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Inhibitory kinetics of DABT and DABPT as novel tyrosinase inhibitors

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4-Dimethylaminobenzaldehyde-thiosemicarbazone (DABT) and 4-dimethylaminobenzaldehyde-*N*-phenyl-thiosemicarbazone (DABPT) were synthesized and established by ^{1}H and ^{13}C NMR and mass spectrum. Both compounds were evaluated for their inhibition activities on mushroom tyrosinase and their anti-tyrosinase kinetics was investigated. The results showed that both compounds exhibited significant inhibitory effects on activity of monophenolase and diphenolase; DABT and DABPT decreased the steady-state rate with 1.54 μM and 1.78 μM as their IC_{50} values respectively. The inhibitory effects of diphenolase activity exhibited sharp in a dose-dependent manner and their IC_{50} values were estimated as 2.01 μM and 0.80 μM, respectively. Kinetic analysis showed that their inhibition mechanism was reversible. The inhibition type of DABT was mix-type with inhibition constants K_I = 1.77 μM and K_{IS} = 6.49 μM, while that of DABPT displays non-competitive with the inhibition constant K_I = 0.77 μM.

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[**Key words:** Mushroom tyrosinase; Benzaldehyde thiosemicarbazone derivatives; Inhibition; Kinetics; Synthesis]

Tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase (PPO), is a type-3 dicopper multi-functional oxidase that catalyzes the hydroxylation of monophenol to *o*-diphenol and the oxidation of *o*-diphenol to *o*-quinones, which undergoes further non-enzymatic polymerization leading to the formation of melanin (1,2). Tyrosinase can be found in almost all of the species, ranging from prokaryocytes to eukaryotes and involved in many physiological processes. For human, tyrosinase plays an important role in production of melanin pigment, which is of importance in the prevention of UV-induced skin injuries (3) and is responsible to various hyper-pigmentation disorders, such as melasma, age spots, and sites of actinic damage (4). Therefore, regulation of melanin synthesis via inhibition of tyrosinase is a current research topic in the context of preventing hyper-pigmentation. Also, tyrosinase participates in the undesired enzymatic browning progresses in fruits and vegetables, impairs the quality and value of such food products (5). To the best of our knowledge, several tyrosinase inhibitors, mainly benzoic acids and their derivatives have been used as anti-browning agents (6). Furthermore, the host defense, wound healing, molting and sclerotization process of insects affected by inhibitors had been considered as an alternative target for insect control (7,8).

Many efforts had been addressed to pursue efficient and feasible tyrosinase inhibitors and many natural and synthetic tyrosinase inhibitors had been reported (9–18). In our previous papers, some tyrosinase inhibitors, several benzaldehyde derivatives (19,20), benzoic acid derivative (21) and *o*-diphenols (22) were found to possess slight to significant inhibitory effects on mushroom tyrosinase. Also our previous research revealed that phenyl thioureas and alkyl thioureas could exhibit moderate to tyrosinase activity. Recently, benzaldehyde thiosemicarbazone derivatives had been reported to be efficient tyrosinase inhibitors (23,24).

In this study, we synthesized two kinds of thiosemicarbazone derivatives: 4-dimethylaminobenzaldehyde-thiosemicarbazone (DABT) and 4-dimethylamino-benzaldehyde-*N*-phenyl-thiosemicarbazone (DABPT). It was found that both of them are potent reversible inhibitors of tyrosinase. The aim of this paper is, therefore, to carry out a kinetic study of the inhibition on the enzyme. The anti-tyrosinase kinetic study will provide a better understanding of the inhibition mechanism of thiosemicarbazone derivatives and a basis for the future design of potent tyrosinase inhibitors.

MATERIALS AND METHODS

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

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Abbreviations: DABPT, 4-dimethylaminobenzaldehyde-*N*-phenyl-thiosemicarbazone; DABT, 4-dimethylaminobenzaldehyde-thiosemicarbazone; DMSO, dimethyl sulfoxide; L-DOPA, L-3,4-dihydroxyphenylalanine; 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; Tyr, L-tyrosine.

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Synthesis Compounds were prepared by the reaction of 4-dimethylaminobenzaldehyde with thiosemibazide or 4-phenylthiosemibazide in an acidic solution of ethanol. A mixture of 4-dimethylaminobenzaldehyde (10 mM) with corresponding thiosemibazide derivatives (10 mM) in 40 ml of ethanol with 2 ml of acetic acid solution was refluxed until the reaction was complete. The reaction mixture was cooled to room temperature and then filtrated. The precipitates were collected and washed with cold ethanol and then purified by recrystallization from 50% ethanol. The purified products were identified by ESI-MS (ESQUIRE-LC ion trap mass spectrometry) and ^{1}H NMR analysis (Bruker AV400 400 MHz NMR spectrometer).

Enzyme activity assay Monophenolase and diphenolase activities of mushroom tyrosinase were determined as previously reported (25) with modification, by measuring the rate of dopachrome formation at 475 nm (ε = 3700 M^{-1} cm^{-1}). In this investigation, L-tyrosine (L-Tyr) was used as substrate for monophenolase activity assay and L-DOPA was used as substrate for diphenolase activity assay. The activity assay was 300 μl reaction media containing 2 mM L-tyrosine 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., USA.

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 (26).

RESULTS

Chemical synthesis of DABT and DABPT 4-Dimethylamiobenzaldehyde-thiosemicarbazone is a light yellow powder. Yield rate is 90.4%. ^{1}H NMR (DMSO-d_6, TMS, 400 MHz): δ (ppm) 11.18 (1H, s, HN), 8.00 (1H, s, CH), 7.93 ~ 7.57 (4H, m, C_6H_4), 6.71 (2H, s, NH_2), 2.96 (6H, s, Me_2N), ESI-MS: m/z 223.1 (M + H^+, CH_3OH). 4-Dimethylaminobenzaldehyde-*N*-phenyl-thiosemicarbazone is also a light yellow powder and yield rate is 95.9%, ^{1}H NMR (DMSO-d_6, TMS, 400 MHz): δ (ppm) 11.62 (1H, s, HN), 9.94 (1H, s, HN), 8.01 (1H, s, CH), 6.72 ~ 7.71 (4H, m, C_6H_4), 7.18–7.61 (5H, m, N_6H_5), 2.98(6H, s, Me_2N). ESI-MS: m/z 298.1 (M + H^+, CH_3OH). The structures are shown in Fig. 1.

Effect of DABT and DABPT on the monophenolase activity of mushroom tyrosinase The inhibitory effects of the different concentrations of DABT and DABPT 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 (curve 0 in Fig. 2a). The system reached a constant rate (the steady-state rate) after the lag period, which was estimated by extrapolation curve to the abscissa. With the increasing inhibitor concentration, the kinetic course of the oxidation of L-Tyr were shown in Fig. 2a, curves 1–6, and as shown in Fig. 2b and c, the lag time prolonged with increasing inhibitor concentration, and the steady-state rate

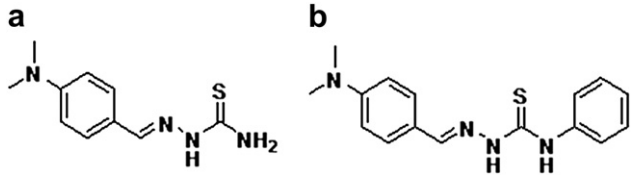

FIG. 1. Chemical structure of 4-dimethylaminobenzaldehyde-thiosemicarbazone (a) and 4-dimethylaminobenzaldehyde-*N*-phenyl-thiosemicarbazone (b).

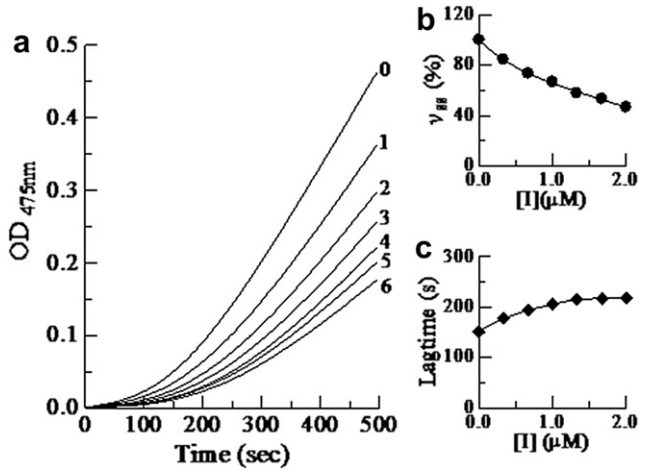

FIG. 2. Inhibition effects of DABPT on monophenolase activity of mushroom tyrosinase. (a) Progress curves for the oxidation of L-Tyr by the enzyme. The concentrations of inhibitor for curves 0–6 are 0, 0.33, 0.67, 1, 1.33, 1.67 and 2 μM, respectively. (b) Effects of DABPT on the steady-state rates of monophenolase. (c) Effects of DABPT on the lag time of mushroom tyrosinase.

decreased distinctly and dose-dependently. The inhibitory effects of DABPT on the oxidation of L-Tyr follow the same mechanism. The concentration lead to 50% loss of enzyme activity (IC_{50}) was determined to be 1.54 μM and 1.78 μM, respectively.

Effect of DABT and DABPT on the diphenolase activity of mushroom tyrosinase The effect of DABT and DABPT on the activity of mushroom tyrosinase for the oxidation of L-DOPA was also estimated. When the diphenolase activity of tyrosinase was assayed with 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 2.02 μM and 0.80 μM, respectively.

The inhibition mechanism of DABT and DABPT on the diphenolase activity of mushroom tyrosinase was reversible The inhibition mechanism on mushroom tyrosinase by DABT and DABPT for the oxidation of L-DOPA was probed. Both inhibitors followed the same manner. Fig. 3 illustrates the relationship between enzyme activity and its concentration in the presence of DABT. 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 just inhibited the enzyme activity and did not reduce the amount of enzyme. Both compounds were reversible tyrosinase inhibitors.

The inhibition type of DABT on the diphenolase activity of mushroom tyrosinase was mixed-type Under the conditions employed in the present investigation, the oxidation reaction of L-DOPA by mushroom tyrosinase followed Michaelis–Menten kinetics. Fig. 4a illustrates the inhibitory type of DABT on the diphenolase activity of mushroom tyrosinase. Lineweaver–Burk double reciprocal plots yield a group of lines intercept in the second quadrant, indicating DABT is a mixed-type inhibitor. The equilibrium constant for inhibitor binding with the free enzyme and the enzyme–substrate complex, K_I and K_{IS}, were obtained from the secondary plot (Fig. 4b and c) and their values were 1.77 μM and 6.49 μM.

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