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Inhibitory Effects of Methyl *trans*-Cinnamate on Mushroom Tyrosinase and Its Antimicrobial Activities

QIAN-SHENG HUANG,[†] YU-JING ZHU,[†] HUA-LIANG LI,[†] JIANG-XING ZHUANG,[†] CHUN-LE ZHANG,[†] JING-JING ZHOU,[†] WEN-GANG LI,*[,‡] AND QING-XI CHEN*[,†,§]

Key Laboratory of Ministry of Education for Cell Biology and Tumor Cell Engineering, School of Life Sciences, Xiamen University, Xiamen 361005, China, Xiamen First Hospital attached to Fujian Medical University, Xiamen, 361004, China, and Key Laboratory for Chemical Biology of Fujian Province, Xiamen University, Xiamen 361005, China

The control of food browning and growth of disease-causing microorganisms is critical to maintaining the quality and safety of food. Tyrosinase is the key enzyme in food browning. The inhibitory effect of methyl *trans*-cinnamate on the activity of tyrosinase has been investigated. Methyl *trans*-cinnamate can strongly inhibit both monophenolase and diphenolase activity of mushroom tyrosinase. When the concentration of methyl *trans*-cinnamate reached 2.5 mM, the lag time was lengthened from 32 to 160 s and the steady-state activity was lost about 65%. The IC_{50} value was 1.25 mM. For the diphenolase activity, the inhibition of methyl *trans*-cinnamate displayed a reversible and noncompetitive mechanism. The IC_{50} value was 1.62 mM, and the inhibition constant (K_I) was determined to be 1.60 mM. Moreover, the antibacterial activity against *Escherichia coli, Bacillus subtilis and Staphyloccocus aureus* and antifungal activity against *Candida albicans* were tested. The results showed that methyl *trans*-cinnamate possessed an antimicrobial ability.

KEYWORDS: Tyrosinase; methyl *trans*-cinnamate; monophenolase activity; diphenolase activity; antimicrobial activity

INTRODUCTION

In most foods, including fruits, vegetables and some seafoods (crustacea), the discoloration and browning process has two components: enzymatic and nonenzymatic oxidation. The enzymatic oxidation is considered to be deleterious to the color quality of foods, especially to bruised or cut fruits and vegetables. This unfavorable darkening from enzymatic oxidation has been of great concern (*1*). The enzymatic oxidation is mainly caused by tyrosinase (EC 1.14.18.1) and is usually initiated by the enzymatic oxidation of monophenols into *o*-diphenols and *o*-diphenols into quinones, which undergo further nonenzymatic polymerization leading to the formation of pigments. Thus, tyrosinase inhibitors could be useful as antibrowning agents. Several inhibitors of tyrosinase have been used, mainly benzoic acids and their derivatives. Diamine derivatives of coumarin and 4-hexylresorcinol are effective inhibitors of black-spot formation in shrimp (*2*).

We have reported some tyrosinase inhibitors, such as cetylpyridinium chloride (*3*), Cupferron (*4*), flavonoids (*5*), hexylresorcinol and dodecylresorcinol (*6*), alkylbenzaldehydes (*7*), fluorobenzaldehydes (*8*), salicylic acid family (*9*) and some *o*-diphenols which are the substrate analogues (*10*). All of these compounds showed inhibitory effects on the activity of tyrosinase.

Incidences of foodborne illnesses are still a major problem. An estimated 76 million people contract foodborne illnesses each year in the United States; the estimated costs related to foodborne diseases in the United States is between $10 billion and $83 billion annually (data from United State Food and Drug Administration). Microbes are the main pathogens of foodborne illnesses. Microbial contamination of food is a major concern for the food industry, regulatory agencies and consumers. Therefore, it is of great importance to search for compounds which possess antimicrobial activity for food preservation.

The control of food browning and growth of disease-causing microorganisms is critical to maintaining the quality and safety of food. However, the incompatibility between the use of commercially available browning inhibitors (reducing agent-based) and conventional antimicrobial treatments (oxidant-based) presents a major technical challenge to the industry. Sulfites have long been used as a low cost

* Author for corresponding. Tel/fax: +86 592 2185487. E-mail address: chenqx@xmu.edu.cn (Q.-X.C.); lwg11861@163.com (W.-G.L.).

[†] Key Laboratory of Ministry of Education for Cell Biology and Tumor Cell Engineering, School of Life Sciences.

[‡] Xiamen First Hospital attached to Fujian Medical University.

[§] Key Laboratory for Chemical Biology of Fujian Province, Xiamen University.

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additive in food products for their antibrowning and antimicrobial activity. However, their use in foods was banned by the USA Food and Drug Administration (FDA) in 1986 in view of their potential health risks to sensitive individuals (*11*).

A lot of tyrosinase inhibitors have been used commercially (*12*), but the safety regulation limits their applications. Methyl *trans*-cinnamate (Chemical Abstracts Service Registry Number, 103-26-4; European Inventory of Existing commercial Chemical Substances Number, 203-093-8) is a fragrance ingredient used in many fragrance compounds and decorative cosmetics. Different kinds of experiments have been done to confirm its safety, such as acute toxicity, skin irritation, mucus membrane irritation, skin sensitization. Its use worldwide is in the region of 10−100 metric tonnes per annum (*13*). Though methyl *trans*-cinnamate is widely used as fragrance, there is no report about its inhibitory mechanism on tyrosinase and its antimicrobial ability.

This article studies the inhibition of methyl *trans*-cinnamate on tyrosinase and its antibacterial, antifungal effects in order to present a potential use of methyl *trans*-cinnamate as an antibrowning and antimicrobial food additive.

MATERIALS AND METHODS

Reagents. Methyl *trans*-cinnamate, L-3,4-dihyroxyphenylalanine (L-DOPA), L-tyrosine (L-Tyr) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Mushroom tyrosinase (EC1.14.18.1) was also obtained from Sigma-Aldrich, with 6680 U/mg of its enzymatic activity. *Escherichia coli*, *Bacillus subtilis*, *Staphyloccocus aureus*, and *Candida albicans* were kept by our laboratory. All other reagents were local products of analytical grade. The water used was redistilled and ion-free.

Enzymatic Activity Assay. The enzymatic activity assay was performed as reported by Chen et al. (*6*). We used L-Tyr as the substrate for the monophenolase activity assay, and L-DOPA as the substrate for the diphenolase activity assay. The reaction media (3 mL) for activity assay contained 2.0 mM L-Tyr or 0.5 mM L-DOPA in 50 mM Na_2HPO_4−NaH_2PO_4 buffer (pH 6.8). The final concentration of mushroom tyrosinase was 33.3 μg/mL for the monophenolase activity and 6.67 μg/mL for the diphenolase activity. The reaction was carried out at a constant temperature of 30 °C. Methyl *trans*-cinnamate was first dissolved in DMSO and then added to the reaction media. The final concentration of DMSO in the test solution was 3.3%. Thus, 3.3% DMSO without inhibitor was used as control (*14*, *15*). All the above measurements were performed on a Bechman UV-650 spectrophotometer. The extent of inhibition by the addition of the sample was expressed as the percentage necessary for 50% inhibition (IC_{50}). The inhibition type was assayed by the Lineweaver−Burk plot, and the inhibition constant was determined by the second plot of the apparent K_m/V_m or $1/V_m$ versus the concentration of the inhibitor.

Antimicrobial Assay. The antimicrobial activity of methyl *trans*-cinnamate was evaluated against several bacterial species, including Gram-negative *E. coli*, Gram-positive *B. subtilis*, *St. aureus* and fungal species *C. albicans*. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were tested by broth macro dilution methods (*16*). Briefly, serial 2-fold dilutions of methyl *trans*-cinnamate were prepared in DMSO, and 30 μL of each dilution was added to 3 mL of the Luria−Bertani liquid medium with an inoculum of $1-2 \times 10^5$ cells/mL. After the cultures were incubated at 37 °C for 24 h, MIC was determined as the lowest concentration of the test compound that showed no visible growth. After the determination of the MIC, 100-fold dilutions with compound-free medium from each tube showing no turbidity were incubated at 37 °C for 48 h. The MBC was the lowest concentration of the test compound that showed no visible growth in the compound-free cultivation. *C. albicans* was cultured using potato dextrose agar (PDA) medium, at 28 °C with an inoculum of $1-2 \times 10^5$ colony forming units (CFU)/mL. Other processes were the same with bacteria. For the inhibitory effects of growth curve, bacteria were cultured in Luria−Bertani liquid medium, at 37 °C with an organism inoculum of $1-2 \times 10^5$ cells/mL. The inoculum was in the exponential phase. *C. albicans* was cultured using potato dextrose agar (PDA) medium, at 28 °C with an inoculum of $1-2 \times 10^5$ colony forming units (CFU)/mL. The inhibition of methyl *trans*-cinnamate on the growth curve of the microbes was determined as usual. Briefly, the microbes were cultured with different concentrations of methyl *trans*-cinnamate in the medium. The concentrations of methyl *trans*-cinnamate were chosen according to the MIC and MBC/MFC values. During the growth course, the number of viable cell numbers is measured over time and is plotted as a graph of the log10 viable cell numbers against time. The number of viable cell numbers was measured by spreading the liquid culture on the solid plate (*12*, *17*). 0, 2.5, 5, 8, 11, 16, and 24 h time points of the growth period were chosen; and for fungal *C. albicans*, the time was 0, 2, 6, 9, 18, 24, 32, 36 and 48 h. For each time point, the data was expressed as mean viability (log CFU/mL) ± SE ($n = 3$). DMSO, which methyl *trans*-cinnamate was dissolved in, was used as the negative control; and for the positive control, gentamycin sulfate (2000 U/mL) was used for the tested bacteria and K_2CrO_4 (1 mg/mL) was used for the tested fungi.

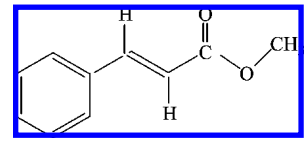

Figure 1. Chemical structure of methyl *trans*-cinnamate.

RESULTS

Effect of Methyl *trans*-Cinnamate on the Monophenolase Activity of Mushroom Tyrosinase. The tyrosinase can catalyze the hydroxylation of monophenols (monophenolase activity). Thus, 2.0 mM L-Tyr was used as a substrate to assay the effect of methyl *trans*-cinnamate on the monophenolase activity of mushroom tyrosinase. The effects of methyl *trans*-cinnamate (see **Figure 1** for structure) on the oxidation of L-Tyr by the enzyme were assayed at different concentrations. The kinetic courses of the oxidation of the substrate in the presence of methyl *trans*-cinnamate are shown in **Figure 2a**. A marked lag period, characteristic of monophenolase activity, was observed. Estimated by extrapolation of the linear portion of the product accumulation curve to the abscissa, the lag time increased with the increasing concentration of methyl *trans*-cinnamate as we can see from the curves. After the reaction system reached the steady state, the curve of product increased with increasing reaction time; the slope of the line denoted the steady-state rate. In the presence of different concentrations of methyl *trans*-cinnamate, the steady-state rate and the lag time were determined, and the results are showed in **Figure 2b,c**. The steady-state rate decreased distinctly. When the concentration of methyl *trans*-cinnamate increased from 0 to 2.5 mM, the remaining activity was 35% of the initial activity. The IC_{50} value for the monophenolase activity inhibited by methyl *trans*-cinnamate was 1.25 mM. The lag time was prolonged from 32 to 160 s with increasing inhibitor concentration. The above results indicate the strong inhibition of methyl *trans*-cinnamate for the monophenolase of tyrosinase.

Effects of Methyl *trans*-Cinnamate on the Diphenolase Activity of Mushroom Tyrosinase. Methyl *trans*-cinnamate was used as an effector on the activity of mushroom tyrosinase for the oxidation of L-DOPA. **Figure 3** showed that the enzyme activity markedly decreased with increasing concentra-

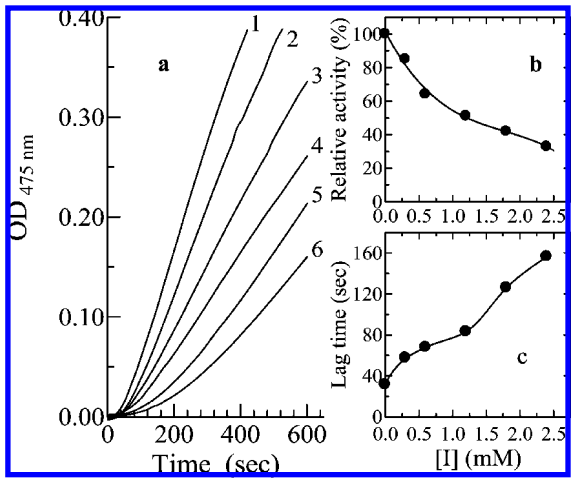

Figure 2. Progress curves for oxidation of L-Tyr by monophenolase of mushroom tyrosinase in the present of different concentrations of methyl *trans*-cinnamate (**a**), steady-state velocity of monophenolase activity of mushroom tyrosinase (**b**), and effect of methyl *trans*-cinnamate on the lag time for oxidation of Tyr (**c**). The concentrations of methyl *trans*-cinnamate for curves 1−6 were 0, 0.3, 0.6, 1.2, 1.8 and 2.4 mmol/L respectively.

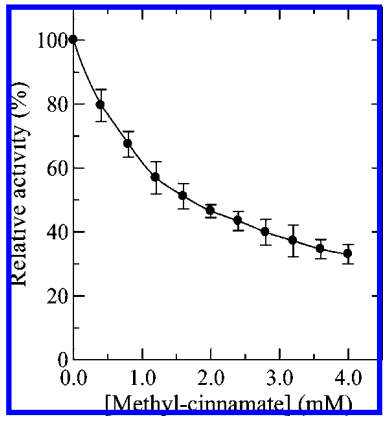

Figure 3. Effects of methyl *trans*-cinnamate on the activity of mushroom tyrosinase for the catalysis of L-DOPA at 30 °C. The reaction media (3 mL) for relative activity assay contained 0.5 mM L-DOPA in 50 mM Na_2HPO_4−NaH_2PO_4 buffer (pH 6.8), 6.67 μg/mL tyrosinase and different concentrations of methyl *trans*-cinnamate. The activity assayed without methyl *trans*-cinnamate was defined as 100% relative activity.

tion of the methyl *trans*-cinnamate. The IC_{50} value for the diphenolase activity inhibited by methyl *trans*-cinnamate was 1.62 mM.

The Inhibition Mechanism of Methyl *trans*-Cinnamate on Diphenolase Activity. The inhibition mechanism on the enzyme by methyl *trans*-cinnamate for oxidation of L-DOPA was investigated. The result is shown in **Figure 4**. The plots of the remaining enzyme activity versus the concentrations of enzyme in the presence of different concentrations of methyl *trans*-cinnamate gave a family of straight lines, which all passed through the origin, indicating that the inhibition of methyl *trans*-cinnamate on the diphenolase was a reversible reaction course. The presence of methyl *trans*-cinnamate did not bring down the amount of the efficient enzyme, but just resulted in the descending of the activity of the enzyme.

Methyl *trans*-Cinnamate Showed Noncompetitive Inhibitory Type on the Diphenolase Activity. Under the conditions employed in the present investigation, the oxidation reaction of L-DOPA by mushroom tyrosinase followed Michaelis−

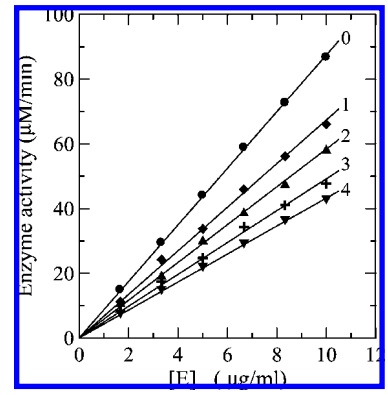

Figure 4. Determination of the inhibitory mechanism of methyl *trans*-cinnamate on mushroom tyrosinase. The concentrations of methyl *trans*-cinnamate for curves 0−4 were 0, 0.25, 0.50, 0.75, and 1.0 mmol/L, respectively.

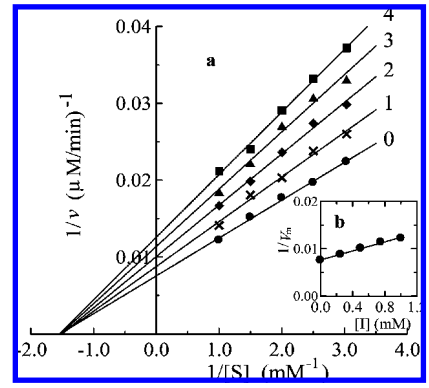

Figure 5. (**a**) Lineweaver−Burk plots for inhibition of methyl *trans*-cinnamate on mushroom tyrosinase for the catalysis of L-DOPA at 30 °C, pH 6.8. The concentrations of methyl *trans*-cinnamate for curves 0−4 were 0, 0.25, 0.50, 0.75 and 1.0 mmol/L respectively. The inset (**b**) represents the secondary plot of K_m versus concentration of methyl *trans*-cinnamate to determine the inhibition constant K_I.

Menten kinetics. **Figure 5a** illustrates the inhibitory type of methyl *trans*-cinnamate on the diphenolase activity of mushroom tyrosinase. The plots of $1/v$ versus 1/[S] give a family of lines with different slope and intersect one another in the *X*-axis, indicating that methyl *trans*-cinnamate was a noncompetitive inhibitor of diphenolase. The equilibrium constant (K_I) for methyl *trans*-cinnamate binding to the free enzyme was obtained from the secondary plot (**Figure 5b**). The value of K_I was 1.60 mM.

The Antimicrobial Activity of Methyl *trans*-Cinnamate. *E. coli*, *B. subtilis*, *S. aureus* and *C. albicans* were used as experimental microbes, which resulted in the decay of food. The antimicrobial activity of methyl *trans*-cinnamate was studied. The MIC and MBC of methyl *trans*-cinnamate for the microbes were tested. The MIC values for *E. coli*, *B. subtilis*, *St. aureus* and *C. albicans* were 0.5 mg/mL, 0.25 mg/mL, 0.25 mg/mL and 1 mg/mL respectively; and the MBC (MFC for *C. albicans*) values were 1 mg/mL, 0.5 mg/mL, 0.5 mg/mL, and 2 mg/mL respectively. Methyl *trans*-cinnamate was effective against the four kinds of representative microbes.

It was found that methyl *trans*-cinnamate can inhibit the proliferation of these four different kinds of microbes in different concentrations. **Figure 6** showed the inhibitory effects of methyl *trans*-cinnamate for the growth curve of the four microbes. For curves 1, 2, 3 and 4, the concentration of methyl *trans*-cinnamate was 0, 1/2 of MIC, MIC and MBC respectively. As shown in

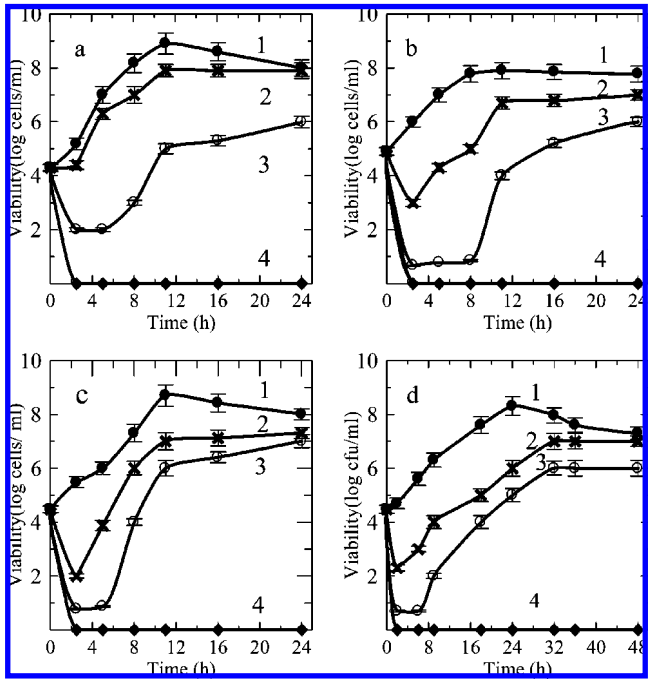

Figure 6. Inhibitory effects of methyl *trans*-cinnamate for the growth curve of the four microbes. The concentrations of methyl *trans*-cinnamate for curves 1−4 were 0, 0.125, 0.25 and 0.5 mg/mL for *St. aureus* (**a**) and *B. subtilis* (**b**), 0, 0.25, 0.5, 1 mg/mL for *E. coli* (**c**), and 0, 0.5, 1, 2 mg/mL for *C. albicians* (**d**).

the figure, there was not a distinct lag phase of growth curve as the inoculum was in exponential phase. Curve 1 showed the normal growth of microbes without inhibitor. The inhibitor concentration for curve 4 was equal to MBC, thus the microbes could not grow. Curves 2 and 3 showed the obviously inhibitory effects of methyl *trans*-cinnamate for the four kinds of microbes. DMSO has no obvious inhibition on the proliferation of these four different kinds of microbes.

DISCUSSION

Tyrosinase is a multifunctional oxidase with activity of both monophenolase and diphenolase. Taking L-DOPA and L-Tyr as substrates, the effects of methyl *trans*-cinnamate on the diphenolase activity and the monophenolase activity of tyrosinase were first studied. The results showed that methyl *trans*-cinnamate could inhibit the monophenolase activity and the diphenolase activity of mushroom tyrosinase. Methyl *trans*-cinnamate was a reversible noncompetitive type inhibitor for the diphenolase activity. The value of K_I was about the same as K_{IS}, indicating that the appetency of inhibitors to enzyme−substrate complexes was the same as to the free enzyme. Cinnamic acid is also a reversible noncompetitive inhibitor with IC_{50} of 1.99 mM (*18*). Methyl *trans*-cinnamate has more potent inhibitory effect than cinnamic acid.

The monophenolase activity of mushroom tyrosinase was assayed using L-Tyr as substrate. The lag time could be estimated by extrapolation of the linear portion of the product accumulation curve to the *X*-axis. As was shown in **Figure 2**, methyl *trans*-cinnamate could decrease the steady state rate of the monophenolase activity and prolonged the lag time by 4 times. Methyl *trans*-cinnamate was obviously an inhibitor of monophenolase.

Methyl *trans*-cinnamate has antimicrobial activity against *E. coli*, *B. subtilis*, *St. aureus* and *C. albicans*. It was found that methyl *trans*-cinnamate could inhibit the proliferation of these four different kinds of microbes to different extents. The result shows that the antimicrobial activity of methyl *trans*-cinnamate is broad-spectrum. It can inhibit G^+ bacteria, G^- bacteria and fungi.

In conclusion, first, methyl *trans*-cinnamate could inhibit both monophenolase activity and diphenolase activity of tyrosinase. Second, it has a broad-spectrum antimicrobial ability. Third, it is a widely used safe fragrance. Thus, we believe that methyl *trans*-cinnamate could be a potential compound used in anti-browning food additive.

ABBREVIATIONS USED

DMSO, dimethyl sulfoxide; L-DOPA, L-3,4-dihydroxyphenylalanine; L-Tyr, L-tyrosine; IC_{50}, the inhibitor concentration leading to 50% activity lost; CFU, colony forming unit; MIC, the minimum inhibitory concentration; MBC, the minimum bactericidal concentration; MFC, the minimum fungistatic concentration; PDA, potato dextrose agar medium; K_I, the equilibrium constant of the inhibitor combining with the free enzyme; K_{IS}, the equilibrium constant of the inhibitor combining with the enzyme−substrate complex.

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