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Inhibitory effects of cefotaxime on the activity of mushroom tyrosinase

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Tyrosinase (EC 1.14.18.1) catalyzes both the hydroxylation of tyrosine into *o*-diphenols and the oxidation of *o*-diphenols into *o*-quinones that form brown or black pigments. In the present paper, cefotaxime, a cephalosporin antibacterial drug, was tested as an inhibitor of tyrosinase. The results show that cefotaxime inhibits both the monophenolase and diphenolase activities of tyrosinase. For the monophenolase activity, cefotaxime increased the lag time and decreased the steady-state activity with an IC_{50} of 3.2 mM. For the diphenolase activity, the inhibition by cefotaxime is reversible and mix-I type with an IC_{50} of 0.14 mM. The inhibition constants (K_I and K_{IS}) were determined to be 0.14 and 0.36 mM, respectively. The molecular mechanism of inhibition of tyrosinase by cefotaxime was determined by fluorescence quenching and molecular docking. The results demonstrated that cefotaxime was a static quencher of tyrosinase and that cefotaxime could dock favorably in the active site of tyrosinase. This research may offer a lead for designing and synthesizing novel and effective tyrosinase inhibitors in the future.

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[**Key words:** Tyrosinase; Cefotaxime; Inhibition; Fluorescence quenching; Docking]

Tyrosinase (EC 1.14.18.1) is a key enzyme in melanin biosynthesis in plants and animals, and is thus involved in determining the color of mammalian skin and hair (1,2). Melanin is produced by melanocytes through the conversion of the amino acid L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA), which is then oxidized to yield dopaquinone (3). Abnormal Tyrosinase expression is responsible for a variety of dermatological disorders, such as melasma age spots, and sites of actinic damage (4). It also contributes to neuromelanin formation in the human brain and the neurodegeneration associated with Parkinson's disease (5). Tyrosinase induction causes the formation of reactive oxygen species in the cytosol and mitochondria, and induces neurotoxicity via activation of apoptotic stress-activated protein kinases and caspase 3 (6). Thus, the discovery of tyrosinase inhibitors is of great importance. Tyrosinase inhibitors would be clinically useful for the treatment of some dermatological disorders associated with melanin hyperpigmentation and would also be important in cosmetics for whitening and depigmentation (7,8). Some existing drugs have an inhibitory effect on tyrosinase activity, as has been reported for captopril, which is used in the treatment of hypertension and heart failure (9), and the antithyroid drug methimazole (10,11).

Although many inhibitors including synthetic and natural extracting compounds have already been reported (2,12), the safety limit their applications. Cefotaxime is a third generation cephalosporin that is widely used to treat bacterial infections, mostly respiratory and urinary infections, because it is active against many bacteria (13,14). Cefotaxime is important in the pharmaceutical or clinical area owing to its crucial role in numerous pathological processes as it could inhibit the synthesis of the peptidoglycan layer from the cell wall (15). Asif et al. (16) reported that cefotaxime could prevent microbial contamination and improve microspore embryogenesis in wheat and triticale. Meanwhile, Reiss et al. (17) synthesized cefotaxime-derived Schiff bases and investigated their antimicrobial activities.

In the present investigation, cefotaxime was found to have previously unreported inhibitory effects on mushroom tyrosinase. We report here a kinetic study of the inhibition of the diphenolase and monophenolase activity of tyrosinase by cefotaxime, evaluate the kinetic parameters and inhibition constants characterizing the system and investigate the mechanism of inhibition. Previous studies have emphasized the use of tyrosinase inhibitors in preparations for the prevention and/or treatment of hyperpigmentation, and we suggest cefotaxime could be used in creams to treat and prevent cutaneous hyperpigmentation. In addition, these data may provide the basis for developing novel tyrosinase inhibitors.

MATERIALS AND METHODS

Reagents and materials Tyrosinase (EC 1.14.18.1) from mushroom was the product of Sigma–Aldrich (St. Louis, MO, USA). The specific activity of the enzyme was 6680 U/mg. Dimethyl sulfoxide (DMSO), L-Tyrosine (L-Tyr) and L-3,4-dihydroxyphenylalanine (L-DOPA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Cefotaxime (Fig. 1) was purchased from National Institute for the

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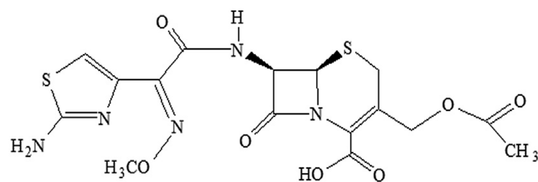

FIG. 1. Chemical structure of cefotaxime.

Control of Pharmaceutical and Biological Products. All other reagents were local and of analytical grade. The water used was re-distilled and ion-free.

Monophenolase activity The monophenolase activity assay was performed as previously reported using L-tyrosine as the substrate (18). The reaction (3 mL) contained 1.0-mM L-Tyr in 50-mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8); 100 μL of an aqueous solution of the enzyme (containing 100 μg) was used to assay the activity. The final concentration of mushroom tyrosinase was 33.33 μg/mL. The enzyme activity was monitored by measuring the optical density at 475 nm (ε = 3700 M^{-1} cm^{-1}) each 5 s for 10 min. The reaction was carried out at a constant temperature of 30°C.

Diphenolase activity The diphenolase activity of mushroom tyrosinase was performed as previously reported using L-DOPA as the substrate (18,19). The reaction (3 mL) contained 0.5-mM L-DOPA and 20 μg of the enzyme in 50-mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8). Enzyme activity was determined by following the increase in optical density at 475 nm accompanying the oxidation of L-DOPA to dopachrome. The reaction was carried out at a constant temperature of 30°C.

The effects of inhibitors on enzyme activity were determined as follows. The inhibitor was first dissolved in DMSO and diluted 30-fold in the assay to give a final concentration of DMSO in the test solution of 3.3%. In this method, 0.1 mL of DMSO solution containing different concentrations of inhibitor was first mixed with 2.8 mL of substrate solution, then 100 μL of enzyme solution was added into this mixture and the residual activity was determined. Controls, without inhibitor but containing 3.3% DMSO, were routinely carried out. The extent of inhibition by the addition of the sample is expressed as the concentration necessary for 50% inhibition (IC_{50}).

The inhibition type of cefotaxime on the enzyme was determined using Lineweaver–Burk plots and the inhibition constant was determined from secondary plots of the apparent K_m/V_{max} or $1/V_{max}$ versus the concentration of the inhibitor. A Beckman UV-650 spectrophotometer was used for absorbance measurements.

Fluorescence quenching Fluorescence spectra were measured by the method of Ionita et al. (20) and Cui et al. (21) with some modification. Fluorescence spectroscopy experiments were performed on an Cary Eclipse fluorescence spectrophotometer. The excitation wavelength was set at 280 nm, and the fluorescence emission spectra were scanned from 300 to 420 nm. Assays of 2.0-mL final volume contained tyrosinase (0.2 mg/mL) and 2-μL cefotaxime of different concentrations. Each measurement was recorded in triplicate.

The fluorescence quenching data were analyzed by fitting to the Stern–Volmer equation:

$$F_0/F = 1 + K_{SV}[Q] = 1 + K_q\tau_0[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, [Q] is the concentration of the quencher, K_{SV} is the Stern–Volmer quenching constant, K_q is the bimolecular quenching constant and τ_0 (10^{-8} s) is the lifetime of the fluorophore in the absence of the quencher. Hence, K_q was obtained from the slope of plots of F_0/F versus [Q].

There are two fluorescence-quenching mechanisms: static quenching is caused by complex formation while dynamic quenching is caused by collisional processes (22). For static quenching, the K_{SV} value cannot be larger than 100 L/M and the apparent binding constant (K_A) and the binding sites (n) were estimated by plots of log[(F_0–F)/F] versus log[Q] with the following equation (4,23):

$$\log[(F_0 - F)/F] = \log K_A + n\log[Q] \quad (2)$$

Molecular docking study Molecular operation environment 2008 software (MOE) was used to dock cefotaxime with tyrosinase. The structure of tyrosinase from *Agaricus bisporus* PPO3 (pdb entry 2Y9W) (24) with Cu^+ was used as the initial model for docking simulation after the caddie protein, the exogenous ions and water molecules were removed. The 3D structure of cefotaxime was drawn using ChemDraw 7.0 and exported to MOE. The structures of tyrosinase and cefotaxime were energy-minimized before docking. During molecular docking, the refinement was set to force field and the retention threshold of both the first and second scoring was set to 10. Docking poses were ranked by the MM/GBVI binding free energy score. Parameters not mentioned here used the default settings of the MOE software. The mode of binding used for analysis was based on the docked conformation, which had the highest score (25,26).

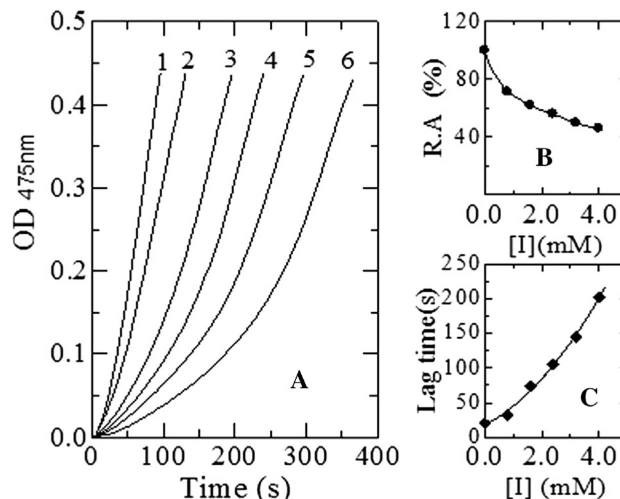

FIG. 2. Progress curves for the inhibition of monophenolase activity of mushroom tyosinase by cefotaxime. (A) The concentrations of cefotaxime for curves 1–6 were 0, 0.8, 1.6, 2.4, 3.2 and 4.0 mM, respectively. (B) The dependence of the steady-state rate on inhibitor concentration. (C) The dependence of the lag time on inhibitor concentration.

RESULTS

Effect of cefotaxime on the monophenolase activity of mushroom tyrosinase In the presence of different concentrations of cefotaxime, the monophenolase activity of mushroom tyrosinase was assayed using L-tyrosine as the substrate (Fig. 2A). The steady-state rate and lag time were determined and were both found to be affected by cefotaxime (Fig. 2B and C). The lag time increased from 20 s in the inhibitor-free control reaction to 202 s in the presence of 4.0-mM cefotaxime. The steady state rate of monophenolase activity decreased; with 4.0-mM cefotaxime the monophenolase had reduced to 46.0% of the uninhibited rate. The IC_{50} for inhibition of monophenolase activity by cefotaxime was determined to be 3.2 mM.

Effect of cefotaxime on the diphenolase activity of mushroom tyrosinase The assay for diphenolase activity used L-DOPA as the substrate. The progress curve of the enzyme reaction was a straight line passing through the origin without a lag period so that the formation of product was proportional to reaction time. The effect of cefotaxime on the oxidation of L-DOPA by mushroom tyrosinase was examined, and was found to inhibit the diphenolase activity (Fig. 3A). The diphenolase activity of mushroom tyrosinase decreased with added cefotaxime in a concentration dependent manner. Using the data from Fig. 3A, the concentration of cefotaxime causing a 50% loss of activity (IC_{50}) was estimated to be 0.14 mM.

The mechanism of inhibition of L-DOPA oxidation by mushroom tyrosinase by cefotaxime was studied. The relationship between the remaining enzyme activity and enzyme concentration in the presence of different concentrations of cefotaxime is a family of straight lines that all pass through the origin (Fig. 3B). Increasing the inhibitor concentration resulted in the lowering of the slope of the line, indicating that the inhibition by cefotaxime was reversible: the presence of inhibitor did not reduce the amount of active enzyme, but just resulted in a decrease in the rate of enzyme turnover. Double–reciprocal plots yielded a family of straight lines, which intersected in the 2nd quadrant (Fig. 3C). The values of K_m and V_{max} decreased with increasing inhibitor concentration. Thus, cefotaxime is a competitive-uncompetitive mixed-I type inhibitor. The uncompetitive effect is weaker than the competitive effect, indicating that cefotaxime inhibited the free enzyme more strongly

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