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Inhibitory effects of α-cyano-4-hydroxycinnamic acid on the activity of mushroom tyrosinase

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ABSTRACT

The effects of α-cyano-4-hydroxycinnamic acid (HCCA) on the activity of mushroom tyrosinase have been studied. Results showed that HCCA could inhibit both the monophenolase activity and diphenolase activity of mushroom tyrosinase. For the monophenolase activity, the lag phase was obviously lengthened, and the steady-state activity of the enzyme decreased sharply. When the concentration of HCCA reached to 80 μM, the lag time was lengthened from 20 s to 150 s and the steady-state activity was lost by about 75%. The IC_{50} value was estimated to be 48 μM. For the diphenolase activity, the inhibitory effect of HCCA was also dose-dependent and the IC_{50} value was estimated to be 2.17 mM. The kinetic analyses showed that the inhibition of HCCA on the diphenolase activity was reversible and competitive with the inhibition constants (K_I) determined to be 1.24 mM.

1. Introduction

Tyrosinase (EC 1.14.18.1), a copper containing enzyme, can cause enzymatic browning in some vegetables and fruits. It catalyzes the hydroxylation of monophenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity), both depending on molecular oxygen (Martynez & Whitaker, 1995; Prota, 1988). *o*-Quinones evolve nonenzymatically to yield several unstable intermediates, which then polymerize to render melanin. The browning is an undesirable reaction that is responsible for less attractive appearance and loss in nutritional quality, and therefore becomes a major problem in the food industry and one of the main causes of quality loss during post-harvest handling and processing. The degree of browning among different fruits and vegetables is variable because of differences in phenolic content and tyrosinase activity. Therefore, the control of the tyrosinase activity is of importance in preventing the synthesis of melanin in the browning of mushrooms and other vegetables and fruits.

Tyrosinase inhibitors have recently attracted a lot of interest (Friedman, 1996) due to decrease of the hyper pigmentation resulting from the enzyme action. Hence, tyrosinase inhibitors are supposed to have broad applications in medicinal (Mosher, Pathak, & Fitzpatrick, 1983) and cosmetic (Maeda & Fukuda, 1991) products. Many efforts have been spent in the search for feasible and effective tyrosinase inhibitors. There is a concerted effort to search for naturally occurring tyrosinase inhibitors from plants, because plants constitute a rich source of bioactive chemicals and many of them are largely free from harmful adverse effects (Kubo, Yokokawa, & Kinst-Hori, 1995; Lee & Lee, 1997), but their individual activity is not sufficiently potent to be of practical use, which limits their in vivo use, so laboratory synthesis (Kubo et al., 2000) has been applied to resolve the problem.

It is well known that tyrosinase can be inhibited by aromatic aldehydes (Jiménez, Chazarra, Escribano, Cabanes, & Garcia-Carmona, 2001) and aromatic acids (Robit, Rouch, & Cadet, 1997), tropolone (Valero, Garcia-Moreno, Varon, & Garcia-Carmona, 1991) and kojic acid (Cananes, Chazarra, & Garcı′a-Carmona, 1994). Our front-stage work had found several groups of effective inhibitors: cinnamic acids (Shi, Chen, Wang, Song, & Qiu, 2005), benzoic acids (Huang et al., 2006; Xie, Song, Qiu, Huang, & Chen, 2007), salicylic

Abbreviation: HCCA, α-cyano-4-hydroxycinnamic acid; DMSO, dimethyl sulfoxide; L-DOPA, L-3,4-dihydroxyphenylalanine; Tyr, L-tyrosine; IC_{50}, the inhibitor concentrations leading to 50% activity lost.

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acids (Han et al., 2008; Zhang, Chen, Song, & Xie, 2006), phloridzin dihydrate (Wang et al., 2007), 3,5-dihydroxystilbene (Song et al., 2006). In our continuing search for tyrosinase inhibitors, α-cyano-4-hydroxycinnamic acid (HCCA), a substrate analogue, was found to have obvious inhibitory effects on the monophenolase activity and the diphenolase activity of mushroom tyrosinase. The aim of the present paper is, therefore, to carry out a study of the inhibitory kinetics of this compound on the enzyme activity and to evaluate the kinetic parameters and the inhibition mechanisms in order to provide the basis for development novel effective tyrosinase inhibitors.

2. Materials and methods

2.1. Reagents

Mushroom tyrosinase (EC 1.14.18.1) was purchased from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), α-cyano-4-hydroxycinnamic acid (HCCA), L-tyrosine (Tyr) and L-3,4-dihydroxyphenylalanine (L-DOPA) were the products of Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade. The water used was re-distilled and ion-free.

2.2. Enzyme activity assay

Monophenolase and diphenolase activities of mushroom tyrosinase were determined as previously reported (Chen & Song, 2006) by following the increase in absorbance at 475 nm accompanying the oxidation of the substrates with a molar absorption coefficient of 3700 M^{-1} cm^{-1} for the product (Jiménez et al., 2001). One unit of enzymatic activity was defined as the amount of enzyme transforming 1 μM of L-DOPA or L-tyrosinase per minute. The monophenolase assay conditions were 3 ml reaction system containing 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8), 0.5 mM L-tyrosine, 33.3 μg/ml of enzyme. The diphenolase reaction media (3 ml) contained 0.5 mM L-DOPA in 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8); a portion of 100 μl of the natural enzyme (containing 20 μg) was used to assay the activity. The temperature was controlled at 30 °C. Absorption and kinetic measurements were carried out on a Beckman UV-650 spectrophotometer. The compositions of the assay media for monophenolase and diphenolase activities of tyrosinase are given in the legends of the figures. The reaction was started by addition of the enzyme.

2.3. Effects of inhibitors on the enzyme activity

Inhibitor, HCCA, was first dissolved in DMSO and used for the experiment after a 30-folds dilution. The final concentration of DMSO in the test solution was 3.33%. In this method, 0.1 ml of DMSO solution with different concentrations of the inhibitors, was first mixed with 2.8 ml of substrate solution contained 0.5 mM L-DOPA in 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8), then, a portion of 100 μl of enzyme solution (20 μg) was added to this blend and the residual activity determined. The final concentration of DMSO in the test solution was 3.3%. Controls, without inhibitor but containing 3.3% DMSO, were routinely carried out (Chen, Song, Qiu, Liu, & Huang, 2005). The extent of inhibition by the addition of the sample was expressed as the percentage necessary for 50% inhibition (IC_{50}).

2.4. Determination of the inhibition type and the inhibition constant

The inhibition type was assayed by the Lineweaver–Burk plot, and the inhibition constant was determined by the second plots of the apparent K_m/V_m or $1/V_m$ versus the concentration of the inhibitor (Zhang et al., 2006).

3. Results

3.1. Effect of HCCA on the monophenolase activity of mushroom tyrosinase

When the enzymatic oxidation reaction used L-tyrosine as substrate, a marked lag period, characteristic of monophenolase activity, was observed simultaneously with the appearance of the first stable product, dopachrome (Fig. 1). The system reached a constant rate (the steady-state rate) after the lag period, which was estimated by extrapolation of the curve to the abscissa (Xie, Chen, Huang, Liu, & Zhang, 2003). The inhibitory effects of the different concentrations of HCCA on the oxidation of Tyr by the enzyme were studied. The kinetics course of the oxidation of the substrate in the presence of different concentrations of HCCA is shown in Fig. 2. With increasing the concentration of HCCA, the lag period was increased exponentially. The lag period was estimated to be 20 s in the absence of this inhibitor, and extended to 150 s in the presence of 80 μM of HCCA, which lengthened about seven times. The

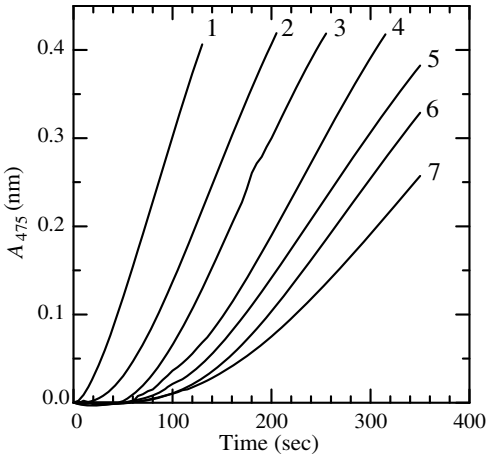

Fig. 1. Course of the oxidation of L-tyrosine by mushroom tyrosinase in the presence of different concentrations of HCCA. Concentration of HCCA for curves 1–7 was 0, 16, 32, 40, 48, 64 and 80 μM, respectively. The monophenolase activity assay conditions were 3 ml reaction system containing 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8), 0.5 mM L-tyrosine, 33.3 μg/ml of enzyme. The temperature was controlled at 30 °C.

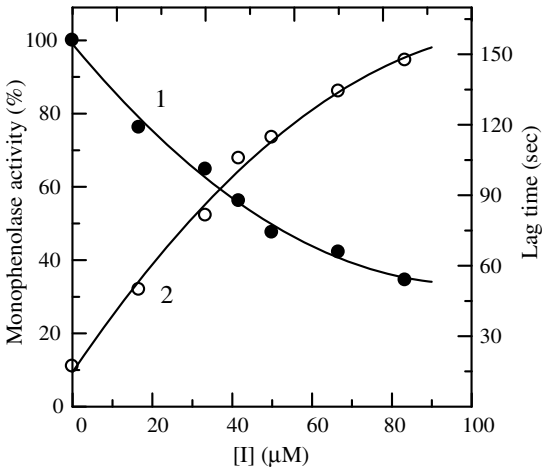

Fig. 2. Effect of HCCA on the steady-state rate (curve 1, ●) and the lag time (curve 2, ○) of mushroom tyrosinase for the oxidation of L-tyrosine. The activity assay conditions were the same as Fig. 1.

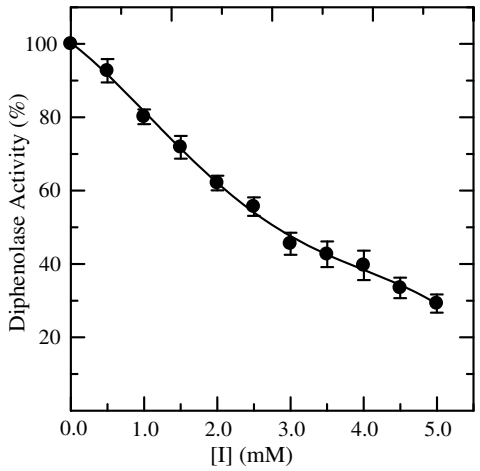

Fig. 3. Effects of HCCA on the diphenolase activity of mushroom tyrosinase. The diphenolase reaction media (3 ml) contained 0.5 mM L-DOPA in 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8). The enzyme concentration was 6.67 μg/ml. The temperature was controlled at 30 °C.

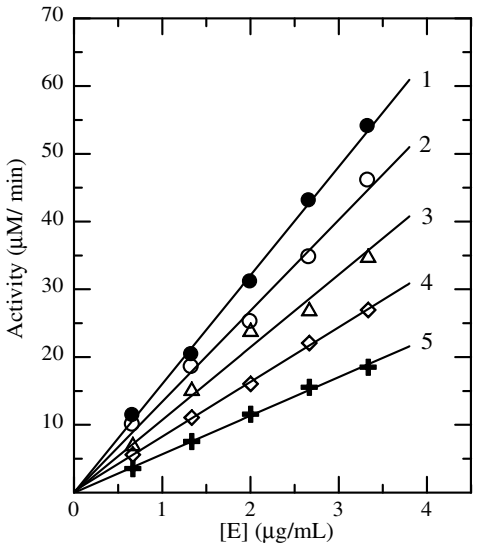

Fig. 4. The effect of concentrations of mushroom tyrosinase on its activity for the oxidation of L-DOPA. The activity assay conditions were the same as Fig. 3. The concentrations of HCCA for lines 1–5 were 0 (●), 1.25 (○), 2.50 (△), 3.75 (◇), 5.00 (+) mM, respectively.

steady-state rate decreased with increasing the concentration of HCCA. When the concentration of this inhibitor reached 80 μM, the remaining enzyme activity was determined to be 35%. The inhibitor concentration leading to 50% activity lost (IC_{50}) was estimated to be 48 μM. The results indicated that HCCA inhibited the monophenolase activity of mushroom tyrosinase obviously.

3.2. Effect of HCCA on the diphenolase activity of mushroom tyrosinase

We probed the effect of HCCA on the activity of mushroom tyrosinase for the oxidation of L-DOPA. When the diphenolase activity of tyrosinase was assayed by using L-DOPA as substrate, the reaction course immediately reached a steady-state rate. HCCA can inhibit the diphenolase activity of tyrosinase with dose-dependence as shown in Fig. 3. With increasing concentrations of HCCA, the remaining enzyme activity gently decreased. When the concentrations of HCCA were enhanced to 5.0 mM, the remaining activity of the enzyme was determined to be 29.1%. The inhibitor concentration leading to 50% activity lost (IC_{50}) was estimated to be 2.17 mM.

3.3. The inhibition mechanism of HCCA on the diphenolase activity of mushroom tyrosinase

The inhibition mechanism on mushroom tyrosinase by HCCA for the oxidation of L-DOPA was first studied. Fig. 4 shows the relationship between enzyme activity and its concentration in the presence of HCCA. The plots of the remaining enzyme activity versus the concentrations of enzyme at different inhibitor concentrations gave a family of straight lines, which all passed through the origin. Increase of inhibitor concentration resulted in a decrease of the slope of the line, indicating that the inhibition of HCCA on the enzyme was reversible.

3.4. Inhibition of HCCA on the diphenolase activity of mushroom tyrosinase following a competitive mechanism

The inhibitory type of HCCA on the diphenolase activity, during the oxidation of L-DOPA, was determined from Lineweaver–Burk double reciprocal plots. In the presence of HCCA, the kinetics of the enzyme is shown in Fig. 5. The plots of $1/v$ versus $1/[S]$ gave a family of straight lines with different slopes but they intersected one another in the Y-axis. The values of V_{max} remained the same

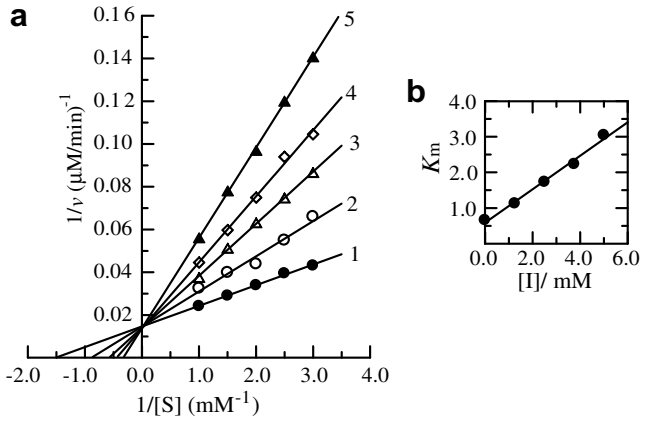

Fig. 5. Determination of the inhibitory type and inhibition constants of HCCA on the diphenolase activity of mushroom tyrosinase. (a) Lineweaver–Burk plots. The concentrations of HCCA for curves 1–5 were 0 (●), 1.25 (○), 2.50 (△), 3.75 (○) and 5.00 (▲) mM, respectively. (b) represents the secondary plot of K_m versus concentrations of HCCA to determine the inhibition constant.

and the value of K_m increased with increasing concentrations of the inhibitor, which indicates that HCCA is a competitive inhibitor. The result showed that HCCA could only bind with free enzyme. The inhibition constant for the inhibitor binding with the free enzyme (E), K_I, was obtained from the secondary plot (Fig. 5b) as 1.24 mM.

4. Discussion

This paper reported the effect of HCCA (Fig. 6a) on the activity of mushroom tyrosinase. The results showed that HCCA had inhibitory effect on the monophenolase activity of the enzyme. It could delay the lag time from 20 s to 150 s at 80 μM, about 7.5 times. At the same time, the monophenolase activity decreased sharply. We used HCCA as the substrate to test the enzyme activity. HCCA (5 mM) was mixed with 33.3 μg/ml of enzyme for 10 min in the reaction system. The absorbency at 475 nm was kept the same and the colour did not change. The results showed that the enzyme

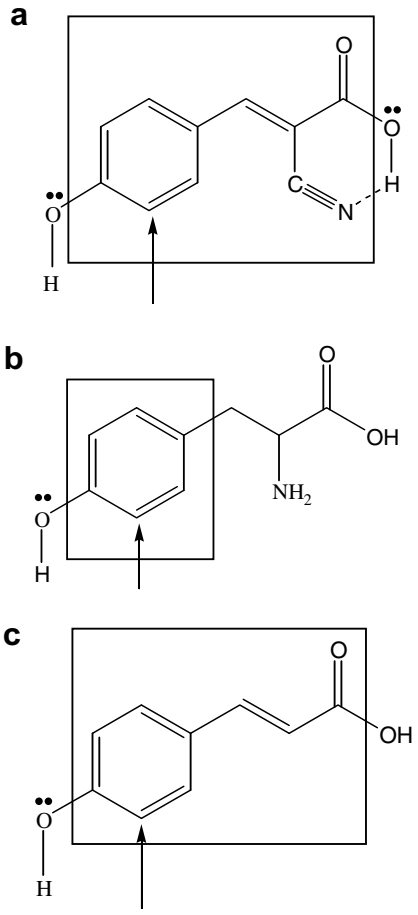

Fig. 6. Chemical structures of HCCA, (a) tyrosine (b) and 4-hydroxycinnamic acid (c).

cannot catalyze the oxidation reaction of HCCA. So HCCA could not be the substrate of tyrosinase. HCCA is an analogue of monophenolase substrate, L-tyrosine (Fig. 6b). 4-Hydroxy group could combine to the cuprum which is at the active center of tyrosinase (E_{oxy}), and therefore compete with L-tyrosine. Once HCCA connects with the cuprum, L-tyrosine cannot combine to the active site of the enzyme. So, HCCA can inhibit the monophenolase activity (Huang, Liu, & Chen, 2003). The ethylenic linkage strengthened the effect of competitive inhibition. Therefore, HCCA could effectively regulate the tyrosinase activity at the beginning of the approach which leads to brown or black pigments.

Arbutin (hydroquinone glucopyranoside) is one of inhibitors of tyrosinase, which has been used widely in the cosmetic industry. The IC_{50} value and K_I for the diphenolase activity were measured to be 5.30 and 2.98 mM, respectively (Song, Qiu, Huang, & Chen, 2003). In our research, the IC_{50} and K_I of HCCA were 2.17 and 1.24 mM. It indicated that HCCA could inhibit the diphenolase activity of tyrosinase at a lower concentration than that of arbutin. HCCA was more effective inhibitor of the diphenolase activity than arbutin. The inhibition of HCCA was reversible. Its inhibition type was determined to be competitive. According to the competitive inhibition, HCCA inhibits the diphenolase activity of tyrosinase by combining with the active site of the enzyme.

4-Hydroxycinnamic acid (Fig. 6c) was an inhibitor of tyrosinase (Shi et al., 2005). Its derivatives had similar effects on tyrosinase (Chen, Lin, & Song, 2007). L-Tyrosine, the monophenolase substrate, has a benzene ring. The six carbons on the benzene ring create the π-conjugated compound (marked by the circle of Fig. 6b). Tyrosinase can catalyze the hydroxylation of the 3-site of the benzene ring, and turn tyrosine to L-3,4-dihydroxyphenylalanine. While α-cyano-4-hydroxycinnamic acid (HCCA) is a 12 elements large π-conjugated compound (marked by the circle of Fig. 6a) which is made up of a benzene ring, an ethylenic linkage, an α-cyano-group and a carboxyl group. This structure is quite stable and difficult to be hydroxylated at the 3-site of the benzene ring. So HCCA is considered to be an inhibitor, not a substrate. 4-Hydroxycinnamic acid is ten elements large π-conjugated compound (marked by the circle of Fig. 6c) which is made up of a benzene ring, a ethylenic linkage and a carboxyl group. This structure is also stable and difficult to be hydroxylated at the 3-site of the benzene ring. Because the π-conjugated framework of hydroxycinnamic acid is much smaller than that of HCCA, hydroxycinnamic acid has lesser steric hindrance to combine with the enzyme active site. So, hydroxycinnamic acid is a more potent inhibitor than HCCA. The presence of the cyano-group on the α-site of HCCA makes this compound difficult to bind to the active site of tyrosinase. HCCA is a 4-hydroxycinnamic acid analogue with a cyano-group on its α-site. The IC_{50} values of 4-hydroxycinnamic acid and HCCA for diphenolase activity were 0.50 and 2.17 mM, respectively. Due to the presence of the cyano-group and the ethylenic linkage, HCCA matched to the active site of diphenolase not as exactly as 4-hydroxycinnamic acid. The binding affinity of HCCA to the diphenolase was not as tight as 4-hydroxycinnamic acid's. So the inhibition of HCCA on the diphenolase was weaker than 4-hydroxycinnamic acid. The inhibition types of 4-hydroxycinnamic acid and HCCA on the diphenolase activity of tyrosinase were the same, and both are competitive inhibitors. The results indicated that the 4-hydroxy group was connected with the inhibition mechanism.

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