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Inhibition Kinetics of Chlorobenzaldehyde Thiosemicarbazones on Mushroom Tyrosinase

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2-Chlorobenzaldehyde thiosemicarbazone (2-Cl-BT) and 4-chlorobenzaldehyde thiosemicarbazone (4-Cl-BT) were synthesized, and their inhibitory kinetics on the activity of mushroom tyrosinase were investigated. Results showed that these compounds exhibited significant inhibitory potency on both monophenolase activity and diphenolase activity of tyrosinase. For the monophenolase activity, both compounds could decrease the steady-state activity of the enzyme sharply, without any influence on the lag period. The IC_{50} values of them were estimated to be 15.4 μM and 6.7 μM, respectively. For the diphenolase activity, both compounds belonged to reversible inhibitors, but their mechanisms were different: 2-Cl-BT was a noncompetitive type inhibitor, while 4-Cl-BT was a mixed-type inhibitor. Their inhibition constants were determined and compared.

KEYWORDS: Mushroom tyrosinase; monophenolase activity; diphenolase activity; chlorobenzaldehyde thiosemicarbazones; inhibition kinetics; synthesis

INTRODUCTION

Tyrosinase (EC 1.14.18.1), a type-3 dicopper multifunctional oxidase widely distributed in nature, plays a crucial role in melanogenesis. It catalyzes the key step of the formation of melanin, the hydroxylation of monophenol to *o*-diphenol and the oxidation of diphenol to *o*-quinones (*1*, *2*). Melanogenesis is an important physiological process that results in the production of melanin pigment, which is of importance in the prevention of UV-induced skin injuries (*3*). However, excessive accumulations of epidermal pigmentation can cause various hyperpigmentation disorders, such as melasma, age spots, and sites of actinic damage (*4*). Therefore, the regulation of melanin synthesis via the inhibition of tyrosinase is a current research topic in the context of preventing hyperpigmentation. Also, tyrosinase participates in the undesired enzymatic browning progress in plants, which may drastically decrease the quality and value of such food products (*5*). Furthermore, tyrosinase distributed in insects is involved in the host defense, wound healing, molting and sclerotization process of insects (*6*, *7*). Thus, tyrosinase inhibitors are of great concern to scientists in clinical medicinal (*8*−*10*) and cosmetic research (*11*) in relation to hyperpigmentation, food preservation technology (*12*) and alternative insect control (*13*).

Many efforts have been addressed to screening efficient and safe tyrosinase inhibitors from natural materials and synthetic methods (*14*). To date, numerous novel tyrosinase inhibitors, with inhibitory potency ranging from slight to significant, have been reported (*15*−*17*). Several thiourea derivatives were reported to have moderate antityrosinase activity, and their inhibitory mechanisms were elucidated (*18*). Xue et al. reported the 3D-QSAR and molecular docking studies of benzylaldehyde thiosemicarbazone derivatives as phenoloxidase inhibitors; results showed that these thiosemicarbazone compounds possess much higher inhibitory activity (*19*, *20*). Also our previous work showed that *trans*-cinnamaldehyde thiosemicarbazone exhibits significant tyrosinase inhibitory activity (*21*).

In this study, 2-chlorobenzaldehyde thiosemicarbazone (2-Cl-BT) and 4-chlorobenzaldehyde thiosemicarbazone (4-Cl-BT) were synthesized, and their inhibitory kinetics on the activity of mushroom tyrosinase were investigated.

MATERIALS AND METHODS

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

Synthesis. Compounds were prepared by a simple one-step reaction of corresponding chloride substituted benzylaldehyde with thiosemicarbazide in an acidic solution of ethanol, as previous described (*21*). A mixture of the corresponding benzylaldehyde (10 mM) with thiosemicarbazide (10 mM) in 40 mL of ethanol with 2 mL of acetic acid solution was refluxed until the reaction was complete and then cooled to room temperature. The precipitates were collected and washed with cold ethanol. The products were purified by recrystallization from 50% ethanol and were identified by

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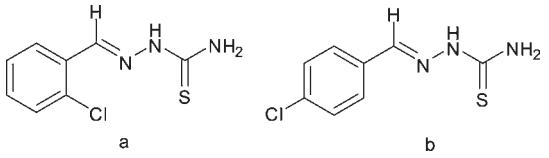

Figure 1. Chemical structure of 2-Cl-BT (**a**) and 4-Cl-BT (**b**).

ESI-MS and ^{1}H NMR analysis. ESI-MS data were performed on ESQUIRE-LC ion trap mass spectrometry.^{1}H NMR data were acquired on a 400 MHz NMR spectrometer (Bruker AV400). The purity was confirmed by elemental analysis.

Enzyme Activity Assay. Monophenolase and diphenolase activities of mushroom tyrosinase were determined as previously reported (*22*) with modification, by measuring the rate of dopachrome formation at 475 nm ($\varepsilon = 3700\ M^{-1}\cdot cm^{-1}$). In this investigation, L-Tyr was used as substrate for monophenolase activity assay and L-DOPA was used as substrate for diphenolase activity assay. The activity assay used 300 μL of reaction medium containing 2 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 tyrosinase was 13.33 μg/mL for monophenolase activity and 6.67 μg/mL for diphenolase activity. The reaction was controlled at a constant temperature of 30 °C. The assay was performed by SPECTRAMAX M2e from Molecule Device Co.

Effects of Inhibitors on the Enzyme Activity. Inhibitors were first dissolved in DMSO and used for the test after a 30-fold dilution. In this assay, 10 μL of DMSO solution with different concentrations of inhibitors was first mixed with 240 μL of substrate solution (contained 0.5 mM L-DOPA or 2 mM L-Tyr in 50 mM $Na_2HPO_4-NaH_2PO_4$ buffer, pH 6.8); then, a portion of 50 μL of enzyme solution was added to this blend and the residual activity was determined. The final concentration of DMSO in the test solution was 3.33%. Controls, without inhibitor but containing 3.33% DMSO, were routinely carried out (*23*). The measurement was performed in triplicate for each concentration and averaged before further calculation. The extent of inhibition by the addition of the sample was expressed as the percentage necessary for 50% inhibition (IC_{50}).

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 (*23*).

RESULTS AND DISCUSSION

Chemical Synthesis of 2-Cl-BT and 4-Cl-BT. 2-Cl-BT recrystallized as a light yellow powder. Yield: 92.5%. ^{1}H NMR (DMSO-d_6, TMS, 400 MHz): δ (ppm) 11.20 (1H, s, HN), 7.91 (1H, s, CH=N), 8.04, 7.74 (2H, s, NH_2), 9.48−7.19 (4H, m, C_6H_4). ESI-MS: m/z (100%) (M + Na^+, methanol) 236.0; (M + H^+, methanol) 214.2. Anal. Calcd: C, 44.97; H, 3.77; N, 19.66. Found: C, 45.22; H, 3.799; N, 19.56. 4-Cl-BT recrystallized as a light yellow powder, too. Yield: 88.1%. ^{1}H NMR (DMSO-d_6, TMS, 400 MHz): δ (ppm) 11.42 (1H, s, NH), 7.95 (1H, s, CH=N), 8.02, 7.40 (2H, s, NH_2), 8.21, 8.04 (4H, d, C_6H_4). ESI-MS: m/z (100%) (M + Na^+, methanol) 236.0; (M + H^+, methanol) 214.2. Anal. Calcd: C, 44.97; H, 3.97; N, 19.66. Found: C, 45.00; H, 3.769; N, 19.79. (See **Figure 1** for the structure.)

Effects of 2-Cl-BT and 4-Cl-BT on the Monophenolase Activity of Mushroom Tyrosinase. The inhibitory effects of the different concentrations of 2-Cl-BT on the oxidation of L-Tyr by the enzyme were studied. From the progress curve of the oxidation of L-Tyr without inhibitor, an apparent lag period, the characteristic of monophenolase activity was observed (**Figure 2a**, curve 1). The system reached a constant rate (the steady-state rate) after the lag period, which was estimated by extrapolating the curve to the abscissa (*24*). With increasing inhibitor concentration, the kinetic course of the oxidation of L-Tyr is shown in **Figure 2a**, curves 2−7. The lag time and the steady-state rate were determined, and the data are shown in **Figure 2b** and Figure**2c**, respectively. The lag time did not change with increasing inhibitor concentration, but

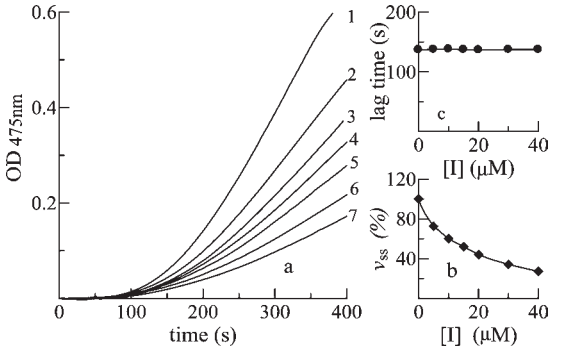

Figure 2. Inhibition effects of 2-Cl-BT on monophenolase activity of mushroom tyrosinase. (**a**) Progress curves for the oxidation of L-Tyr by the enzyme. The concentrations of inhibitor for curves 0−7 were 0, 5, 10, 15, 20, 30, and 40 μM, respectively. (**b**) Effects of 2-Cl-BT on the steady-state rates of monophenolase. (**c**) Effects of 2-Cl-BT on the lag time of mushroom tyrosinase.

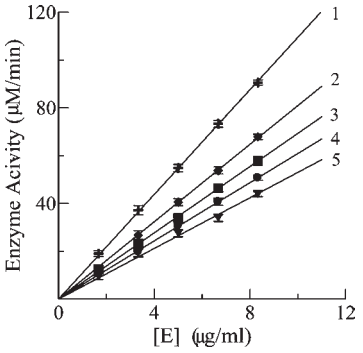

Figure 3. Determination of the inhibitory mechanism of 2-Cl-BT on mushroom tyrosinase. The concentrations of inhibitor for curves 0−5 were 0, 0.3, 0.6, 0.9, and 1.5 μM, respectively.

the steady-state rate decreased distinctly and dose-dependently. The inhibitory effects of 4-Cl-BT on the oxidation of L-Tyr follow the same mechanism. The concentration leading to 50% loss of enzyme activity (IC_{50}) was determined to be 15.4 μM and 6.7 μM, respectively.

Effects of 2-Cl-BT and 4-Cl-BT on the Diphenolase Activity of Mushroom Tyrosinase. We probed the effects of 2-Cl-BT and 4-Cl-BT 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. Both compounds can inhibit the diphenolase activity of tyrosinase in a dose-dependent manner. With increasing concentrations of inhibitors, the remaining enzyme activity decreased exponentially. The inhibitor concentration leading to 50% activity lost (IC_{50}) was estimated to be 1.22 μM and 1.82 μM, respectively.

The Inhibition Mechanism of 2-Cl-BT and 4-Cl-BT on the Diphenolase Activity of Mushroom Tyrosinase Was Reversible. The inhibition mechanism on mushroom tyrosinase by 2-Cl-BT and 4-Cl-BT for the oxidation of L-DOPA was investigated. Both inhibitors behaved in the same manner. **Figure 3** showed the relationship between enzyme activity and its concentration in the presence of 2-Cl-BT. 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, indicating that the presence of inhibitor did not reduce the amount of enzyme, but just resulted

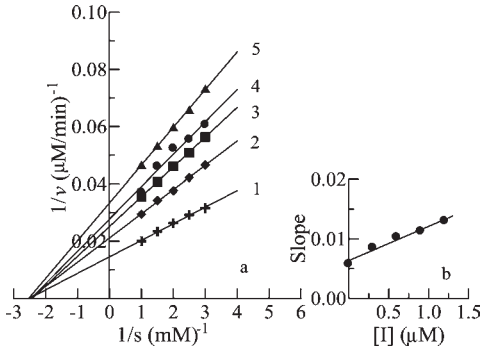

Figure 4. Determination of the inhibitory type and inhibition constants of 2-Cl-BT. (**a**) Lineweaver−Burk plots for inhibition of 2-Cl-BT on mushroom tyrosinase. The concentrations of inhibitor for curves 0−5 were 0, 0.3, 0.6, 0.9, and 1.2 μM, respectively. (**b**) The plot of slope versus the concentration of 2-Cl-BT for determining the inhibition constants K_I and K_{IS}.

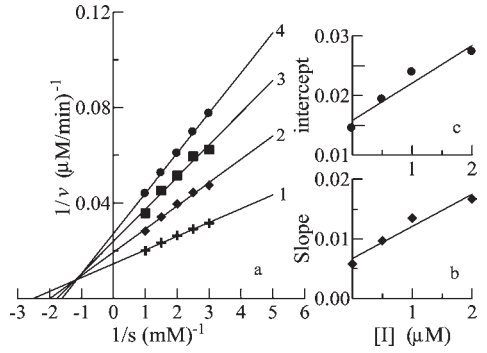

Figure 5. Determination of the inhibitory type and inhibition constants of 4-Cl-BT. (**a**) Lineweaver−Burk plots for inhibition of 4-Cl-BT on mushroom tyrosinase. The concentrations of inhibitor for curves 0−4 were 0, 0.5, 1.0, and 2.0 μM, respectively. (**b**) The plot of slope versus the concentration of 4-Cl-BT for determining the inhibition constants K_I. (**c**) The plot of intercept versus the concentration of 4-Cl-BT for determining the inhibition constants K_{IS}.

in the inhibition of enzyme activity. Both compounds were reversible tyrosinase inhibitors.

The Inhibition Type of 2-Cl-BT on the Diphenolase Activity of Mushroom Tyrosinase Was Noncompetitive. The inhibitory type 2-Cl-BT on the diphenolase activity, during the oxidation of L-DOPA, was determined from Lineweaver−Burk double reciprocal plots. In the presence of 2-Cl-BT, the kinetics of the enzyme is shown in **Figure 4**. The plots of $1/v$ versus 1/[S] gave a family of straight lines with different slopes which intersected one another in the X-axis, indicating that 2-Cl-BT is a noncompetitive inhibitor. The equilibrium constants for inhibitor binding with the free enzyme and the enzyme−substrate complex, K_I and K_{IS}, were obtained from the secondary plot (**Figure 4b**) as 1.20 μM and 1.20 μM.

The Inhibition Type of 4-Cl-BT on the Diphenolase Activity of Mushroom Tyrosinase Was of Mixed Type. In contrast, the kinetics of the enzyme in the presence of 4-Cl-BT is shown in **Figure 5**. Results showed 4-Cl-BT was of competitive−uncompetitive mixed type inhibitor, for the Lineweaver−Burk double reciprocal plots yield a group of lines that intercept in the second quadrant. The inhibitor constants (K_I and K_{IS}) were estimated to be 1.25 μM and 2.49 μM, respectively. The inhibitor constants are summarized in **Table 1** for comparison.

Tyrosinase exhibits both monophenolase and diphenolase activities. Here, we used L-DOPA as substrate for the diphenolase activity and L-Tyr for the monophenolase activity of the enzyme.

Table 1. Inhibition Constants of 2-Cl-BT and 4-Cl-BT with Mushroom Tyrosinase

constants	2-chlorobenzaldehyde thiosemicarbazone	4-chlorobenzaldehyde thiosemicarbazone
IC_{50}		
monophenolase	15.4 μM	6.7 μM
diphenolase	1.22 μM	1.82 μM
inhibition mechanism	reversible	reversible
inhibition type	noncompetitive	mixed type
K_I	1.20 μM	1.25 μM
K_{IS}	1.20 μM	2.49 μM

The results show that 2-Cl-BT and 4-Cl-BT could inhibit both the diphenolase activity and the monophenolase activity of mushroom tyrosinase. For diphenolase activity, the inhibition was reversible, while the inhibition types were determined to be of noncompetitive and competitive−uncompetitive mixed type, respectively. As shown in **Table 1**, the IC_{50} values of 2-Cl-BT and 4-Cl-BT for the diphenolase activity were similar. Kinetic study reveals that both compounds exhibit similar K_I, which means the binding affinity to free enzymes is about the same, while their affinity to enzyme−substrate complexes is different. For monophenolase activity, both compounds could inhibit the steady-state activity drastically, without changing the lag time.

There are two copper ions in the active center of tyrosinase, and a lipophilic long-narrow gorge exists close to the active center (*25*, *26*). According to the 3D structure of tyrosinase, the copper ions were of great importance for tyrosinase activity, and slight change in the dicopper center may lead to activity loss. Benzylaldehyde thiosemicarbazone is a kind of Schiff base compound that exhibits strong affinity to copper ions. Thus, when mixed with tyrosinase and its substrate, a benzylaldehyde thiosemicarbazone derivative may form a complex with tyrosinase molecule by its sulfur atom and nitrogen atom though a hydrogen bond and a coordinate bond. This tight complex could make the free oxygen molecule decrease its reaction ability and even unable to take part in the hydroxylation with monophenols and in the oxidation with *o*-diphenols, as the free oxygen molecule was surrounded closely by two copper ions from tyrosinase and two hydrogen atoms from two benzylaldehyde thiosemicarbazone molecules. Therefore tyrosinase would lose its catalyzing ability (*27*).

This paper investigated the kinetics of 2-Cl-BT and 4-Cl-BT on the activity of mushroom tyrosinase. Results showed that both compounds had a strong inhibitory effect on the monophenolase activity and diphenolase activity of the enzyme. Compared with the most used antityrosinase agents, arbutin (IC_{50} = 30 mM) and kojic acid (IC_{50} = 23 μM), both compounds showed much stronger activities (IC_{50} = 1.22 μM and 1.82 μM, respectively). Such tyrosinase inhibitors may have broad application in cosmetic, medicinal, food preservation and insect control area.

ABBREVIATIONS USED

DMSO, dimethyl sulfoxide; L-DOPA, L-3,4-dihydroxyphenylalanine; L-Tyr, L-tyrosine; IC_{50}, the inhibitor concentrations leading to 50% activity lost; K_I, equilibrium constant of the inhibitor combining with the free enzyme; K_{IS}, equilibrium constant of the inhibitor combining with the enzyme−substrate complex.

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