International Journal of Biological Macromolecules 50 (2012) 257–262

Contents lists available at SciVerse ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Effect of hesperetin on tyrosinase: Inhibition kinetics integrated computational simulation study

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ARTICLE INFO

Article history:
Received 13 October 2011
Received in revised form 29 October 2011
Accepted 2 November 2011
Available online 9 November 2011

Keywords:
Tyrosinase
Inhibition kinetics
Hesperetin
Flavonoid
Docking simulation

ABSTRACT

Tyrosinase inhibitors have potential applications in medicine, cosmetics and agriculture to prevent hyperpigmentation or browning effects. Some of the flavonoids mostly found in herbal plants and fruits are revealed as tyrosinase inhibitors. We studied the inhibitory effects of one such flavonoid, hesperetin, on mushroom tyrosinase using inhibition kinetics and computational simulation. Hesperetin reversibly inhibited tyrosinase in a competitive manner with $K_i = 4.03 \pm 0.26$ mM. Measurements of ANS-binding fluorescence showed that hesperetin induced the hydrophobic disruption of tyrosinase. For further insight, we used the docking algorithms to simulate binding between tyrosinase and hesperetin. Simulation was successful (binding energies for Dock6.3: −34.41 kcal/mol and for AutoDock4.2: −5.67 kcal/mol) and showed that a copper ion coordinating with 3 histidine residues (HIS61, HIS85, and HIS259) within the active site pocket was chelated via hesperetin binding. Our study provides insight into the inhibition of tyrosinase in response to flavonoids. A combination of inhibition kinetics and computational prediction may facilitate the identification of potential natural tyrosinase inhibitors such as flavonoids and the prediction of their inhibitory mechanisms.

1. Introduction

Tyrosinase (EC 1.14.18.1) is a ubiquitous enzyme with diverse physiological roles related to melanin production. Tyrosinase catalyzes the pigmentation of skin [1,2], the browning of vegetables [3,4], wound healing [5,6], and cuticle formation in insects [7]. Therefore, tyrosinase inhibitors have potential applications in medicine, cosmetics (e.g., as whitening agents), and agriculture (as bio-insecticides) to prevent hyperpigmentation by inhibiting enzymatic oxidation. Structurally, tyrosinase belongs to the type 3 copper protein family [8,9], with two copper ions each coordinately bound to a distinct set of three histidine residues within the active site. These copper ions participate directly in the hydroxylation of monophenols to *o*-diphenols (cresolase activity) and in the oxidation of *o*-diphenols to *o*-quinones (catechol oxidase activity) [10]. The tyrosinase mechanism is complex in that this enzyme can catalyze multiple reactions.

A number of tyrosinase inhibitors have been discovered and the majority of them consist of a phenol structure or of metal chelating agents [11–16]. However, only a few of these are used today because of associated side effects and low activity, thus it is necessary to identify new candidates that effectively inhibit tyrosinase without concurrent side effects. In this regard, metabolites biosynthesized by plants become potential alternatives to the synthetic analogues [17,18]. Hesperetin (3′,5,7-trihydroxy-4′-methoxyflavanone) belongs to the class of flavanones that is used in traditional medicine [19–21]. Hesperetin occurs ubiquitously in citrus fruits as hesperidin (its glycoside form), which acts as a prodrug [22]. Hesperetin has biological and pharmacological activities including antioxidant, anti-inflammatory, anticarcinogenic, antihypertensive and anti-atherogenic properties [23–25]. Hesperetin is ingested from the hesperidin by breaking glycosidic bond with releasing glycone.

Previous studies have shown that phenolic compounds are efficient antioxidants that act as free radical terminators of metal chelators [26–28]. Among them, flavonoids, which are derivatives of benzopyrone, are particularly interesting as natural

Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; ANS, 1-anilinonaphthalene-8-sulfonate.

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doi:10.1016/j.ijbiomac.2011.11.001

antioxidants for cosmetics. In addition, flavonoids are effective tyrosinase inhibitors. Several flavonoids have been verified to have tyrosinase inhibitory ability, such as quercetin, galangin, fisetin, morin, and kurarinol; most of these flavonoids are competitive inhibitors of the enzyme [14,29–31].

In this study, we investigated the mechanism of tyrosinase inhibition by hesperetin using kinetic analysis and computational simulation. The inhibitory activity of hesperetin is due to its structure and its antioxidant potency. Experimentally, hesperetin exerted a competitive type of inhibition on tyrosinase. Kinetic parameters suggested that hesperetin displaces L-DOPA from the active site and the results of computational simulations supported this result. Our study suggests that hesperetin is an effective tyrosinase inhibitor. Additionally, the strategy for predicting tyrosinase inhibition based on evaluating phenolic compounds coupled with computational simulations may prove useful for the screening of potential natural tyrosinase inhibitors.

2. Materials and methods

2.1. Materials

Tyrosinase (M.W. 128 kDa), L-DOPA, and hesperetin were purchased from Sigma–Aldrich. When L-DOPA was used as a substrate in our experiments, the purchased tyrosinase had a K_m of 1.78 ± 0.33 mM ($V_{max} = 0.217 \pm 0.033$ mmol min^{-1}) in our conditions according to a Lineweaver–Burk plot.

2.2. Tyrosinase assay

A spectrophotometric tyrosinase assay was performed as previously described [32,33]. To begin the assay, a 10 μL sample of enzyme solution was added to 1 mL of reaction mix. Tyrosinase activity (v) was recorded as the change in absorbance per min at 475 nm using a Shimadzu UV-1800 spectrophotometer.

2.3. Kinetic analysis for competitive-type inhibition

To describe the competitive inhibition mechanism, the Lineweaver–Burk equation in double reciprocal form can be written as:

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)\frac{1}{[S]} + \frac{1}{V_{max}} \quad (1)$$

Secondary plots can be constructed from

$$K_m^{app} = \frac{K_m[I]}{K_i} + K_m \quad (2)$$

As such, the K_i, K_m, and V_{max} values can be derived from the above equations. The secondary replot of the apparent K_m vs. [I] is linearly fitted, assuming a single inhibition site or a single class of inhibition site.

2.4. Intrinsic and ANS-binding fluorescence measurements

Fluorescence emission spectra were measured with a Hitachi F-4500 fluorescence spectrofluorometer using a cuvette with a 1 cm path length. Tryptophan fluorescence was measured following excitation at 280 nm, and the emission wavelength ranged between 300 and 420 nm. Changes in the ANS-binding fluorescence of tyrosinase were measured following excitation at 390 nm, and the emission wavelength ranged from 420 to 600 nm. The tyrosinase was labeled with 40 μM ANS for 30 min prior to measurements. All kinetic reactions and measurements in this study were performed in 50 mM glycine–NaOH buffer (pH 9.2).

2.5. Determination of the binding constant and the number of binding sites

According to a previous report [34], when small molecules are bound to equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by the following equation:

$$\frac{F_0}{F_0 - F} = \frac{1}{n} + \frac{1}{K}\frac{1}{[Q]} \quad (3)$$

where F_0 and F are the relative steady-state fluorescence intensities in the absence and presence of quencher, respectively, and [Q] is the concentration of quencher (in this case, hesperetin). The values for the binding constant (K) and number of binding sites (n) can be derived from the intercept and slope of a plot based on Eq. (3).

2.6. Molecular docking of tyrosinase and hesperetin

Among the many tools available for protein–ligand docking, DOCK6.3 and AutoDock4.2 were applied because of their automated capability. We used the crystal structure of *Agaricus bisporus* tyrosinase (PDB ID: 2Y9X) to simulate the tyrosinase structure [35]. Because the docking programs use a set of predefined 3D grids of the target protein with a systematic search technique [36], we selected the previously defined 3D grids for tyrosinase interacting with tropolone at the binding pocket. The docking simulation scores are approximate binding energies, and the docking score between the ligand and the receptor is described by energy terms such as electrostatic energy, Van der Waals energy and solvation energy. For ligand–protein docking, the Chimera program (http://www.cgl.ucsf.edu/chimera/) was used to manipulate the tyrosinase structure using functions for deleting solvent, adding charge, and replacing incomplete side chains. The hesperetin structure was derived from the PubChem database (Compound ID: 72281, http://pubchem.ncbi.nlm.nih.gov/), and it was manipulated using the ChemOffice program (http://www.cambridgesoft.com). The following steps were taken: (1) conversion of 2D structures to 3D structures, (2) calculation of charges, (3) addition of hydrogen atoms, and (4) location of pockets. From the docking result, we assessed possible hydrogen bonding interactions and residues using the LigandScout [37] program.

3. Results

3.1. Effect of hesperetin on tyrosinase activity

Tyrosinase activity was conspicuously inactivated by hesperetin in a dose-dependent manner (Fig. 1). The inhibitor concentration leading to 50% hesperetin activity loss (IC_{50}) was estimated to be 11.25 ± 1.73 mM ($n = 3$). Tyrosinase was almost completely inactive at 50 mM hesperetin (Fig. 1A). When hesperetin was absent from the assay system, the tyrosinase activity remained at 80%, even with 50 mM hesperetin in the pre-incubation step, indicating that hesperetin reversibly inhibited tyrosinase (Fig. 1B). To confirm the reversibility of hesperetin-mediated inhibition, plots of the remaining activity vs. [E] at different inhibitor concentrations were constructed (Fig. 1C). The results showed straight lines passing through the origin. Increasing the inhibitor concentrations resulted in a decrease in the slope of the line, indicating that the inhibition of hesperetin on the enzyme was reversible.

Next, the time–courses for tyrosinase inhibition at different concentrations of hesperetin were compared (Fig. 2). The results showed that the changes in catalytic rate were not detectable