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The effect of oxaloacetic acid on tyrosinase activity and structure: Integration of inhibition kinetics with docking simulation

Lin Gou[a,1], Jinhyuk Lee[b,c,1], Hao Hao[d], Yong-Doo Park[d], Yi Zhan[e,*], Zhi-Rong Lü[d,*]

[a] College of Biology and Science, Sichuan Agriculture University, Chengdu 611130, PR China
[b] Korean Bioinformation Center (KOBIC), Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Republic of Korea
[c] Department of Nanobiotechnology and Bioinformatics, University of Sciences and Technology, Daejeon 305-350, Republic of Korea
[d] Zhejiang Provincial Key Laboratory of Applied Enzymology, Yangtze Delta Region, Institute of Tsinghua University, Jiaxing 314006, PR China
[e] College of Life Sciences, Zhejiang Sci-Tech University, Hangzhou 310018, PR China

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ABSTRACT

Oxaloacetic acid (OA) is naturally found in organisms and well known as an intermediate of citric acid cycle producing ATP. We evaluated the effects of OA on tyrosinase activity and structure *via* integrating methods of enzyme kinetics and computational simulations. OA was found to be a reversible inhibitor of tyrosinase and its induced mechanism was the parabolic non-competitive inhibition type (IC_{50} = 17.5 ± 0.5 mM and K_i = 6.03 ± 1.36 mM). Kinetic measurements by real-time interval assay showed that OA induced multi-phasic inactivation process composing with fast (k_1) and slow (k_2) phases. Spectrofluorimetry studies showed that OA mainly induced regional changes in the active site of tyrosinase accompanying with hydrophobic disruption at high dose. The computational docking simulations further revealed that OA could interact with several residues near the tyrosinase active site pocket such as HIS61, HIS259, HIS263, and VAL283. Our study provides insight into the mechanism by which energy producing intermediate such as OA inhibit tyrosinase and OA is a potential natural anti-pigmentation agent.

1. Introduction

Tyrosinase (EC 1.14.18.1) is a multifunctional copper-containing metalloenzyme that is critical for melanin pigment production [1]. The multifunction catalytic functions of tyrosinase can be briefly summarized as hydroxylation of monophenols and oxidation of diphenols to form quinines [2,3] and thus, tyrosinase has broad substrate specificity toward many kinds of phenols and catechols that are useful in industrial applications [4]. Since tyrosinase has two copper ions at the catalytic active site pocket in which each copper bound to a set of three histidines, structurally it belongs to the type 3 copper protein family [5].

In human, tyrosinase catalyzes the pivotal process in melanogenesis of skin and eye and is directly related to pigmentation disorder such as oculocutaneous albinism when it is mutated [6]. In insects, tyrosinase is required for cuticle formation compared to phenoloxidase *via* importantly production of DOPA that is required for both cuticle pigmentation (tanning) and immune-associated melanization [7]. Tyrosinase also induced unfavorable browning side effect of plant-derived foods causing a decrease in nutritional quality and economic loss and therefore, tyrosinase inhibition is important for various applications [8].

Considerable many efforts have been tried by researchers to develop naturally derived tyrosinase inhibitors that effectively down-regulated melanin pigment with avoiding harmful side effects, not only for the clinical purpose but also for the commercial cosmetic purpose [9,10]. Among the tyrosinase inhibitors derived from natural sources, a structural distinctive that contains hydroxyl groups in their structures including flavonoids and polyphenols was turned to be effective for tyrosinase inhibition due to chelating copper ion as well as binding critical residues affecting substrate access at the active site pocket. Those inhibitors displayed different binding mechanisms and various inhibition types: i) reversible and competitive types, which were mostly interacts with histidine residues of binding coppers located in the active site [11,12]; ii) chelation of copper forming apo-tyrosinase *via* competing with the copper chaperon and result in a lysosomal mistargeting [13]; suicide inactivation of the active site of tyrosinase [14,15]; mixed-

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

* Corresponding authors.
E-mail addresses: zhanyi_1006@hotmail.com (Y. Zhan), lzr7899@163.com (Z.-R. Lü).

[1] These authors contributed equally to this work.

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type [16,17], non-competitive [18,19], and uncompetitive [20,21] inhibitions of tyrosinase.

Oxaloacetic acid (OA) is naturally found in organisms and well known as a metabolic intermediate of various processes such as citric acid cycle, urea cycle, gluconeogenesis, and glyoxylate cycle. OA also participates in amino acid synthesis and fatty acid synthesis. Recent evidences showed that OA could be possibly applied to treat the neurodegenerative diseases [22,23]. The neuroprotective effect of OA against ischemic injury as a glutamate scavenger has been suggested for the treatment of ischemic stroke patients obtained from animal models [24,25].

To find an effective and safe tyrosinase inhibitor for the various applications, and to validate the inhibition effect of naturally derived compounds having carboxyl groups on tyrosinase, we performed the effect of OA on tyrosinase activity and structure by using inhibition kinetic analyses integrating with computational simulations in this study. We found that the structure of OA having carboxylic group was effective for its inhibitory activity and induced reversible non-competitive inhibition of tyrosinase. OA binds to free tyrosinase and structural changes mostly in the regional active site of the enzyme. The kinetic results were confirmed using computational simulations in which we explored binding site of OA-binding tyrosinase. Our study indicated that OA is an effective and safe tyrosinase inhibitor and suggested the new potent application of OA on dermatologic usage.

2. Materials and methods

2.1. Materials

Tyrosinase (MW 128 kDa) from *Agaricus bisporus*, oxaloacetic acid (OA), and L-DOPA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tyrosinase, OA, and L-DOPA were dissolved in 50 mM sodium phosphate buffer (pH 7.0). All other chemicals were obtained locally and were of the highest analytical grade.

2.2. Tyrosinase activity assay

A spectrophotometric assay method for measuring DOPA oxidase activity of tyrosinase was performed as previously described [26,27]. The tyrosinase activity (v) was determined in the presence of L-DOPA substrate by monitoring the change in absorbance per min at 475 nm using a Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto).

2.3. Inhibition kinetic analysis

For non-competitive type inhibition, the Lineweaver-Burk equation can be expressed in double reciprocal form, as follows:

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

Secondary plots can be constructed from:

$$Y - \text{intercept} = \frac{1}{V_{max}} + \frac{1}{K_i V_{max}}[I] \quad (2)$$

The K_i value was derived from these equations. The secondary replot of *Y-intercept vs.* [I] was linearly fitted, assuming it is a pure non-competitive type with a single inhibition site or a single inhibition site class. However, the secondary replot is not a linear fit; it is parabolic in shape, which indicates a complex non-competitive type inhibition with multiple inhibition sites or structural conformational changes. K_i cannot be determined directly from the usual equations due to the parabolic relationship; thus, Eq. (3) was applied based on the previous reports [28,29]:

$$Y - \text{intercept} = \frac{1}{V_{max}} + \frac{1}{V_{max}}\left(\frac{1}{K_i}[I]\right)^2 \quad (3)$$

where K_i is a more complex function of Eq. (2), which varies with [I] calculated from the *Y-intercept* of the replot.

2.4. Intrinsic and ANS-binding fluorescence measurements

Intrinsic fluorescence was measured *via* tryptophan fluorescence that was measured upon excitation at 280 nm where the emission wavelength ranged between 300 and 400 nm. To measure changes in the ANS-binding fluorescence of tyrosinase, tyrosinase was labeled with 40 μM ANS for 30 min prior to all measurements. The following excitation at 390 nm with the emission wavelength ranged from 420 to 600 nm. All reactions and measurements were performed in 50 mM sodium phosphate buffer (pH 7.0) and all fluorescence emission spectra were measured with a Hitachi F-4500 fluorescence spectrofluorometer using a cuvette with a 1-cm path length (Hitachi, Tokyo, Japan).

2.5. Secondary replotting of determining the binding constant and the number of binding sites

Based on the previous report [30], the binding constant of OA binding to tyrosinase and the number of binding sites were calculated by the following equation:

$$\frac{F_0}{F_0 - F} = \frac{1}{n} + \frac{1}{K}\frac{1}{[Q]} \quad (4)$$

where F_0 and F are the relative steady-state fluorescence intensities in the absence and presence of quencher, respectively, and [Q] is the quencher (OA) concentration. The values for the binding constant (K) and the number of binding sites (n) can be derived from the intercept and slope of Eq. (4).

2.6. Computational molecular dynamics (MD) and docking simulations

The crystal structure of *Agaricus bisporus* tyrosinase has been reported [31]; thus, we used this structure for molecular docking (PDB: 2y9w) between OA and tyrosinase. The OA structure was obtained from the PubChem database (compound ID: 970, http://pubchem.ncbi.nlm.nih.gov/) and manipulated using Marvin software (ChemAxon; http://www.chemaxon.com; 5.11.4, 2012). Computational docking simulation was performed using AutoDock Vina [32]. We found 57 pocket residues. Ten docking simulations with different random seeds were performed each pocket residue. Imaginary box with each box dimension of 15 Å was considered around a pocket residue to prevent OA out of the box. Total 1600 conformations of OA were generated around tyrosinase. They were grouped into a cluster based on their center of geometry. The lowest energy and largest number clusters are chosen for further analyses.

3. Results

3.1. Inhibition effect of OA on the DOPA oxidase activity of tyrosinase

We found that OA significantly inhibited the L-DOPA oxidation of tyrosinase in a dose-dependent manner (Fig. 1). When OA was added into the substrate mixture as same corresponding to the incubating reaction, the concentration of OA that yielded a 50% reduction in tyrosinase activity (IC_{50}) value was measured to be