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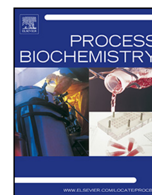

Inhibitory mechanism of cardanols on tyrosinase

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ABSTRACT

Cashew nut shell liquid (CNSL), extracted from cashew nut shell, is an abundant natural resource. Cardanols were the major phenolic components isolated from CNSL. In this research, we reported on the inhibitory mechanism of cardanols on tyrosinase for the first time. We studied the functions of cardanols and revealed the underlying mechanism of cardanols as tyrosinase inhibitors. Cardanol triene, cardanol diene and cardanol monoene could decrease the steady-state rate of the tyrosinase diphenolase activity efficiently. The IC_{50} values of three cardanol compounds were determined to be 40.5 ± 3.7, 52.5 ± 3.2 and 56.0 ± 3.6 μM (n = 3), respectively. Meanwhile, the kinetic analysis and the intrinsic/ANS-binding fluoresecence-quenching showed that one cardanol might enter into one tyrosinase. The characteristic values further revealed that cardanols could interact with tyrosinase. Besides, computational study with molecular docking implied that cardanols might affect the amino acid residues of the tyrosinase active site. Collectively, cardanols could moderate inhibitory activities on tyrosinase effectively.
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1. Introduction

Tyrosinase (EC 1.14.18.1) is widely distributed in nature as *o*-diphenol-oxygen oxidoreductase, copper-containing polyphenol oxidase [1]. Tyrosinase from various organisms have similar structures and characteristics. Two copper irons with three states (E_{oxy}、E_{met}、E_{deoxy}) are the point of the tyrosinase active site [2]. There were two key reactions, the hydroxylation of *p*-monophenolic amino acid L-tyrosine (L-Tyr) (monophenolase activity of tyrosinase) and the oxidation of *o*-diphenolic amino acid L-3, 4-dihydroxyphenylalanine (L-DOPA) to the corresponding *o*-quinone that ultimately transforms to melanin (diphenolase activity of tyrosinase). At present, how to control the melanogenesis becomes a hot topic of life science. Disordered or excessive accumulation of pigmentation leads to various dermatological disorders, such as melasama, age spots and actinic damage [3]. Tyrosinase inhibitors are very important for therapy of clinical phenotypes. A certain amount of inhibitors are not available for clinic treatment for their insolubility, high toxicity or low activity [4]. Therefore, tyrosinase inhibitors extracted from natural products will become a new research direction with broad application prospects [5].

Cashew nut shell liquid (CNSL) is an efficiently available material. CNSL was treated as industrial waste [6]. In recent years, studies show that CNSL has been widely applied in enormous industrial products, polymerization products and combination with other materials. CNSL mainly consists of cardanols (60–65%), cardols (15–20%), polymeric material (10%), and traces of methyl cardol. There are some efficient methods for the separation of different components from technical CNSL [7–9]. Their structures were determined by nuclear magnetic resonance spectroscopic analyses (NMR) [10,11]. In previous studies, ELMER-RICO EMOJICA [12] and ISAO KUBO [13] reported that CNSL showed the significant inhibition of tyrosinase activity. There were many researches on its components, such as anacardic acids and cardols except cardanols [14–16].

Abbreviations: L-DOPA, L-3,4-dihydroxyphenylalanine; L-Tyr, L-tyrosine; CNSL, cashew nut shell liquid; ANS, 1-anilinonaphthalene-8-sulfonate.

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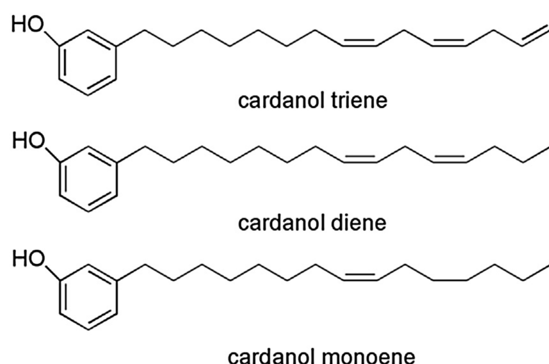

Fig. 1. Chemical structures of cardanols from cashew nut shell liquid (CNSL).

Though cardanols and various their derivatives have been associated with various of biological effects [15,17,18], the inhibitory mechanism on regulating tyrosinase activity is still unknown. Cardanols are rich sources of long-chain alkyl substituted salicylic acid and resorcinol [19,20]. Their chemical structures were showed in Fig. 1. We suggested that they might suppress the generation of melanin by specifically inhibiting tyrosinase. Zhuang et al. [21] have demonstrated that the inhibitory mechanism of cardol triene on tyrosinase. In this work, we continued to investigate the inhibitory kinetic course of tyrosinase by cardanols and explored their interaction mechanism. This study will provide a comprehensive understanding of the inhibitory regulation by cardanols in vivo.

2. Materials and methods

2.1. Materials

Cardanols were separated and purified from CNSL [21], which was provided by Xiamen Welso Co., Ltd. Mushroom tyrosinase (Agaricus bisporus) was the product of Sigma-Aldrich (St. Louis, MO, USA). The specific activity of the enzyme was 6680 U/mg. L-DOPA, L-Tyr, dimethylsulfoxide (DMSO) were also obtained from Sigma-Aldrich. Other reagents were to be analytical grade. The water used was redistilled and ion-free.

2.2. Enzyme activity assay

The reaction media (3 mL) for enzyme activity assay was previously reported [21,22]. It contained 0.5 mM L-DOPA and 100 μL different concentrations of inhibitors dissolved in 3.3% DMSO. The reaction was added the substrate (L-DOPA). Along with the oxidation of L-DOPA, the density at 475 nm ($\varepsilon = 3700\ M^{-1}\ cm^{-1}$) was checked at each concentration of inhibitors. Tyrosinase was firstly incubated with inhibitors using a Beckman DU800 spectophotometer to make the absorbance and kinetic measurements [23]. Temperature was controlled at 37 °C to keep the stability of the system [2]. All were performed in 50 mM sodium phosphate buffer (pH 6.8).

In this method, the effects of inhibitors can be evaluated [24]. The reaction at each concentration was carried out along with controls of 3.3% DMSO without inhibitors. The values of IC_{50} (the inhibited 50% of the enzyme activity) were calculated from triplicate measurements to express the inhibitory effects of inhibitors on the enzyme. The inhibition types (irreversible & reversible) and the inhibition constants were obtained using the method described by Chen et al. [25,26].

2.3. UV scanning study

The experiment contained 3 mL reaction media and was measured described by Jiménez-Atiénzar et al. [27]. The mixture included 0.5 mM L-DOPA and 100 μL of inhibitors (dissolved in DMSO) in 50 mM sodium phosphate buffer (pH 6.8). The oxidation process of L-DOPA was recorded at the absence and presence of inhibitors. These assays were monitored for the formation of dopachrome at 475 nm using a Beckman DU-800 spectrophotometer [28]. The final concentration of tyrosinase was 16.67 μg/mL.

2.4. Inhibitory kinetic course of tyrosinase

The experiment was performed based on the method in a previous study [21]. L-DOPA was used as the substrate. The substrate-enzyme reaction was tested to detect the inhibitory kinetic, and kinetic course of the enzyme was carried out on the following conditions. The 3.0 mL reaction contained 50 μL of enzyme and 100 μL of different concentrations of inhibitors. All tests were conducted at a constant temperature of 30 °C, and reacted in 0.1 M sodium phosphate buffer (pH 6.8). During the oxidation of L-DOPA, the absorbance at 475 nm ($\varepsilon = 3700\ M^{-1}\ cm^{-1}$) was monitored to determine the enzyme activity. The final concentration of tyrosinase was 16.67 μg/mL. The kinetic constants were obtained with reference to Chen et al. [29].

2.5. Intrinsic and ANS-binding fluorescence measurement

Fluorescence spectra were measured by the methods of Kim [30] and Ioniţă [31] with some modification. To investigate the interaction of inhibitors and tyrosinase, the fluorescence intensities were recorded using a Varian Cary Eclipse fluorophotometer with an excitation wavelength (λ_{ex}) of 280 nm and a range of emission wavelength (λ_{em}) from 300 to 450 nm. The excitation and emission slit widths were both 5 nm, and the scan speed was 600 nm/min. Assays of system contained 2.0 mL mixture with tyrosinase and different concentrations of inhibitors [32]. The change of the fluorescence emission intensity was measured with 1 min blending. Each measurement was recorded in triplicate. The final concentrations of tyrosinase were 33.33 μg/mL.

In addition, the ANS-binding fluorescence intensity of tyrosinase was studied by the emission wavelength ranged from 400 to 600 nm with an excitation wavelength of 350 nm. Tyrosinase was labled with 100 μM ANS for 5 min first, and the other measurements were the same with the intrinsic fluorescence experiments [33,34].

2.6. Molecular docking with ligand

The molecular operation environment software (MOE) is an effective tool to study the interaction of tyrosinase and ligands. In the docking, the parameters should be set accurately consulted with the previous research [26]. After the parameter setting, the tertiary structures of inhibitors and tyrosinase were energy minimized. The receptor and site denote the receptor atoms and dummy atoms, respectively. The docked conformations are improved with the highest score.

3. Results and discussion

3.1. Effects of cardanols on the diphenolase activity of tyrosinase

Effects of cardanols on the oxidation of L-DOPA by tyrosinase were studied. Cardanols inhibited tyrosinase activities dramatically with a dose-dependent manner. The relative activities of enzyme were all reduced to around 40% by cardanol triene, cardanol diene

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