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Anti-tyrosinase kinetics and antibacterial process of caffeic acid *N*-nonyl ester in Chinese Olive (*Canarium album*) postharvest

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ABSTRACT

Enzymatic browning and bacterial putrefaction are mainly responsible for quality losses of Chinese Olive (*Canarium album*) postharvest and lead to very short shelf life on average. Screening anti-browning and anti-bacterial agents is important for preservation of Chinese Olive. Caffeic acid *N*-nonyl ester (C-9) and caffeic acid *N*- Heptyl ester (C-7) was synthesized as inhibitors of tyrosinase, which is a key enzyme in browning process. The compound of C-9 could inhibit the activity of tyrosinase strongly and its IC_{50} value was determined to be 37.5 μM, while the compound of C-7 had no inhibitory ability. Kinetic analyses showed that compound of C-9 has been a reversible inhibitory mechanism below 50 μM and been irreversible mechanisms above 50 μM. For the reversible inhibitory mechanism, the values of inhibitory constants (K_I and K_{IS}) were determined to be 24.6 and 37.4 μM, respectively. The results of Chinese Olive fruit postharvest showed that the compound of C-9 could effectively anti-browning and anti-bacterial putrefaction. In addition, this compound had strong antibacterial activities against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis* and *Salmonella*. Therefore, C-9 could be a potential anti-browning and anti-bacterial reagent.

1. Introduction

Chinese Olive (*Canarium album*), a native species in the southeast of China, has been found with relatively broad in traditional medicine material that has some pharmacological functions [1]. Some previous researches reported that the dried fruits of Chinese Olive have activity of an anti-bacterium, anti-virus, anti-inflammation and detoxification [2]. Like the Mediterranean olive (*Olea europaea* L.), Chinese olive fruit was a fusiform drupe, the fruit flesh has the characteristics of strong bitter and astringent tastes [3]. Chinese olive (*Canarium album* L.), one native and well-known tropical fruit tree in the southeast of China, contain a large amount of phenolic compounds and possess great pharmacological activities [4]. Browning and bacterial putrefaction were mainly responsible for the quality loss of Chinese Olive postharvest [5].

Caffeic acid, an analogue of cinnamic acid, has been found in plants such as the seeds of *Cuscuta chinensis* [6]. Recently, various functions have been identified for caffeic acid and its derivatives, including anti-tumour [7], anti-inflammatory [8], and anti-oxidation [9] effects and as a prophylactic for malathion-induced neuropeptides [10]. In addition, many caffeic acid analogues also had great potential as anti-fungal and anti-bacterial agents [11]. Huleihel M et al. found that the caffeic acid phenethyl ester could effect some biomarker in bacteira by FTIR microspectroscopy [12]. Caffeic acid also could present antibacterial properties by pH dependent [13]. In particular, caffeic ester derivatives, such as caffeic acid phenethyl ester, with diverse functions had attracted considerable attention recently [14]. We had synthesized many types of caffeic ester analogues and studied the different biological activities of analogues. In those studies, we observed an effect of C-9 and C-7 on tyrosinase.

Abbreviations: C-9, caffeic acid *n*-nonyl ester; C-7, caffeic acid *n*-heptyl ester; DMAP, 4-dimethylaminopyridine; EDC·HCl, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; DMSO, dimethyl sulphoxide; L-DOPA, 3,4-dihydroxy-L-phenylalanine; 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; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

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Tyrosinase (EC 1.14.18.1) was a multifunctional copper-containing oxidase that was widely founded in microorganisms [15]. Tyrosinase was found in many species of bacteria and usually associated with melanin synthesis [16]. Tyrosinase was ubiquitously distributed in most species and essential for pigmentation. The active site of tyrosinase was characterized by a pair of antiferromagnetically coupled copper ions [17]. Tyrosinase catalysed the hydroxylation of phenols to catechols and the oxidation of catechols to quinones [18]. This catalytic process was very important. Enzymatic browning was a major factor in the Maillard reaction. Browning contributes to quality loss in foods and usually reduces the sensory properties of foods. There were associated changes in colour, smell and nutrients, which resulted in a short shelf-life and market value [19]. Tyrosinase inhibitors had been established as important constituents of anti-browning agents and had potential uses as food additives [20]. In our previous papers, alkyl 3,4-dihydroxybenzoates [21], chlorobenzaldehyde thiosemicarbozones [22] and cardol triene [23] had strong inhibitory effects on mushroom tyrosinase.

Due to the importance of tyrosinase inhibitors, the aim of our work was to identify novel compounds that inhibit tyrosinase. The inhibition of the diphenolase activities of tyrosinase was an important criterion used in the evaluation of tyrosinase inhibitors. Therefore, we synthesized new types of caffeic acid derivatives and investigated the kinetics of the inhibition on tyrosinase by C-9, which was an unvalued derivative of caffeic acid. We measured the kinetic parameters and inhibition constants characterizing the mechanism of inhibition. Furthermore, we found that C-9 has two different inhibitory mechanisms within two concentration ranges. That was quite different from other tyrosinase inhibitors we previously studied [23–25]. We also found that C-9 had stronger bacteriostatic activity than previously identified compounds [26]. These data may provide the basis for novel tyrosinase inhibitors and potent new food additives or antibacterial agents.

2. Materials and methods

2.1. Materials

2.1.1. Raw materials

Olive fruits for the main analysis and bioassays were generously provided by Prof. He-Tong Lin from the collection of varieties maintained at Fuzhou (Fujian Agriculture and Forestry University, Fujian Province, China).

2.1.2. Reagents

Mushroom tyrosinase (EC 1.14.18.1) was purchased from Sigma-Aldrich (St. Louis, MO). The activity of the enzyme is 5037 U/mg. 3,4-dihydroxy-L-phenylalanine (l-DOPA) was purchased from Aldrich (St. Louis, MO, USA). Caffeic acid and *n*-nonyl alcohol were the products of TCI Chemicals Co. Ltd. (Shanghai, China). The purities of these chemical compounds are 99.99% and 99.99%, respectively. The positive control Streptomycin Solution was purchased from Becton Dickinson Medical Devices Co. Ltd. (Shanghai). *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Agrobacterium tumefaciens* and *Salmonella* were collected from a colony preserved at −80 °C at the Fujian Academy of Agricultural Sciences. All other reagents were analytical grade products obtained from Aldrich (St. Louis, MO, USA). The water was redistilled and ion-free.

2.2. Methods

2.2.1. Synthesis method

This compound was prepared by a reaction of caffeic acid with nonyl ester in a solution of acetone and trichloromethane. A mixture of caffeic acid (30 mM) with nonyl ester (30.5 mM) in 60 mL of acetone and trichloromethane with 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) (30 mM) and 4-dimethylaminopyridine (DMAP) (0.05 mM) solution was refluxed at 85 °C for 5–7 h and then cooled to 25 °C. The yellow viscous oily liquid was collected and purified on a silica column. The products were purified by recrystallization from trichloromethane and methanol and were identified by ESI–MS and ^{1}H NMR data. ESI–MS data were obtained on a Bruker Esquire-LC. The ^{1}H NMR data were acquired on a 600 MHz NMR spectrometer (AV600) from Bruker.

2.2.2. Anti-tyrosinase assay

The activities of mushroom tyrosinase were determined by a previously reported method [27] using a Beckman UV-800 spectrophotometer. C-9 was first dissolved in dimethyl sulphoxide (DMSO) and diluted into DMSO solutions of the inhibitors in different concentrations. The inhibitory effects of the inhibitors on the diphenolase activity of tyrosinase were determined by a previously reported method [28]. The effect of different concentrations of mushroom tyrosinase on the catalysis of DOPA at different concentrations of C-9 was determined as follows. The assay conditions were a 3-mL reaction system containing 0.05 M phosphate sodium buffer, pH 6.8, 0.5 mM l-DOPA and different concentrations of C-9 (0, 10, 20, 30, 40, 50, 60 and 70 μM). The final concentration of DMSO was kept at 3.3% in the reaction media. The inhibition types of the inhibitors on the diphenolase activity of mushroom tyrosinase were determined as follows. The concentrations of inhibitors were unchanged, and the concentrations of substrates (l-DOPA for the diphenolase activity assay) were changed. The concentrations of l-DOPA ranged from 0.20 to 1.00 mM and 0.25 to 0.67 mM. The final concentration of mushroom tyrosinase was 6.67 μg/ml in the diphenolase activity assay. Controls without inhibitor but containing 3.3% DMSO were routinely included. The inhibition types of the inhibitors on the enzyme were assayed by Lineweaver-Burk plots, and the inhibition constants were measured by the plot of the intercept versus the concentration of the inhibitor. The extent of inhibition by the addition of the sample was expressed as the percentage necessary for 50% inhibition (IC_{50}). The inhibition type was assayed by a Lineweaver-Burk plot, and the inhibition constant was determined by the second plots of the apparent Km/Vm or 1/Vm versus the concentration of the inhibitor. The reactions were executed at a constant temperature of 30 °C.

2.2.3. Fluorescence quenching

Fluorescence quenching means the decrease of fluorescence intensity between the fluorescent and solute molecules. It was usually used to study the interaction between conformation of protein molecules and small molecules [29]. Cary Eclipse fluorescence spectrophotometer was used to record the fluorescence intensities with an excitation wavelength of 280 nm and emission slit widths of 5 nm [30]. C-7 and C-9 did not have any fluorescence phenomenon at this excitation wavelength. In this study, the compound was added in 0.2 mg/ml tyrosinase solution to detect the fluorescence intensity changes and the final concentrations of inhibitor range from 25 to 75 μM.

2.2.4. Preservation of Chinese Olive

The Olive fruits were harvested from Min-Hou district of Fuzhou country of Fujian province, China, at mature stage. Then, it was transported the fruits under ambient conditions within 4 h to the laboratory. Any mechanical damage Olive fruits were excluded and the size of fruits was selected. The fruits were washed with bacteria free water in 3 times and air-dried. The selected fruits were dipped in each solution for 15 mins and air-dried, then packed in 0.015 mm thick polyethylene bags(30 fruit per bag) and stored at (37 ± 1) °C and 80% ± 5% relative humidity. The fruits dipped in bac-