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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.

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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

and 200 s respectively. Regeneration was carried out for 30 s with 10 mM glycine pH 2.0 at 30 μl/min [53]. The data analysis was done with T200 evaluation software ver. 2.0 and was fit to two state binding [54].

3. Results and discussion

Laccase has a great potential for industrial and biotechnological applications. It has ability to metabolize phenolic compounds in the field of bioremediation [55,56]. A number of immobilized laccase biosensors have been developed and studied for the determinations of phenolics [24,25,33,41–47,57–63]. All reported biosensors consist of an electrode for immobilization of laccase enzyme which showed stability for one to two months only. Therefore in this study we have used SPR as a biosensor for immobilization of laccase on chip surface which can be used for hundreds of times without losing enzyme biological activity under proper maintenance. Here the chip surface was used for detection of binding affinity with different small molecules by thiol coupling method. SPR is a real time, label free, quick method compared with others reported in the literature because SPR has the ability to detect several compounds within a short time [33,45] and with great sensitivity and reproducibility. Laccase with broad substrate specificity towards various phenolics was studied using electrode surfaces as laccase biosensor for detection, monitoring and oxidation of phenolic compounds [39,25,46]. The affinity of phenolics towards immobilized laccase and their detection will develop new approaches for characterization of bioactive phenolics binding to laccase. In this study, compounds were chosen with increasing number of hydroxyl groups in their structure as laccase has affinity towards mono, di, and tri hydroxyl phenols. Also these compounds are important in pharmaceutical and food industry for their use as antioxidants, coloring and flavoring agents, indicators of food quality, as anticancer agent etc.

In the present study, the steady state affinity constants (K_D) followed by binding affinity (Fig. 1) of compounds were evaluated with SPR. For very small compounds it is very difficult to estimate kinetics as on/off rates are very fast. The studied compounds showed affinity towards immobilized laccase (*T. versicolor*). Laccase showed binding constants (K_D) for ascorbic acid (8.331×10^{-8} M), kojic acid (6.411×10^{-7} M), phloroglucinol (1.532×10^{-8} M), crocin (3.204×10^{-3} M) and saffron (3.466×10^{-7} M). All these molecules showed higher affinity towards laccase except crocin. This indicates that laccase oxidizes above compounds with characteristics similar to *p*-diphenol. Laccase activity reduces by oxidizing these compounds which was confirmed by calculating percentage inhibition and their IC_{50} values. The IC_{50} value of ascorbic acid and kojic acid was observed 34.66 μM and10.33 μM respectively (Fig. 2). It has been reported in literature that fungal laccase oxidize ascorbic acid [64]. *T. versicolor* laccase [42] was used to determine the phenolic acid including ascorbic acid present in blood plasma. In food industry, laccase was used in monitoring ascorbic acid [13] as it is a relevant indicator of food quality. Acting as an antioxidant due to action of polyphenols, ascorbic acid can improve the color and palatability of food products. While IC_{50} value for kojic acid reported by Refs. [65] and [66] was 0.13 μM and 175 μM respectively. The IC_{50} value of phloroglucinol was 0.26 μM (Fig. 3). *T. versicolor* laccase with slow oxidation rate showed stability in the presence of phloroglucinol [67]. Phloroglucinol with more number of hydroxyl groups on the aromatic ring can aid in decolorization of indigo carmine as efficient laccase mediators due to which its phenoxy radicals make them more stable [68]. IC_{50} value for crocin was 0.17 μM and saffron 0.19 μM (Fig. 3). Ascorbic acid, kojic acid and phloroglucinol are well studied compounds with laccase enzyme. Crocin is a well known inhibitor of monophenolase activity of polyphenol oxidase (PPO) and also found to be inhibiting diphenolase laccase enzyme. As the compounds described above have applications in the food and pharmaceutical industry, it might be advantageous to investigate them using SPR. The biosensor was thus successful in revealing binding of the compounds with immobilized laccase in a very easy manner without using any labeling step. The present study demonstrates for the first time the detection of phenolics and their oxidation by immobilized laccase showing their affinity constants with SPR.

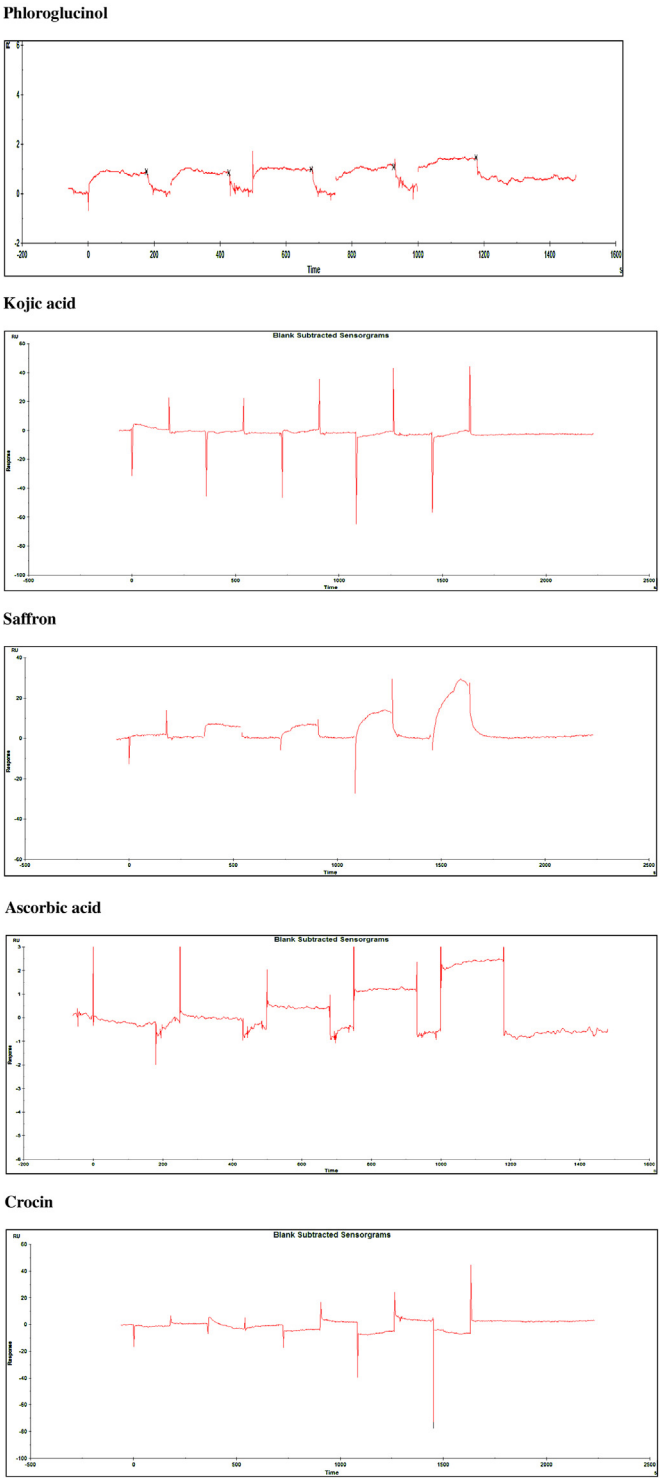

Fig. 1. Binding sensorgrams of phloroglucinol, kojic acid, saffron, ascorbic acid and crocin with immobilized laccase.

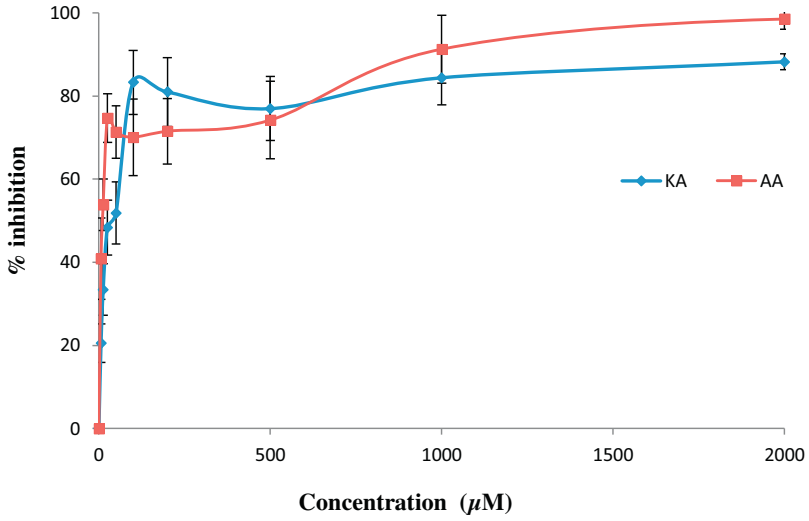

Fig. 2. Effect of increasing concentrations (μM) of compounds on laccase enzyme and their % inhibition.

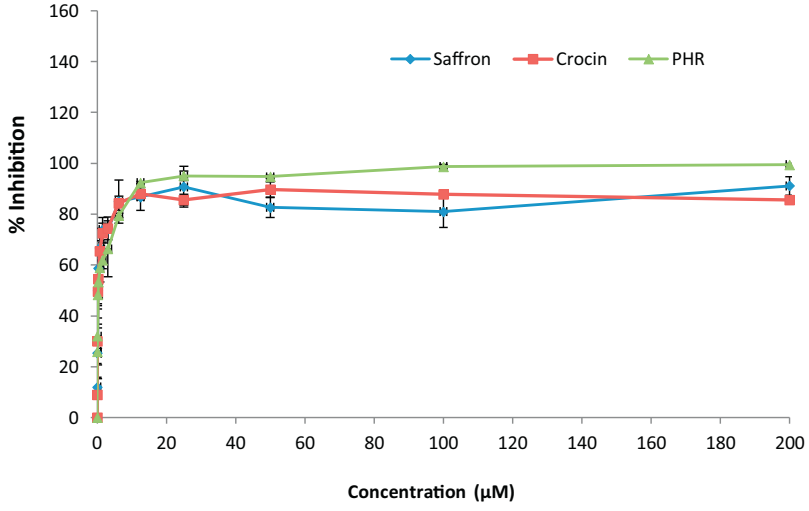

Fig. 3. Effect of increasing concentrations (μM) of compounds on laccase enzyme and its % inhibition.

4. Conclusions

Here we can conclude that, SPR is a label free, real time method used for detection of phenolics. SPR based biosensor with immobilized laccase showed binding affinity constants which were confirmed by enzyme inhibition assay of laccase. The proposed method is very simple and has potential applications for the development of SPR biosensors related characterization of inhibitors and modulators of laccase activity. Detection of various phenolics present in fruit juices, wine, analysis of lignin in wastewater and to check the levels of tannins at different stages of tea production, determination of neurological transmitters in plasma and urine samples will be taken up in our later studies.

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