0.0068	5.697×10^{-4} ± 4.9E − 4	0.003641 ± 0.0017	8.331×10^{-8} M
Crocin	154.2 ± 15	0.6043 ± 0.025	2.052×10^{-4} ± 1.9E − 5	9.191×10^{-4} ± 1.5E − 4	3.204×10^{-3} M