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Investigating the inhibitory activity and mechanism differences between norartocarpetin and luteolin for tyrosinase: A combinatory kinetic study and computational simulation analysis

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ABSTRACT

Flavonoids are an important type of natural tyrosinase inhibitor, but their inhibitory activity and mechanism against tyrosinase are very different because of their different structures. In this study, the inhibitory activity and mechanism differences between norartocarpetin and luteolin for tyrosinase were investigated by a combination of kinetic studies and computational simulations. The kinetic analysis showed that norartocarpetin reversibly inhibited tyrosinase in a competitive manner, whereas luteolin caused reversible noncompetitive inhibition. Both norartocarpetin and luteolin showed a single type of quenching and a static-type quenching mechanism. A computational simulation indicated that the hydroxyl groups of the B ring of norartocarpetin interacted with tyrosinase residues Asn81 and His85 in the active pocket, while the hydroxyl groups of the B ring of luteolin bound residues Asn81 and Cys83. HPLC and UPLC-MS/MS further confirmed that luteolin acted as a substrate or a suicide inhibitor, yet norartocarpetin acted as an inhibitor.

1. Introduction

Tyrosinase (EC 1.14.18.1) is a type-3 copper protein containing two copper ions coordinately bonded with a distinct set of three histidine residues within the active site (Si et al., 2013). It catalyses both the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones (Olivares, Jimenez-Cervantes, Lozano, Solano, & Garcia-Borron, 2001). Tyrosinase plays a vital role in melanin formation in bacteria, fungi, plants, and mammals (Sánchez-Ferrer, Rodríguez-López, García-Cánovas, & García-Carmona, 1995). In human being, it catalyses the synthesis of dopaquinone from L-DOPA in the active site of the enzyme and then forms melanin to protect the skin from ultraviolet radiation (Kim & Uyama, 2005; Wang & Hebert, 2006). However, the overproduction of melanin results in age spots, freckles, melasma, and even malignant melanoma of skin (Solano, Stefania, Picardo, & Ghanem, 2006). In the food industry, the undesirable browning in fruits, vegetables, and beverages caused by tyrosinase impairs their colour and sensory properties, shortens shelf life, reduces market value, and increases the loss of nutritional value during postharvest handling processes (Artés, Castañer, & Gil, 1998). Tyrosinase inhibitors can inhibit undesirable enzymatic browning in food products to maintain their quality attributes and have whitening effects on skin. Thus, they have garnered great attention.

Flavonoids are abundant in fruits, vegetables, spices, tea, soy-based food stuffs, and traditional herbal medicine. Many flavonoids have been identified and exploited as tyrosinase inhibitors in recent years (Chang, 2009; Kim & Uyama, 2005). Some flavonoids have been identified as potent tyrosinase inhibitors from the wood and twigs of *Artocarpus heterophyllus*, the root of *Morus nigra* and *M. australis*, the twigs of *Cudrania tricuspidata* and *Broussonetia papyrifera*, and the stems of *C. cochinchinensis* in our previous works (Zheng, Cheng, Chao, Wu, & Wang, 2008; Zheng, Tan, Chen, & Wang, 2013; Zheng, Tan, & Wang, 2012; Zheng, Zhu, Fan,

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Tan, & Wang, 2011; Zheng et al., 2009; Zheng et al., 2010). Some of these compounds, such as norartocarpetin, steppogenin, artocarpanone, artocarpesin, 2,4,2′,4′-tetrahydroxychalcone, and morachalcone A, are potent natural tyrosinase inhibitors with great promise. Although great progress has already been made in the discovery of natural flavonoid tyrosinase inhibitors, the lack of understanding of the interaction between tyrosinase and flavonoid inhibitors has seriously limited the application of these available natural tyrosinase inhibitors and affected the discovery of new ones. Therefore, it is important to exploit the inhibition mechanism of tyrosinase inhibitors at a molecular level by investigating tyrosinase-inhibitor interactions.

Norartocarpetin (5, 7, 2′, 4′-tetrahydroxyflavone) is widely distributed in Moraceae plants and is especially rich in *Morus* and *Artocarpus* plants (Jeong et al., 2009; Likhitwitayawuid, Sritularak, & De-Eknamkul, 2000). Compared to norartocarpetin, luteolin (5, 7, 3′, 4′-tetrahydroxyflavone) is a more common flavone that is extensively found in many plant species. The difference in structures of these compounds is only a difference in the hydroxyl substituted position. The former has a 2′, 4′-dihydroxyl group substitution in the B ring, while the latter has a 3′, 4′-dihydroxyl group substitution. A recent study suggested that luteolin is an uncompetitive tyrosinase inhibitor with weaker inhibitory activity than kojic acid (Xie, Chen, Huang, Wang, & Zhang, 2003), whereas norartocarpetin showed strong tyrosinase inhibitory activity in a reversible competitive manner (Ryu et al., 2008). However, so far, no studies have been conducted to reveal the causes of the difference in the tyrosinase inhibitory activity and the mechanism of norartocarpetin and luteolin. Thus, it would be very interesting and significant to explore the causes in this difference.

The tyrosinase mechanism is complex because tyrosinase exhibits wide substrate specificity and can catalyse multiple reactions (Fenoll, Garciäa-Ruiz, Varoän, & García-Cänovas, 2003; Hu et al., 2012). Therefore, exploring the enzyme mechanism demands a combination of a variety of kinetic, computational methods and chromatography techniques to derive the relationships among substrates and ligands of the enzyme (Hu et al., 2012). In this study, a combination of kinetic studies and computational simulations was employed to reveal the inhibitory activity and mechanistic differences in tyrosinase between norartocarpetin and luteolin.

2. Materials and methods

2.1. Materials

Norartocarpetin (purity > 98.0%) was isolated from the wood of *A. heterophyllus*. Mushroom tyrosinase (5771 units/mg), L-DOPA, luteolin (purity ⩾ 98.0%), and kojic acid were purchased from Sigma Chemical Co. (St. Louis, USA). Dimethyl sulfoxide (DMSO), methanol (MeOH), sodium dihydrogen orthophosphate ($NaH_2PO_4 \cdot 2H_2O$), and anhydrous di-sodium hydrogen phosphate (Na_2HPO_4) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Suzhou, PR China). All stock solutions were stored at 4 °C.

2.2. Mushroom tyrosinase inhibitory test

The tyrosinase inhibition activity of the compounds was measured using L-DOPA as a substrate according to the modified method of our previous study (Zheng et al., 2010). The compounds were first dissolved in DMSO at a concentration of 1.0 mM and then diluted to different concentrations with DMSO. At the same time, the substrate and tyrosinase solutions were dissolved in 50 mM sodium phosphate buffer (pH 6.8) at a substrate concentration of 0.5 mM and an enzyme concentration of 0.04 mg/mL. The reaction mixtures (3.0 mL), which included 2.8 mL of substrate solution, 100 μL of different concentrations of the compounds and 100 μL of mushroom tyrosinase, were mixed by vortexing. The initial absorbance at 475 nm was measured using a Cary 50 spectrophotometer (Varian, USA) to monitor the formation of dopachrome. After incubation for 10 min at 30 °C, the final absorbance at the same wavelength was taken. Meanwhile, kojic acid was used as a positive control. The IC_{50} values, which represent the concentrations of compounds that caused 50% of the tyrosinase activity to be inhibited, were determined. The percent inhibition of tyrosinase activity was calculated as follows:

$$\%\ \text{Inhibition} = [(A_2 - A_1) - (B_2 - B_1)]/(A_2 - A_1) \times 100$$

where A_1 is the absorbance at 475 nm of the blank at 0 min, A_2 is the absorbance at 475 nm of the blank at 10 min; B_1 is the absorbance at 475 nm of the test sample at 0 min, and B_2 is the absorbance at 475 nm of the test sample at 10 min.

2.3. Kinetic analysis of tyrosinase inhibition

The kinetic analysis for the compound-induced inhibition was performed to determine the type of inhibition caused by norartocarpetin and luteolin. The Lineweaver-Burk equation in double-reciprocal form and the secondary replots were applied to determine the inhibition types and to evaluate the dissociation constants for the inhibitors (K_i), using various concentrations of L-DOPA as the substrate. The formulas for the Lineweaver-Burk equation in double reciprocal form are as follows:

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

$$\textit{Noncompetitive} \quad \frac{1}{v} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)\frac{1}{[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_i}\right) \tag{2}$$

A secondary plot can be constructed from

$$\textit{Slope} = \frac{K_m[I]}{K_i} + K_m \tag{3}$$

The values for K_i, K_m, and V_{max} can be derived from the above equations. [*S*] and [*I*] are the concentrations of substrate and inhibitor. If the secondary plot of *Slope* versus [*I*] is linear, a single inhibition site or a single class of inhibition site can be concluded (Li et al., 2015).

2.4. Fluorescence measurements

Fluorescence experiments were carried according to the modified method of previous studies (Kim et al., 2006; Xiong, Liu, Zhou, Zou, & Chen, 2016; Zhang & Ma, 2013). To determine the linear concentration range of the fluorescence, flavonoid solutions were first dissolved in DMSO at a concentration of 1.0 mM and then were diluted to 200 μM with sodium phosphate buffer. A series of 2 mL solutions containing 0.2 mL of tyrosinase solution were added to a centrifuge tube and then accurately mixed with different concentrations of flavonoid solutions range from 0 to 100 μM. The fluorescence emissions were measured within 10 min after the solution incubation using a F-2700 spectrofluorophotometer (Hitachi, Japan) in the wavelength range between 300 and 500 nm with an excitation wavelength (λ_{ex}) of 280 nm and excitation and emission slit widths of 10 nm at 30 °C. The final tyrosinase concentration was 0.04 mg/mL. All fluorescence curves were corrected by subtracting the corresponding blanks (samples in sodium phosphate buffer without tyrosinase). Meanwhile, the fluorescence intensities were corrected for inner filter effect, which reabsorbs