Tyrosinase Inhibitory Effects and Antioxidant Properties of Paeonol and Its Analogues

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With the aim to find out structural features for the tyrosinase inhibitory activity, the inhibitory effects of seven paeonol analogues on the diphenolase of mushroom tyrosinase, the interaction between the inhibitors and the copper ions, and the antioxidant activity by DPPH radical were investigated. These paeonol analogues had suggested remarkable inhibition toward tyrosinase. Among them, paeonol (a) exhibited the strongest inhibition activity ($IC_{50}=0.21$ mM) as well as showed potent scavenging activity on the DPPH radical. The inhibition kinetics revealed that paeonol (a) was a mixed-type inhibitor, 2'-hydroxy acetophenone (c), 4'-hydroxy acetophenone (d), and 2,4'-dihydroxy acetophenone (e) were competitive inhibitors, while acetophenone (b), 2'-methoxyacetophenone (f), and 4'-methoxy acetophenone (g) behaved as non-competitive inhibitors. The maximum absorbing wavelengths of compounds (c), (d), and (e) showed different significant blue shifts, which could explain that they exhibited competitive inhibition by forming a chelate with the copper ions at the catalytic domain of the tyrosinase.

Keywords: paeonol, acetophenone, tyrosinase inhibitor, DPPH radical, copper ions chelation

Introduction

Tyrosinase (EC 1.14.18.1), known as polyphenol oxidase, is a multifunctional copper-containing metalloenzyme widely distributed in nature. It mainly catalyzes the *o*-hydroxylation of monophenols to the corresponding catechols, and the oxidation of monophenols to the corresponding *o*-quinones (Yoon *et al.*, 2009). Consequently, a series of high reactive quinones are produced to initiate the pigmentation and excessive activation of tyrosinase can cause various dermatological disorders, such as Parkinson and other degenerative diseases (Asanuma *et al.*, 2003).

One of the reactions catalyzed by the tyrosinase is the oxidation of L-dopa to L-dopaquinone by utilising molecular oxygen. In addition, reactive oxygen species (ROS) and free radical-mediated reactions are associated with various diseases, such as diabetes mellitus, cancer, ageing, skin disorders, and neurodegenerative diseases (Pham-Huy *et al.*, 2008). If the inhibitors we have designed can weaken the tyrosinase activity as well as scavenge ROS effectively, the production of melanin and the damage of ROS to our human beings will be decreased to a great extent. Hence, tyrosinase inhibitors have become increasingly important in cosmetic

(Guevara and Pandya, 2001) and medicinal (Kanost *et al.*, 2004) industry, the food industry (Lunadei *et al.*, 2011) and agriculture (Guerrero and Rosell, 2005) to prevent hyperpigmentation.

In recent years, a large number of naturally occurring and synthetic tyrosinase inhibitors have been reported. There are many tyrosinase inhibitors, such as hydroquinone (Garcia and Fulton Jr, 1996), ascorbic acid (Kameyama *et al.*, 1996), arbutin (Nakajima *et al.*, 1998), kojic acid (Mishima *et al.*, 1994), aromatic aldehydes (Jimenez *et al.*, 2001; Chen *et al.*, 2003), aromatic acids (Chen *et al.*, 2005; Si *et al.*, 2011), aromatic alcohol (Liu *et al.*, 2007; Zhu *et al.*, 2011), and tropolone (Valero *et al.*, 1991). It has been suggested that the addition of a methoxy group at the meta or para position of salicylaldehyde shows different inhibition strength. Alkoxybenzoic acids have been studied for the inhibition of mushroom tyrosinase (Huang *et al.*, 2006), revealing that different alkoxy substitutions induce various inhibition type (Chen *et al.*, 2005). The inhibition of 2'-phenylethanol, 2'-phenylacetaldehyde and 2'-phenylacetic acid on the activity of mushroom tyrosinase indicating that the functional group on the benzene ring group played an important role in the enzyme inhibition (Zhu *et al.*, 2011).

Paeonol (structure was shown in Fig. 1(a)), belonging to aromatic ketone, one of the primary bioactive components

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- (a): R1 = OH, R2 = OCH3; (b): R1 = H, R2 = H;
 (c): R1 = OH, R2 = H; (d): R1 = H, R2 = OH;
 (e): R1 = OH, R2 = OH; (f): R1 = OCH3, R2 = H;
 (g): R1 = H, R2 = OCH3;

Fig. 1. Chemical structures of paeonol and its analogues.

isolated from Chinese national flower-peony, is known to have a series of pharmaceutical properties including anti-inflammatory, anti-oxidant and also known as traditional skin-whitening agent, a potential tyrosinase inhibitor (Gong *et al.*, 2006). However, the inhibitory effects of other aromatic ketones on the enzymatic activity of mushroom tyrosinase are little explored. It has been confirmed that ortho- and para- substitutes of benzene ring produce bigger influence on mushroom tyrosinase for the oxidation of L-DOPA (Liu *et al.*, 2007). Therefore, the aim of the present paper is, to study the inhibitory kinetics of paeonol analogues which all are 2,4 substituted acetophenones on the enzyme activity and evaluate the kinetic parameters and the inhibition mechanisms, to investigate the interaction between the inhibitors and the copper ions, and to measure the antioxidant activity of paeonol analogues and finally come up with the structure-activity relationship. We hope they can be used to provide the basis for developing novel effective tyrosinase inhibitors and searching for new whitening agents in cosmetic preparations or antibrowning agents for food products.

Materials and Methods

Materials Mushroom tyrosinase (EC 1.14.18.1), L-3,4-dihydroxyphenylalanine (L-DOPA), and 2,2-diphenyl-1-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co (St. Louis, MO, USA). The specific activity of the enzyme was 1881 U/mg. Paeonol (a) (see Fig. 1 for structures), acetophenone (b), 2'-hydroxyacetophenone (c), 4'-hydroxyacetophenone (d), 2,4'-dihydroxyacetophenone (e), 2'-methoxyacetophenone (f), and 4'-methoxyacetophenone (g) were obtained from J & K Chemical Co. (Shanghai, China). All other reagents were of analytical grade. The water used was re-distilled and ion-free. All the data were recorded using Shimadzu UV-2450 spectrophotometer (Japan).

Tyrosinase inhibitory activity The assay of the enzyme activity was performed as previously described by Xie *et al.* (2003) with minor modification. 0.1 mL of different

concentrations of effector (dissolved in DMSO solution), was added to 2.8 mL L-DOPA in Na₂HPO₄-NaH₂PO₄ buffer (50 mM, pH 6.8), and then 0.1 mL of aqueous solution of mushroom tyrosinase was added to the assay mixture with a total volume of 3 mL. The final concentration of mushroom tyrosinase was 3.75 µg/mL. The solution was immediately monitored by measuring the dopachrome formation at 475 nm ($\epsilon = 3700 \text{ M}^{-1} \text{ cm}^{-1}$) for 5 min using a Shimadzu UV-2450 spectrophotometer. The reaction was carried out under a constant temperature of 30°C. The extent of inhibition by the addition of the sample was expressed as the percentage necessary for 50% inhibition (IC₅₀). Controls, without inhibitor but containing 3.3% DMSO.

Determination of the inhibition type and inhibition constants The inhibition type was determined by Lineweaver-Burk plots, and the inhibition constants were obtained by the second plots of the apparent Km/V_{mapp} or 1/V_{mapp} versus the concentration of the inhibitor (Chen *et al.*, 2002).

Copper ions interaction study To investigate the interaction between the copper ions and the above substances, stock solutions of compounds (a) – (g) (1 mM) were prepared in methanol. Then 25 µM solutions were prepared in a cuvette containing phosphate buffer (50 mM, pH 6.8), and the absorption spectra were recorded between 200 and 600 nm. Scans with 10, 25, 50, or 100 µM CuSO₄ were taken after 10 s using a UV-2450 spectrophotometer, which were compared to the substances alone.

Radical-scavenging activity assay The radical-scavenging activities of the samples on DPPH were determined according to the modified method described previously by Lee *et al.* (2008). First, 1.0 mL of 1 mM different sample solutions (dissolved in ethanol), and 0.5 mL of ethanolic solution of 0.6 mM DPPH were put into the test tube. Then, 3.5 mL of ethanol was added to get a 5 mL system. 1.0 mL of ethanol was added to the tube in place of scavengers as the control. The decrease in absorbance of DPPH at 517 nm after 30 min at room temperature was measured using a UV-2450 spectrophotometer. The inhibition (%) of DPPH absorbance was calculated based on the expression $(A_{\text{control}} - A_{\text{test}}) \times 100 / A_{\text{control}}$, where A_{control} is the absorbance of the control (DPPH solution in the absence of a test sample) and A_{test} is the absorbance of the test sample.

Results and Discussion

Effects of compounds (a) – (g) on the diphenolase activity of mushroom tyrosinase The inhibition of compounds (a), (b), (c), (d), (e), (f), and (g) on the oxidation of L-DOPA by mushroom tyrosinase was determined, as shown in Fig. 2, indicating paeonol and its analogues inhibited tyrosinase in a dose-dependent manner. The IC₅₀ values were listed in

Fig. 2. Dose-dependent inhibitory effects of paeonol analogues on the diphenolase activity of mushroom tyrosinase.

Table 1. Inhibitory effects and constants of the paeonol analogues on mushroom tyrosinase

Compd	IC ₅₀ (mM)	Inhibition type	Inhibition constants (mM)	
			K _I	K _{IS}
a	0.207	mixed	0.201	1.447
b	0.916	non-competitive	0.686	0.686
c	3.096	competitive	1.574	
d	3.820	competitive	2.265	
e	1.248	competitive	0.826	
f	9.278	non-competitive	6.358	6.358
g	1.674	non-competitive	0.994	0.994

Table 1 for comparison. Among them, compound (a) had the most potent inhibition activity of mushroom tyrosinase with the IC₅₀ value of 0.21 mM, and the inhibitory strength of *p*-methoxy acetophenone was about 6 times stronger than that of *o*-methoxy acetophenone.

It can be speculated that acetophenone exhibited tyrosinase inhibitory effect due to the formation of a Schiff base with the primary amino group of tyrosinase as aromatic aldehydes. Compound (a) showed the strongest inhibitory effect due to the introduction of an electron donor methoxy group with high hydrophobicity at the *para*-position stabilizing the Schiff base and the *ortho* hydroxyl group forming a quasi

Fig. 3. Effects of concentrations of tyrosinase on its activity for the catalysis of L-DOPA at different concentrations of paeonol (a). Concentrations of paeonol for curves 0–4 were 0, 0.05, 0.1, 0.2, and 0.4 mM.

six-member ring through intramolecular hydrogen bonding to produce a more stable structure (Chen *et al.*, 2003). It has been suggested that the more stable the Schiff base is, the more potent the inhibitory activity. The more hydrophobic the electron donor group at the *para* position, the stronger the inhibition. Furthermore, the hydroxyl group of paeonol is able to chelate copper ions in the active site of tyrosinase. Therefore, replacements of *o*-hydroxy and *p*-methoxy moieties of acetophenone are more appreciable for the activity enhancement. Phenolic compounds (c), (d), and (e) did not serve as substrates, they cannot be oxidized by tyrosinase.

The inhibition mechanism of compounds (a) – (g) against the activity of mushroom tyrosinase The inhibition mechanism of compound (a) on mushroom tyrosinase for the oxidation of L-DOPA was shown in Fig. 3. The results showed straight lines passing through the origin, which indicate a reversible inhibition, the other inhibitors (b) – (g) showed the same behavior. Tyrosinase activity was inhibited, but the amount of efficient enzyme was not decreased in the presence of these inhibitors. The results showed that the catalytic rate did not make any change during the variation of the concentration. The inhibitors appeared to bind tyrosinase very quickly and did not induce further conformational change in the protein.

The inhibition pattern and constants of compounds (a) – (g) on the activity of mushroom tyrosinase The inhibition kinetics of the enzyme for oxidation of L-DOPA by selected compounds (a), (e), and (b) were illustrated in Fig. 4 – 6, respectively. The Lineweaver-Burk plots in Fig. 4 gave a family of lines intersected in the second quadrant, indicating that (a) was a mixed-type inhibitor. The constant for inhibitor binding with the free enzyme (K_I) was obtained from a plot of the slopes of the straight lines versus the inhibitor concentrations; the constant for inhibitor binding with the enzyme-

Fig. 4. Lineweaver-Burk plots of the inhibition by paeonol (a) of the mushroom tyrosinase mediated catalysis of L-DOPA (A). The inset represents the second plot of the intercept (B) and the slope (C) versus the concentrations of inhibitor to determine the inhibition constant K_i and K_{is} . The concentrations of paeonol (a) for curves 0 – 4 were 0, 0.1, 0.2, 0.3 and 0.4 mM, respectively.

Fig. 5. Lineweaver-Burk plots of mushroom tyrosinase on the presence of 2,4'-dihydroxy acetophenone (e) during the oxidation of L-DOPA (A). The inset represents the second plot of the K_m (B) versus the concentrations of inhibitor to determine the inhibition constant. The concentrations of 2,4'-dihydroxy acetophenone (e) for curves 0 – 4 were 0, 0.15, 0.3, 0.6, and 1.2 mM, respectively.

Fig. 6. Lineweaver-Burk plots of mushroom tyrosinase for inhibition of acetophenone (b) on the oxidation of L-DOPA (A). The inset represents the second plot of the Intercept (B) versus the concentrations of inhibitor to determine the inhibition constant. The concentrations of acetophenone (b) for curves 0 – 4 were 0, 0.16, 0.32, 0.48, and 0.64 mM, respectively.

substrate complex (K_{is}) was gained from a plot of the vertical intercepts versus the inhibitor concentrations, which are linearly fitted, showing that compound (a) has a single inhibition site or a single class of inhibition site on tyrosinase (Si *et al.*, 2011), as shown in the inset. The inhibition constants are summarized in Table 1 for comparison. Because $K_{is} > K_i$, the affinity of paeonol with the free enzyme is greater than that of the inhibitor with the enzyme-substrate complex.

The inhibitory type of (c), (d), and (e) on the enzyme activity shared the same. Lineweaver-Burk plots of the inhibition kinetics of tyrosinase for selected compound (e) is shown in Fig. 5, as the concentration of compound (e) increased, the K_m for tyrosinase increased in a dose-dependent manner without changing V_{max} , which indicates compound (e) as a competitive inhibitor of tyrosinase. The result showed that these inhibitors could only bind with free enzyme. The inhibition constant for the inhibitor (K_i), was obtained from a plot of the intercept versus the inhibitor concentration, as shown in the inset.

Similarly, The inhibitory type of (b), (f), and (g) on the enzyme activity shared the same. The Lineweaver-Burk plots of compound (b) shown in Fig. 6, gave a family of straight lines, with different slopes but with a common intercept on the $1/[s]$ axis, indicating that they are non-competitive inhibitors. The behaviour observed showed that the inhibitor could combine with both the free enzyme and the enzyme-substrate complex. The inhibition constants (K_i and K_{is}) can be obtained from a plot of the vertical intercept versus the inhibitor concentration as showed in Table 1.

It is evident to summarize that the above compounds with methoxy exhibited non-competitive inhibition type, showed competitive inhibition with mono- or dihydroxy, displayed mixed-type inhibition when both methoxy and hydroxyl existed.

The copper ions chelation ability Many substances have been reported to act as an tyrosinase inhibitor due to their innate function in chelating metal ions, maybe as a chelator to the copper ions in the tyrosinase (Kim *et al.*, 2006; Khatib *et al.*, 2005; Zhu *et al.*, 2009). In order to explore and illustrate the underlying mechanism better, the direct interactions between the inhibitors and the copper ions were assessed routinely by UV-2450 spectrophotometer at pH 6.8. The effect of the increments of the CuSO_4 concentration on the spectral characteristics of each compound is measured in terms of red shifts/blue, as a result of complex formation between the copper ions and the tested compounds. A solution of each compound (a) – (g) (25 μM) in PBS buffer was scanned with 0, 10, 25, 50, or 100 μM CuSO_4 , and the spectra obtained were compared.

The characteristic maximum at 276.5 nm of compound

Fig. 7. Copper ions chelating ability. (A) and (B) are the UV-vis absorption spectra of 4-hydroxy acetophenone (d) and acetophenone (b) at phosphate buffer (50 mM, pH 6.8) after the addition of different concentrations of copper ions, respectively.

(d) showed various significant blue shifts peaks with the addition of different concentrations of copper ions, shifting to 268.5 nm after added 100 μM CuSO_4 (Fig. 7A). Similar results were obtained with compounds (a), (c), and (e), with the addition of copper ions, the maximum absorbing wavelengths λ_{max} were shifted from 311 to 308 nm, from 322.5 to 316 nm and from 321.5 to 314 nm, respectively. However, it is obvious that the characteristic values of compounds (b), (f), and (g) were almost unchanged, as shown in Fig. 7B.

As such, it is concluded that the characteristic maximum absorbing wavelengths of hydroxy substituted acetophenone showed different significant blue shifts while that of methoxy substituted acetophenone didn't make any change. This could explain that the hydroxy compounds (c), (d), and (e) exhibited competitive inhibition by forming a chelate with the copper ions at the catalytic domain of the tyrosinase to prevent L-DOPA binding to the active sites of tyrosinase.

DPPH radical-scavenging activities Tyrosinase is a kind of oxidase, one of the reactions catalyzed by the tyrosinase is the oxidation of L-dopa to L-dopaquinone by using molecular oxygen. The reducers which have strong radical-scavenging activity are all potent inhibitors of tyrosinase. The mechanism is that inhibitors cleared the peroxy radicals, terminated radical chain initiation, and weakened the role of

Fig. 8. DPPH radical-scavenging activities of compounds a-g with an identical final concentration of 200 μM .

oxygen of tyrosinase, which weakened the role of tyrosinase (Chen *et al.*, 2012). Therefore, free radical scavenging compounds could also be used as a cosmetic ingredient to relieve skin aging (Tanaka *et al.*, 2004).

The DPPH radical-scavenging assay was chosen to examine antioxidative properties of compounds (a) – (g). All the compounds were tested at 200 μM final concentration. The results are shown in Fig. 8. In general, among these compounds, paeonol displayed the most potent inhibitory activities; (b), (f), and (g) which contain no hydroxyl group approximately showed no inhibitory activity; Monohydroxyl compounds (c) and (d) exhibited certain inhibitory activities, which was weaker than that of dihydroxyl compounds (e). In contrast to the result of tyrosinase, numbers and position of phenolic hydroxyl groups and methoxy substitutes in acetophenone showed major effect on their inhibitory activities. It has been confirmed that these substance not only acted as tyrosinase inhibitors but also provided antioxidant activity.

In this paper, the effects of paeonol and its analogues on the oxidation of L-DOPA by mushroom tyrosinase were studied. These analogues have been found having remarkable inhibition on mushroom tyrosinase and the inhibition was displayed as reversible. Despite the structural similarities, the inhibition mechanisms and inhibitory effects of the seven compounds are different from each other to some extent. Replacement of *o*-hydroxy and *p*-methoxy moieties of acetophenone is more favorable for the activity enhancement. The kind, the number, and the position of the functional group on the benzene ring group played an important role in the enzyme inhibition activity. The inhibition kinetic of these compounds revealed that the inhibition type was induced by the kind of substitutions at ortho or para position. Previous studies have recognized an apparently potent inhibitory effect of compounds with hydroxyl groups on tyrosinase (Yan *et al.*, 2009; Yamazaki *et al.*, 2009). The hydroxyl groups in compounds carry out an attack on the coppers of tyrosinase

active site, competing with substrate L-DOPA in a complex manner (Munoz-Munoz *et al.*, 2008). Copper ions chelation assay could be used to explain that the hydroxy compounds (c), (d), and (e) exhibited competitive inhibition by forming a chelate with the copper ions at the catalytic domain of the tyrosinase to inhibit the tyrosinase. In conclusion, these results suggest that they may serve as a potential agent for the prevention and treatment of pigmentation and the inhibition of melanogenesis.

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