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Evaluation of crocin and curcumin affinity on mushroom tyrosinase using surface plasmon resonance

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ABSTRACT

Tyrosinase inhibitors have potential applications in the cosmetics and food industries for preventing browning reactions and also as therapeutic drugs for neurodegenerative diseases such as Parkinson's. In this article, crocin and curcumin were evaluated as mushroom tyrosinase inhibitors. Results showed that, both compounds strongly inhibited the diphenolase activity than monophenolase. The IC_{50} values for diphenolase activity were estimated to be 0.11 mM and 0.18 mM for crocin and curcumin respectively. The binding kinetics of crocin and curcumin was studied with mushroom tyrosinase using surface plasmon resonance (SPR). Tyrosinase was immobilized on the gold surface of a Biacore sensor chip through amine coupling. Binding of inhibitors was analyzed by SPR without the need to further modify the surface or the use of other reagents. The binding constant K_D (M) for mushroom tyrosinase obtained was 1.21×10^{-4} M for crocin and 1.64×10^{-4} M for curcumin, while showing a higher affinity for L-DOPA 1.95×10^{-8} M, a substrate for tyrosinase (positive control). The study reveals the SPR sensor's ability to detect binding of the inhibitors.

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1. Introduction

Tyrosinase (E.C. 1.14.18.1) is a ubiquitous enzyme involved in pigmentation and is widely distributed in plant, fungi and animals. It plays a major role in hydroxylation of monophenols and conversion of o-diphenols to the corresponding o-quiniones [1]. Therefore, tyrosinases have a great potential for development in the fields of food, medicine, agricultural industries as well as analytical and environmental purposes [2].

Products from tyrosinase activity can cause deleterious effects, those associated with browning reactions in fruits, vegetables and black spotting of shrimp and lobsters. However, abnormal accumulation of melanin product is responsible for hyperpigmentation which includes melasma, senile lentigines; it would lead to a serious aesthetic problem, also dermatological disorders such as age spots and freckles [3]. Thus, it is a major problem in the food industry and one of the main causes of quality loss during post harvest handling, processing and also responsible for many skin disorders [4].

As a result, tyrosinase inhibitors have become increasingly important in food industry as well as the medical and cosmetic products due to their ability to decrease the hyperpigmentation resulting from enzyme action [4–7]. Until now, large numbers of natural and synthetic tyrosinase inhibitors were screened for effective tyrosinase inhibition [8–10]. But, some of their individual activities are not potent enough to be considered for practical use, following safety regulations for food and cosmetic products [3]. Recently, number of potent tyrosinase inhibitors from various sources such as, from the roots of *Artocarpus heterophyllus* (Artoindonesianin F), components from a desugared sugar cane extract, synthetic inhibitors like azo-resveratrol and azo-oxyresveratrol, resveratrol analogues, arctigenin from *Fructus arctii* and azastilbene analogues, from pomegranate fruit peel, benzaldehyde thiosemicarbazones, curcumin-like diarylpentanoid analogues, hesperetin inhibition kinetics by integrated computational simulation study have been reported [11–20].

Surface plasmon resonance (SPR) with coupling chemistries for enzyme immobilization on the sensor surface facilitates detection and screening of inhibitors [21]. SPR is an optical technique that reports changes in refractive index based on alterations occurring in the dielectric medium within 500 nm from a metal gold surface [22]. SPR makes possible real-time, label-free detection of biomolecular binding events such as ligand–receptor coupling, antibody–antigen interactions, and protein–DNA interactions. Detection of tyrosinase inhibitors using SPR has been reported recently [23,24].

In this study, affinity of crocin and curcumin towards mushroom tyrosinase was detected directly by SPR using surface-immobilized tyrosinase. Change in refractive index indicates binding of

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tyrosinase inhibitors (crocin and curcumin) at different concentrations were monitored by SPR. The sensor was able to detect both inhibitors and corresponding binding affinity constants were generated after the analysis. This approach could be used to evaluate the binding of other tyrosinase inhibitors. It can be used for other proteins which undergo conformational changes on binding of small molecules.

2. Materials and methods

2.1. Chemicals and reagents

Sensor Chips Series S CM5, N-ethyl-N′-(dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), ethanolamine HCl, as well as sampling vials, and caps were obtained from GE Healthcare Life Sciences, Uppsala, Sweden. Mushroom tyrosinase, crocin and curcumin were purchased from Sigma (St. Louis, MO, USA), whereas L-3, 4 dihydroxyphenylalanine (L-DOPA), L-tyrosine from Himedia, India. All other chemicals were of highest purity and analytical grade. Milli Q (Milipore) water was used for preparing buffers and reagents.

2.2. Enzyme activity assay

2.2.1. Diphenolase activity assay

Diphenolase activity of mushroom tyrosinase was performed using L-DOPA as substrate by measuring the dopachrome accumulation at 475 nm ($\varepsilon_{\text{dopachrome}} = 3400\,M^{-1}\,cm^{-1}$) [4]. The reaction media (3 ml) contained 0.5 mM L-DOPA in 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8); a portion of 100 μl of enzyme (20 μg) was used for the activity assay. The reaction was carried out at a constant temperature of 30 °C using Shimadzu UV visible spectrophotometer.

2.2.2. Inhibition of tyrosinase activity

Inhibitory activity assay was measured according to the method of [25], with minor modifications. The crocin and curcumin were first dissolved in 10% DMSO and incubated with 20 μl of mushroom tyrosinase (3130 U/mg) in 50 mM sodium phosphate buffer, pH 6.8. The reaction mixture was incubated for 10 min. Then, 40 μl of 0.5 mM L-DOPA in 50 mM sodium phosphate buffer pH 6.8 was added. The final concentration of DMSO in the reaction mixture was 3.3%. The amount of dopachrome in the mixture was determined by the optical density at 475 nm using Shimadzu UV visible spectrophotometer. Kojic acid is used as positive control agent. The inhibitory percentage of tyrosinase was calculated as follows:

$$\%\,\text{inhibition} = \frac{100[A-B]-[C-D]}{[A-B]}$$

A is the OD at 475 nm without test substance; B is the OD at 475 nm without test substance and tyrosinase; C is the OD at 475 nm with test substance; D is OD at 475 nm with test substance without tyrosinase.

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

2.3. Surface plasmon resonance (SPR) studies

SPR interaction analyses were performed using a Biacore T 200 optical biosensor (GE Healthcare Life Sciences, Bangalore, India). SPR measurements were carried out in phosphate buffer saline (PBS), from analyte stock solutions, working solutions were diluted in PBS prior to flowing them on the sensor surface. Data were collected with the Biacore control software. Experiments were performed by monitoring the refractive index changes as a function of time under constant flow conditions. The relative amount of inhibitor bound to the tyrosinase 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. This change is usually reported in response units (RU). The surface was washed with PBS (running buffer) between each concentration.

Mushroom tyrosinase dissolved (50 μg/ml) in 0.1 M sodium acetate buffer pH 4.5 was immobilized to a CM5 chip using amine coupling. Using a flow rate of 10 μl/min, 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), and subsequently tyrosinase was injected for 7 min, and residual activated carboxy methyl groups on the surface were blocked by a 7 min injection of 1 M ethanolamine, pH 8.5. A total of 3594 (RU) of tyrosinase were immobilized. For this study, flow cell 3 was blank immobilized (without protein) for using as a reference.

To analyze interactions of L-DOPA, curcumin, and crocin with immobilized tyrosinase, compounds were dissolved in 10 mM PBS pH 7.4 containing 0.005% P20 and were injected. The same buffer was used as the running buffer. Flow rate was maintained constant throughout the kinetics experiment (45 μL/min), contact time and dissociation time was kept at 120 s. Regeneration was carried out with 10 mM glycine pH 2.5 for 30 s. Experiments were performed with various concentrations of L-DOPA or crocin or curcumin from 12.5 μM to 200 μM respectively. The data analysis was done with T200 evaluation software ver 2.0 and data was fit to 1:1 binding or two state binding.

3. Results and discussion

Melanin formation is the main cause of enzymatic browning in plants, fruits and human skin. It is known that biosynthesis of melanin leads to undesirable changes in colour; flavour and nutritive values of plant derived foods and beverages. Therefore, tyrosinase inhibitors have become increasingly important in cosmetic, food and pharmaceutical products in relation to hyperpigmentation [26]. Edible mushroom is considered as a clean, enriched, and cheap source of tyrosinase.

In the present study, the inhibition studies and binding kinetics of crocin and curcumin compounds on mushroom tyrosinase activity has been evaluated. Generally, crocin compounds are natural carotenoids found in flowers (*Crocus sativus*). The methanol extract of *Crocus sativus* showed anti-tyrosinase activity and antioxidant activity [27,28]. But the binding kinetics of particular compound in *Crocus sativus* (crocin) was not yet studied. Also curcumin is the principal curcuminoid of the popular Indian spice turmeric. The antioxidant and anti-tyrosinase activity of curcumin analogues were studied using spectral methods [29].

Inhibition studies showed that crocin and curcumin are the very effective inhibitors of diphenolase activity of mushroom tyrosinase. The low IC_{50} value of crocin (0.11 mM) and curcumin (0.18 mM) (Fig. 1) reveals that the compound is more potent than kojic acid (IC_{50} 59.72 mM) [30]. Kojic acid is a competitive inhibitor of the monophenolase activity and a mixed inhibitor for diphenolase activity. It is also reported to chelate copper in the active site [10]. In turn, crocin-1, the constituents of the *Crocus sativus* exhibited more potent inhibitory activity with IC_{50} 140 μM [27]. Also, tyrosinase inhibitory activity was reported by synthesized curcumin analogues [31]. Most of the inhibitors were reported to inhibit the diphenolase activity of mushroom tyrosinase compared to monophenolase. Similarly, in this study both inhibitors showed considerable inhibition of monophenolase activity.

The interaction kinetics between mushroom tyrosinase and various compounds L-DOPA, curcumin and crocin was studied by

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