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Inhibitory effects of cinnamic acid and its derivatives on the diphenolase activity of mushroom (*Agaricus bisporus*) tyrosinase

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Abstract

The effects of cinnamic acid and its derivatives (2-hydroxycinnamic acid, 4-hydroxycinnamic acid and 4-methoxycinnamic acid) on the activity of mushroom tyrosinase have been studied. Results showed that cinnamic acid, 4-hydroxycinnamic acid and 4-methoxycinnamic acid strongly inhibited the diphenolase activity of mushroom tyrosinase and the inhibition was reversible. The IC_{50} values were estimated to be 2.10, 0.50 and 0.42 mM, respectively. 2-Hydroxycinnamic acid had no inhibitory effect on the diphenolase activity of the enzyme. Kinetic analyses showed that the inhibition type of cinnamic acid and 4-methoxycinnamic acid was noncompetitive with the constants (K_I) determined to be 1.994 and 0.458 mM, respectively. The inhibition type of 4-hydroxycinnamic acid was competitive, with the inhibition constant (K_I) was 0.244 mM.

Keywords: Mushroom tyrosinase; Diphenolase activity; Cinnamic acid and its derivatives; Inhibition kinetics

1. Introduction

Tyrosinase (EC 1.14.18.1) is a copper containing enzyme that is widely distributed in microorganisms, animals, and plants. It can cause enzymatic browning in some vegetables and fruits. It catalyzes both the hydroxylation of monophenols and the oxidation of diphenol into quinones, which polymerize to form brown or black pigments (Martynez & Whitaker, 1995; Prota, 1988).

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

Abbreviations: DMSO, dimethyl sulfoxide; L-DOPA, L-3,4-dihydroxyphenylalanine; IC_{50}, the inhibitor concentrations leading to 50% activity lost.

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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 (Jimenez, 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, & García-Carmona, 1994). In our continuing search for tyrosinase inhibitors, the aim of the present paper is to carry out a kinetic study of the inhibition of the activity of mushroom tyrosinase by cinnamic acid and its derivatives that are obtained by laboratory synthesis.

2. Materials and methods

2.1. Reagents

Mushroom tyrosinase (EC 1.14.18.1), cinnamic acid **(a)**, 2-hydroxycinnamic acid **(b)**, 4-hydroxycinnamic acid **(c)** and 4-methoxycinnamic acid **(d)**, were purchased from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) 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

Diphenolase activity of mushroom tyrosinase assay was performed, as previously reported (Chen, Song, Qiu, Liu, & Huang, 2004). The 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 enzyme activity was determined by following the increase of optical density at 400 nm ($\varepsilon = 3700\ M^{-1}\ cm^{-1}$) (Jimenez et al., 2001) accompanying the oxidation of L-DOPA to dopachrome. The reaction was carried out at a constant temperature of 30 °C. A Beckman UV-650 spectrophotometer was used for absorbance and kinetic measurements.

2.3. Effects of inhibitors on the enzyme activity

The inhibitor was first dissolved in DMSO and used for the experiment after a 30-fold dilution. The final concentration of DMSO in the test solution was 3.3%. 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 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.

3. Results

3.1. Effect of cinnamic acid and its derivatives on the diphenolase activity of mushroom tyrosinase

Taking cinnamic acid **(a)**, 2-hydroxycinnamic acid **(b)**, 4-hydroxycinnamic acid **(c)** and 4-methoxycinnamic acid **(d)** (see Fig. 1 for structures) as the effectors, we probed the effects of these compounds on the activity of mushroom tyrosinase for the oxidation of L-DOPA. The results are shown in Fig. 2. With increasing concentrations of these inhibitors, the remaining enzyme activity rapidly decreased, except for compound **(b)** which couldnot inhibit the enzyme. When the concentrations of inhibitors were enhanced to 3.0 mM, the remaining activity of the enzyme was determined to be 42.1% (for compound **a**), 20.5% (for **c**) and 14.5% (for **d**). The inhibitor concentrations (IC_{50}) leading to 50% activity lost were estimated to be 2.10 mM (for **a**), 0.50 mM (for **c**) and 0.42 mM (for **d**), respectively. From Fig. 2, it is evident that 4-methoxy and 4-hydroxycinnamic acid were the most potent inhibitors of tyrosinase activity, and 2-hydroxycinnamic acid the weakest. However, the presence of the methoxy group at position 4 resulted in slightly greater inhibition than with the 4-hydroxycinnamic acid derivative.

3.2. The inhibition mechanism of cinnamic acid *(a)*, 4-hydroxycinnamic acid *(c)* and 4-methoxycinnamic acid *(d)* on the diphenolase activity of mushroom tyrosinase

The inhibition mechanisms on mushroom tyrosinase by cinnamic acid **(a)** and its derivatives **(c, d)** for the oxidation of L-DOPA were studied. Fig. 3 shows the relationship between enzyme activity and its concentration in the presence of **(a)**. 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 descent of the slope of the line,

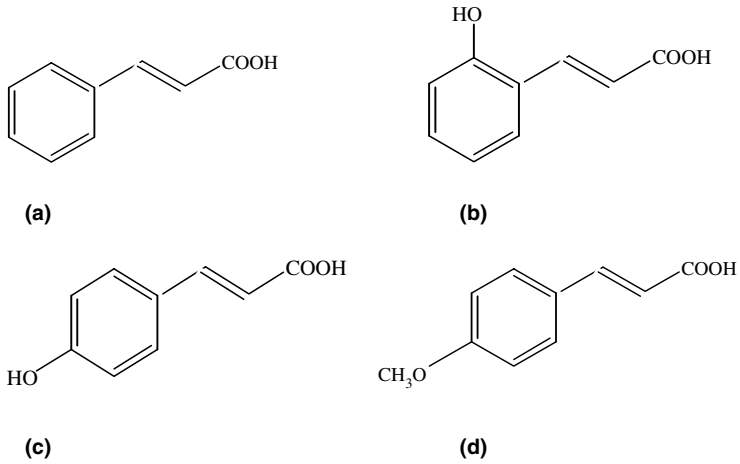

Fig. 1. Chemical structures of cinnamic acid and its derivatives (**a**: cinnamic acid; **b**: 2-hydroxycinnamic acid; **c**: 4-hydroxycinnamic acid; **d**: 4-methoxycinnamic acid).

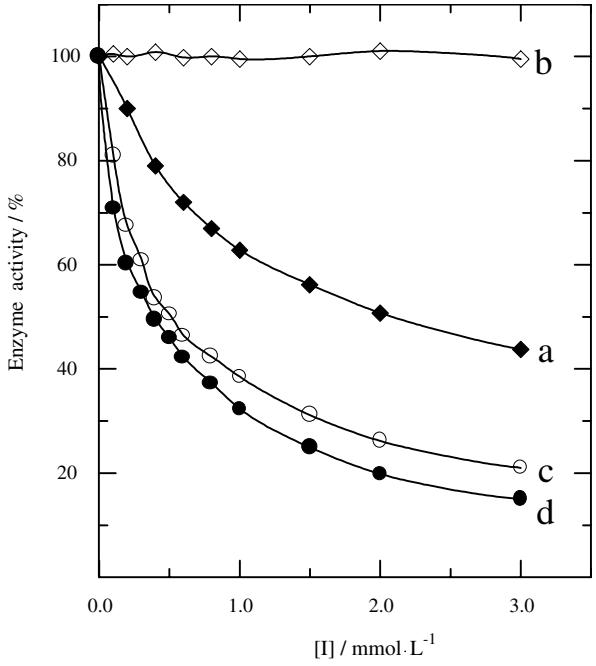

Fig. 2. Effects of cinnamic acid and its derivatives on the activity of mushroom tyrosinase (**a**: cinnamic acid; **b**: 2-hydroxycinnamic acid; **c**: 4-hydroxycinnamic acid; **d**: 4-methoxycinnamic acid).

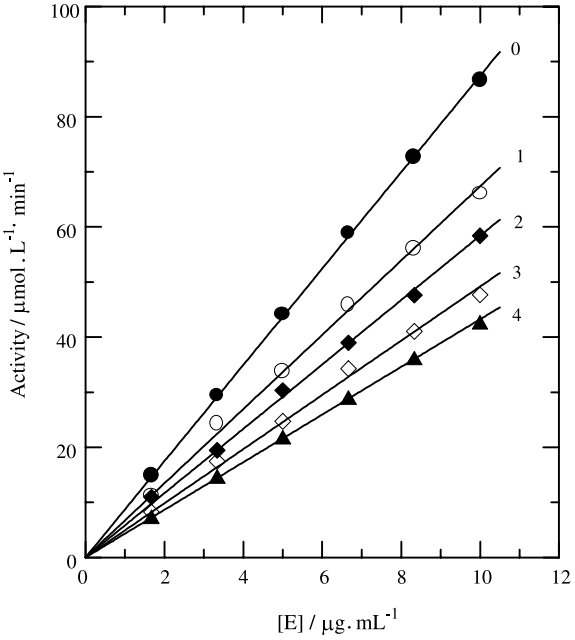

Fig. 3. The effect of concentrations of mushroom tyrosinase on its activity for the oxidation of L-DOPA at different concentrations of cinnamic acid **(a)**. The concentrations of **(a)** for curves 0–4 were 0, 0.1, 0.2, 0.3, 0.4 mM, respectively.

indicating that the inhibition of **(a)** on the enzyme was reversible. We also studied the inhibition mechanisms of **(c)** and **(d)**, as above. Results indicated that both **(c)** and **(d)** were reversible inhibitors of mushroom tyrosinase.

*3.3. Inhibition of cinnamic acid **(a)** and 4-methoxycinnamic acid **(d)** on the diphenolase activity of mushroom tyrosinase following a non-competitive mechanism*

The inhibitory mechanisms of cinnamic acid **(a)** and 4-methoxycinnamic acid **(d)** on mushroom tyrosinase, during the oxidation of DOPA, were determined from Lineweaver–Burk double reciprocal plots. Fig. 4 shows the double-reciprocal plots of the enzyme inhibited by compound **(a)**. The results showed that the plots of $1/v$ versus 1/[S] gave a family of straight lines with different slopes, but they intersected one another in the X-axis. Accompanying the enhancement of the inhibitor concentration, the values of V_{max} descended but the values of K_m remained the same, which indicates that compound **(a)** is a non-competitive inhibitor of the enzyme. This behaviour indicated that cinnamic acid could bind, not only with free enzyme, but also with

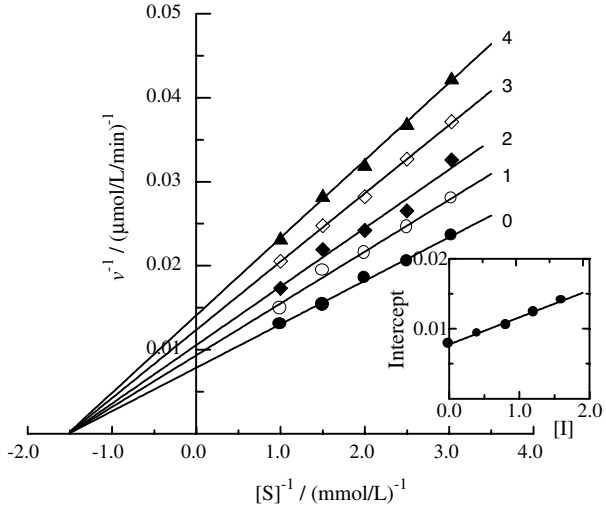

Fig. 4. Lineweaver–Burk plots for inhibition of cinnamic acid **(a)** on mushroom tyrosinase for the catalysis of L-DOPA. Concentrations of **(a)** for curves 1–5 were 0, 0.4, 0.8, 1.2 and 1.6 mM, respectively. The inset represents the secondary plot of $1/V_{max}$ versus concentrations of **(a)** to determine the inhibition constant.

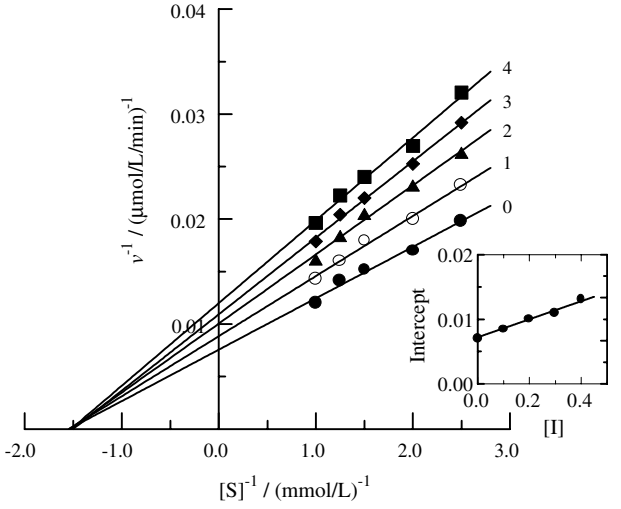

Fig. 5. Lineweaver–Burk plots for inhibition of 4-methoxycinnamic acid **(d)** on mushroom tyrosinase for the catalysis of L-DOPA. Concentrations of **(d)** for curves 1–5 were 0, 0.1, 0.2, 0.3 and 0.4 mM, respectively. The inset represents the secondary plot of $1/V_{max}$ versus concentrations of **(d)** to determine the inhibition constant.

the enzyme–substrate complex, and their equilibrium constants are the same. The inhibition constants for the inhibitor binding with the free enzyme (K_I) and enzyme–substrate complex (K_{IS}) were determined by the plot of the values of $1/V_{max}$ versus the inhibitor concentrations (inset in Fig. 4), and the values obtained are summarized in Table 1. Similar results were obtained with compound of **(d)** (Fig. 5). It is also a non-competitive inhibitor of the enzyme, and the inhibition constants (K_I and K_{IS}) were determined by the same methods and results also summarized in Table 1 for comparison.

3.4. Inhibition of 4-hydroxycinnamic acid (c) on the diphenolase activity of mushroom tyrosinase, following a competitive mechanism

The inhibitory mechanism of 4-hydroxycinnamic acid **(c)** on mushroom tyrosinase, during the oxidation of DOPA, was determined by the same methods. In the presence of **(c)**, the kinetics of the enzyme, by double-reciprocal plots, are shown in Fig. 6. 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 and the values of K_m increased with increasing concentrations of the inhibitor, which indicates that 4-hydroxycinnamic acid is a competitive inhibitor. The result showed that 4-

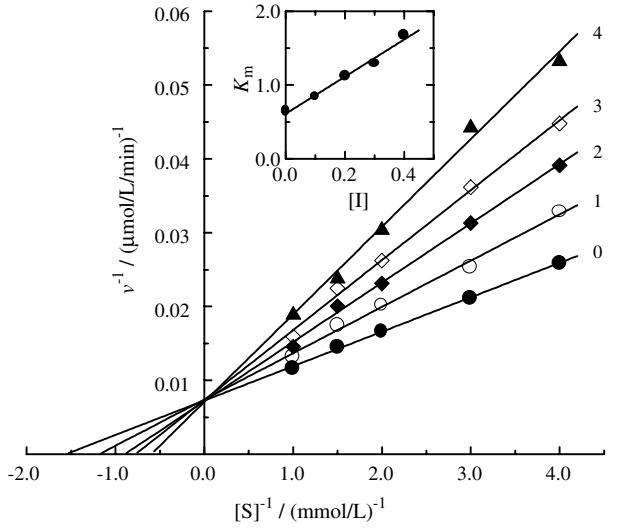

Fig. 6. Lineweaver–Burk plots for inhibition of 4-hydroxycinnamic acid **(c)** on mushroom tyrosinase for the catalysis of L-DOPA. Concentration of **(c)** for curves 1–5 were 0, 0.1, 0.2, 0.3 and 0.4 mM, respectively. The inset represents the secondary plot of $1/V_{max}$ versus concentrations of **(c)** to determine the inhibition constant.

Table 1
Kinetics and inhibition constants of cinnamic acid and its derivatives on the activity of mushroom tyrosinase

	IC_{50} (mM)	Inhibition type	Inhibition constant (K_I) (mM)
Cinnamic acid	2.10	Non-competitive	1.99
2-Hydroxycinnamic acid	Not effective	Not effective	Not effective
4-Hydroxycinnamic acid	0.50	Competitive	0.244
4-Methoxycinnamic acid	0.42	Non-competitive	0.458

hydroxycinnamic acid 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. 6 inset). The values obtained are summarized in Table 1.

4. Discussion

This paper reports the effects of cinnamic acid and its derivatives on the diphenolase activity of mushroom tyrosinase for the oxidation of L-DOPA. The results show that **(a)**, **(c)** and **(d)** had inhibitory effects on the activity of the enzyme, while **(b)** had no inhibitory effects. The inhibitions of **(a)**, **(c)** and **(d)** were reversible. The inhibition types of **(a)** and **(d)** were determined to be non-competitive, while **(c)** was competitive. Cinnamic acid **(a)** had an inhibitory effect on the activity of the enzyme, but 2-hydroxycinnamic acid **(b)** had no inhibitory effect; the reason was probably that 2-hydroxycinnamic acid **(b)** had a 2-hydroxy group which could cause steric hindrance. 4-Hydroxycinnamic acid **(c)** had a competitive inhibitory effect because it was a substrate analogue.

Arbutin is one of inhibitors of tyrosinase, which has been used widely in the cosmetic industry. The IC_{50} and K_I of arbutin were 5.30 and 2.98 mM, respectively (Song, Qiu, Huang, & Chen, 2003). Arbutin is 4-hydroxybenzyl-β-D-glucoside. Arbutin is converted to 4-hydroxycinnamic acid when the glucoside of arbutin is replaced by a crylic acid. Our results in this paper showed that IC_{50} and K_I values of 4-hydroxycinnamic acid were 0.50 and 0.244 mM, respectively. The inhibitory types of these two compounds were competitive type. It is suggested that 4-hydroxycinnamic acid was a stronger inhibitor than arbutin, and that the inhibitory mechanism of these two compounds was decided by the 4-hydroxybenzyl group, while the inhibitory extent was decided by a substitute group.

Liu, Huang, and Chen (2003) reported that benzoic acid had inhibition effects on the diphenolase activity of mushroom tyrosinase, and Kubo and Kinst-Hori (1998) reported that *p*-methoxybenzoic acid had inhibitory effects on the diphenolase activity of mushroom tyrosinase. Huang, Liu, and Chen (2003) reported that benzaldehyde family compounds had inhibitory effects on the diphenolase activity of mushroom tyrosinase. The inhibitory types of these compounds are listed in Table 2. We found that these nine compounds could be classified into three types according to their structures. The first type includes cinnamic acid, benzaldehyde and benzoic acid which all have a phenyl groups. And the first type can be transformed into the second type if the hydrogen of the former is replaced by a hydroxyl in the *para*-position. Likewise, the third type can be transformed to the first type if the hydrogen of the latter is replaced by a methoxy group in the *para*-position. We also found that the inhibitory types of these three compounds were the same. The inhibitory types of the three types were non-competitive, competitive and non-competitive, respectively. These results suggest that the inhibitory effects of these nine compounds were determined by the phenyl and *para*-position substituted groups. The type with a 4-hydroxybenzene structure is an analogue of the monophenol substrate, which can compete with the substrate at the active site. Walker and Wilson (1975) suggested the existence of two distinct sites on the enzyme: one side for the binding of the substrate and another, adjacent site for binding to the inhibitor. The result for the inhibitory type of the first and third type led us to hypothesize that these six inhibitors were attached to a site different from the active site and hindered the binding of substrate to the enzyme through steric hindrance or by changing the protein conformation.

Table 2
Comparison of the inhibition type of some compounds on the diphenolase activity of mushroom tyrosinase

Compounds	Type
Cinnamic acid	Non-competitive
Benzaldehyde	Non-competitive[a]
Benzoic acid	Non-competitive[b]
4-Hydroxycinnamic acid	Competitive
4-Hydroxybenzaldehyde	Competitive[a]
4-Hydroxybenzoic acid	Competitive[c]
4-Methoxycinnamic acid	Non-competitive
4-Methoxybenzaldehyde	Non-competitive[a]
4-Methoxybenzoic acid	Non-competitive[c]

[a] According to Huang et al. (2003).
[b] Liu et al. (2003).
[c] According to Chen et al. (2004).

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