1 **Running title: Tyrosinase inhibition by cefotaxime**

2 **Inhibitory Effects of Cefotaxime on the Activity of Mushroom**

3 **Tyrosinase**

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Abstract:

Tyrosinase (EC 1.14.18.1) catalyzes both the hydroxylation of tyrosine into α -diphenols and the oxidation of α -diphenols into α -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.

Keywords: Tyrosinase; cefotaxime; inhibition; fluorescence quenching; docking

INTRODUCTION

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. reported that cefotaxime could

prevent microbial contamination and improve microspore embryogenesis in wheat and triticale (16). Meanwhile, Reiss et al. synthesized cefotaxime-derived Schiff bases and investigated their antimicrobial activities (17).

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 (**Figure 1**) was purchased from National Institute for the 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 Na₂HPO₄-NaH₂PO₄ 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 ($\epsilon=3700 \text{ M}^{-1} \text{ cm}^{-1}$) 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 $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$ 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 $1/V_{\text{max}}$ 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- μ L cefotaxime of different concentrations. Each measurement was recorded in triplicate.

The fluorescence quenching data were analyzed by fitting to the Stern-Volmer equation: $F_0/F = 1 + K_{SV} [Q] = 1 + K_q \tau_0 [Q]$.

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

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).

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 (**Figure 2a**). The steady-state rate and lag time were determined and were both found to be affected by cefotaxime (**Figure 2b and 2c**). The lag time increased from 20 sec in the inhibitor-free control reaction to 202 sec 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 (**Figure 3A**). The diphenolase activity of mushroom tyrosinase decreased with added cefotaxime in a concentration dependent manner. Using the data from **Figure 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 (**Figure 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 (**Figure 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 than enzyme-substrate complex. The equilibrium constants for inhibitor binding with the free enzyme and the enzyme-substrate complex, K_I and K_{IS} , were obtained from the plots of the slope and the vertical intercept versus the concentration of cefotaxime and were determined to be 0.14 mM and 0.36 mM, respectively.

Analysis of Fluorescence Quenching of Tyrosinase with Cefotaxime The interaction of cefotaxime with tyrosinase was evaluated by measuring the intrinsic fluorescence intensity of the enzyme before and after the addition of cefotaxime (**Figure 4A**). The tyrosinase had strong fluorescence emission with a peak at 334 nm. The fluorescence intensity of the emission peak decreased with increasing concentrations of cefotaxime (**Figure 4B**). The Stern-Volmer quenching constants of cefotaxime were determined from the Stern-Volmer plot (**Figure 4C**), which shows a linear relationship with K_{SV} and K_q determined to be $2.28 \times 10^4 \text{ M}^{-1}$ and $2.28 \times 10^{12} \text{ M}^{-1}\text{s}^{-1}$, respectively. This result indicates that the fluorescence-quenching mechanism between tyrosinase and cefotaxime is of the static type.

$\text{Log}[(F_0-F)/F]$ was plotted against $\text{log}[I]$ for tyrosinase with various concentrations of cefotaxime (**Figure 4D**). Values for the apparent binding constant (K_A) and the binding sites (n) were obtained from the intercept and slope of the plot and were

found to be $1.35 \times 10^5 \text{ M}^{-1}$ and 1.20, respectively. The R^2 of the linear regression was 0.9968.

Molecular Docking Analysis To understand the inhibitory mechanism of cefotaxime on tyrosinase, the docking mode was simulated. The docked model of cefotaxime in the tyrosinase catalytic center is shown in **Figure 5**. The docked conformation revealed that the thiazolyl moiety of cefotaxime could interact with Arg₂₆₈ and the nitrogen-bound hydrogen of the non-lactam amide functionality could interact with the sidechain of Asn₂₆₀. The carbonyl of the carboxyl bonded with the sidechain of Glu₂₅₆ while the carboxyl hydroxy group could interact with the backbone of Glu₂₅₆ and the sidechain of His₂₅₉. The ketone, distal from the thiazolyl group, could interact with the sidechain of His₆₁. Moreover, cefotaxime could form non-hydrogen bonding interactions with the tyrosinase residues Arg₂₆₈, Phe₂₆₄, Ser₂₈₂, Val₂₈₃, His₂₆₃, Thr₈₄, His₆₁, Trp₉₃, His₈₅, His₉₄, Cys₈₃, Phe₉₀, His₂₅₉, Glu₂₅₆, His₂₄₄, Asn₂₆₀ (**Figure 5C**).

DISCUSSION

Tyrosinase is a multifunctional copper-containing oxidase (20) and an important marker of human diseases such as melanoma and the neurodegeneration associated with Parkinson's disease. Discovery of effective tyrosinase inhibitors is of great urgency in the cosmetic and medical fields (27). The results in this paper show that cefotaxime inhibits both the monophenolase and diphenolase activity of mushroom tyrosinase. The IC_{50} value for the monophenolase activity is about twenty-three times greater than that for the diphenolase activity, indicating that cefotaxime inhibits diphenolase activity more strongly than monophenolase activity. L-DOPA is an important neurotransmitter substance. L-tyrosinase could convert to L-DOPA in the

216 action of monophenolase, and L-DOPA converted to DOPA quinone in the action of
217 diphenolase (28).

218 The mechanism of fluorescence quenching between tyrosinase and cefotaxime
219 was static, which indicates that cefotaxime and tyrosinase form a ground state
220 complex that has no fluorescence. It could be concluded that the structure of
221 tyrosinase changed on addition of cefotaxime (23).

222 The molecular docking results showed cefotaxime could dock in the active site of
223 tyrosinase with a calculated binding energy of -12.76 kcal/mol. Cefotaxime was
224 directly involved in tyrosinase inhibition not simply via copper chelation, but in a
225 mixed-type manner. This result is consistent with previous predictions of oxalic acid
226 docking with tyrosinase (29). Yin et al. also reported that phthalic acid bound to
227 tyrosinase in a mixed-type manner rather than binding the copper ion (30).

228 In conclusion, the results in this paper indicate that cefotaxime might be used as
229 an adjunctive therapy for treatment of melanin hyperpigmentation and
230 L-DOPA-related disorders such as Parkinson's disease.

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Legend for figures

Figure 1. Chemical structure of cefotaxime.

Figure 2. Progress curves for the inhibition of monophenolase activity of mushroom tyrosinase 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.

Figure 3. (A) Effect of cefotaxime on the diphenolase activity of mushroom tyrosinase. (B) Determination of the inhibitory mechanism of cefotaxime on mushroom tyrosinase. The concentrations of cefotaxime for curves 1–6 were 0, 0.025, 0.05, 0.075, 0.10 and 0.125 mM, respectively. (C) Determination of the inhibitory type and inhibition constants of cefotaxime on mushroom tyrosinase. (a) Lineweaver-Burk plots for inhibition of mushroom tyrosinase by cefotaxime. The concentrations of cefotaxime for curves 1–6 were 0, 0.025, 0.05, 0.075, 0.10 and 0.125 mM, respectively. Panels (b) and (c) plot the slope and intercept *versus* the concentration of cefotaxime for determining the inhibition constants K_I and K_{IS} , respectively.

Figure 4. Changes in intrinsic tyrosinase fluorescence at different concentrations of cefotaxime. (A) Emission spectra 1-11 of tyrosinase in the presence of cefotaxime for 0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 0.10 mM, respectively. (B) Maximum fluorescence intensity changes. (C) Stern-Volmer plot describing the tyrosinase quenching caused by association with cefotaxime. (D) Plot of $\log[(F_0-F)/F]$ against $\log[I]$ for tyrosinase and various concentration of cefotaxime. F_0 and F are the fluorescence intensities before and after the addition of cefotaxime.

Figure 5. Docking pose of cefotaxime in the binding center of tyrosinase. (A) Tertiary

360 structure for the interaction of cefotaxime with tyrosinase. (B) The interaction of
361 cefotaxime with tyrosinase residues in the active site. (C) 2D structure for the
362 interaction of cefotaxime with tyrosinase residues. In the structures, cefotaxime is
363 depicted by yellow sticks.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5