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Effects of piperonylic acid on tyrosinase: Mixed-type inhibition kinetics and computational simulations

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ABSTRACT

Piperonylic acid is a natural molecule with a benzoic acid group and high antioxidant capacity. Based on its aromatic acid structure and antioxidant properties, we studied the effects of piperonylic acid on tyrosinase by the analysis of its inhibitory kinetics and computational simulations. Piperonylic acid reversibly inhibited tyrosinase through a mixed-type inhibitory mechanism. The time courses of the tyrosinase inhibition showed that piperonylic acid binds to tyrosinase very quickly and the inactivation processes follow first-order kinetics. The continuous substrate reactions indicated that piperonylic acid induced a tight-binding inhibition and the substrate can promote the inactivation process. The ANS-binding fluorescence of tyrosinase suggested that piperonylic acid did not detectably disrupt the tertiary structure of the enzyme. The results of the computational docking and molecular dynamics simulations showed that piperonylic acid closely interacts with three residues and it might block the active site of tyrosinase.

1. Introduction

Tyrosinase (EC 1.14.18.1) is a multifunctional copper-containing enzyme that is widely distributed in plants and animals and is a critical enzyme related to melanin production. This enzyme catalyzes both the *ortho*-hydroxylation of L-tyrosine and the subsequent conversion of 3,4-dihydroxyphenylalanine to DOPA-quinone in the early stage of melanogenesis. DOPA-quinone yields melanin, which is a polymer that is composed of numerous smaller component molecules, such as 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA), and is synthesized through a series of enzymatic reactions, a consequent oxidation and an oxidative cyclization [1]. Structurally, tyrosinase belongs to the type 3 copper protein family [2,3] and has two copper ions, each of which are coordinately bonded with a distinct set of three histidine residues within the active site. These copper atoms participate directly in the hydroxylation of monophenols to *o*-diphenols and in the oxidation of *o*-diphenols to *o*-quinones [4]. Tyrosinase catalyzes the pigmentation of skin and is directly related to pigmentation disorders in mammals [5–7]. This enzyme also causes a browning effect in vegetables [8]. Furthermore, the tyrosinase distributed in insects participates in host defense, wound healing, cuticle formation, molting and the sclerotization process of insects [9–11].

Thus, tyrosinase inhibitors have potential applications in clinical medicine, hyperpigmentation-related cosmetic research, food preservation technology and agriculture as bio-insecticides [12–15]. Therefore, studies on tyrosinase inhibitors have attracted a lot of interest, and many efforts have addressed the screening of efficient and safe tyrosinase inhibitors from natural and synthetic materials. To date, numerous novel tyrosinase inhibitors have been reported, but most have shown poor to moderate activity [16,17]. Various copper chelators, including kojic acid, have been reported to act as tyrosinase inhibitors [18]. Among the copper chelators, aromatic acids containing carboxy groups appear to be potent inhibitory agents for tyrosinase [19] because these molecules have a strong metal chelating ability with ions, such as Cu(II), within the active site. Some aromatic acids, such as benzoic acid, toluic acid, and terephthalic acid, have been verified to be

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

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tyrosinase inhibitors by previous research studies, including our previous research [20–22].

Black pepper (*Piper nigrum* L.), which is one of the most popular spices, has insecticidal properties. Its organic solvent extracts can potentially be utilized as an alternative to synthetic insecticides [23]. Piperine, which belongs to the alkaloid family, represents the major component in the dry fruit of *P. nigrum*. Piperine has been reported to have several pharmacological effects, such as anti-diarrhoeal, anti-inflammatory, hepatoprotective, and analgesic effects [24–26]. In addition, piperine has a high antioxidant activity and is used for the treatment of Alzheimer's diseases [27,28]. The *in vitro* antioxidant activities of piperine and its derivatives were recently evaluated. Piperonylic acid (3,4-methylendioxybenzoic acid), which is synthesized through the alkaline hydrolysis of piperine, was found to have the highest antioxidant activity of the studied molecules [29]. Piperonylic acid is an aromatic acid with a benzoic acid group. Tyrosinase catalyzes the oxidation of *o*-diphenols to *o*-quinones, as previously mentioned; thus, because many antioxidants have the ability to inhibit this reaction, these molecules can act as tyrosinase inhibitors. Our previous research also indicated that the screening of tyrosinase inhibitors from a pool of antioxidants is a promising method [30–32]. Therefore, we hypothesized that piperonylic acid can inhibit melanin formation through a tyrosinase inhibitory action and can act as an anti-pigmentation agent based on its aromatic acid structure and antioxidant capacity.

In this work, we investigated the mechanism underlying the inhibition of tyrosinase by piperonylic acid through kinetic analyses and computational simulations. We hypothesized that piperonylic acid can block the oxidation of 3,4-dihydroxyphenylalanine (L-DOPA) by binding to the tyrosinase active site. The inhibitory activity of piperonylic acid is due to its structure, and we experimentally found that piperonylic acid inhibited tyrosinase through a mixed-type inhibition mechanism. The kinetic parameters suggested that this inhibitory agent bound both to the free enzyme and to the enzyme-substrate complex. We used computational simulations to explore its inhibitory action on tyrosinase further, and the results of the computational simulations confirmed the kinetic results. Our study suggests that piperonylic acid is an effective tyrosinase inhibitor. Additionally, the strategy of predicting tyrosinase inhibitors through the evaluation of aromatic acid compounds coupled with computational simulations might prove useful for the screening of potential tyrosinase inhibitors.

2. Materials and methods

2.1. Materials

Tyrosinase (M.W. 128 kDa), L-DOPA, and piperonylic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Tyrosinase and L-DOPA were dissolved in 50 mM sodium phosphate buffer (pH 7.0). Piperonylic acid was dissolved in 10 mM Tris–HCl buffer (pH 7.2).

2.2. Tyrosinase assay

A spectrophotometric tyrosinase assay was performed as previously described [33,34]. To begin the assay, a 15-μL sample of the enzyme solution was added to 1 mL of the reaction mixture (2 mM L-DOPA in 50 mM sodium phosphate buffer, pH 7.0) at 25 °C. The absorbance of the reaction mixture was determined using a Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto). The tyrosinase activity (v) was recorded as the change in absorbance at 475 nm per min.

2.3. Kinetic analysis

For the general analysis of mixed-type inhibition, the Lineweaver–Burk equation can be written in double reciprocal form:

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

Secondary plots can be constructed from

$$Slope = \frac{K_m}{V_{\max}} + \frac{K_m[I]}{V_{\max}K_i} \quad (2)$$

and

$$Y\text{-}intercept = \frac{1}{V_{\max}^{app}} = \frac{1}{V_{\max}} + \frac{1}{K_{is}V_{\max}}[I] \quad (3)$$

The K_i, K_{is}, K_m, and V_{max} values can be derived from these equations. The secondary re-plot of *Slope* or *Y-intercept vs.* [*I*] is linearly fitted assuming a single inhibition site or a single class of inhibition sites.

For the kinetic analysis of the substrate reaction, the following inactivation model, which is outlined in previous reports [34–36], was applied:

$$\frac{d[E^*]}{dt} = k_{+0}[E \cdot I] - k_{-0}[E^*] = B[E_0] - A[E^*] \quad (4)$$

where $[E_0]$ is the total enzyme concentration. $A = ((k_{+0}/K_I)K_m/K_m(1 + [I]/K_I) + [S])$ and $B = k_{-0}$. A and B are the apparent rate constants for the inactivation and reactivation, respectively. The product formation can be written as:

$$P_t = \frac{k_{-0}v}{A}t + \frac{(A - k_{-0})v}{A^2}(1 - e^{-At}) \quad (5)$$

where P_t is the product formed after reaction time t. v is the initial rate of reaction in the absence of the inhibitor. When t is sufficiently large, the curves become straight lines and the product concentration is written as P_∞, which is the product to be expected from the straight-line portions of the curves:

$$P_\infty = \frac{k_{-0}v}{A}t + \frac{(A - k_{-0})v}{A^2} \quad (6)$$

Combining Eqs. (5) and (6), yields:

$$P_\infty - P_t = \frac{(A - k_{-0})v}{A^2}e^{-At} \quad (7)$$

$$\ln(P_\infty - P_t) = \ln\frac{(A - k_{-0})v}{A^2} - At \quad (8)$$

Plots of $\ln(P_\infty - P_t)$ *vs.* t give a series of straight lines at different concentrations of inhibitor with slopes of $-A$. It can be seen that the apparent forward rate constant (A) is depends on the inhibitor concentration. Therefore, it can be rearranged:

$$\frac{1}{A} = \frac{K_I}{k_{+0}}\left(1 + \frac{[S]}{K_m}\right) + \frac{1}{k_{+0}}[I] \quad (9)$$

The A and k_{+0} were calculated from the plot of $\ln(P_\infty - P_t)$ *vs.* t. The dissociation constant K_I can be obtained from the slope and intercept of the straight line.

For the evaluation of the inactivation kinetics and rate constants, the transition free energy was calculated based on methods described in a previous report with slight modifications [37]. The transition free energy change per second is given by $\Delta\Delta G^\circ = -RT\ln k$. The data were calculated from the semi-logarithmic plots, and k is the monophasic rate constant.

2.4. ANS-binding fluorescence measurements

The fluorescence emission spectra were obtained with a Hitachi F-4500 fluorescence spectrofluorometer using a cuvette with a 1-cm path length (Hitachi, Tokyo). The changes in the ANS-binding fluorescence of tyrosinase were measured following its excitation at 390 nm. The emission wavelength ranged from 400 to 600 nm. Tyrosinase was labeled with 40 μM ANS for 30 min prior to the measurements.

2.5. Computational docking and molecular dynamics simulation

The three-dimensional structure of the tyrosinase from *Agaricus bisporus* [38] was obtained from the Protein Data Bank (PDB ID: 2Y9W) [39]. The piperonylic acid structure was obtained from the PubChem Compound database [40] (CID: 7196) and optimized through energy minimization using the ChemBio3D Ultra 12.0 software (http://www.cambridgesoft.com).

Two programs were used for the computational docking: the Pck software (http://alnitak.u-strasbg.fr/Pck/) and AutoDock Vina [41]. The Pck software was used to select some of the binding points of tyrosinase. This software found several pockets and provided a list of their neighboring residues. Using the list, *in silico* protein-ligand docking simulations were then performed using AutoDock Vina, and 10 docking structures were generated from each pocket residue.

CHARMM [42] was used to validate the docking structures. All of the docking structures were clustered using the CORREL module, and the best clusters were selected for molecular dynamics simulations based on the average docking energy and the number of docking structures. The lowest-energy structures of the chosen clusters were subjected to a 10-ns molecular dynamics simulation, and the structures were saved every 1 ps for trajectory analysis.