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4-Hydroxy cinnamic acid as mushroom preservation: Anti-tyrosinase activity kinetics and application

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ABSTRACT

Tyrosinase is a key enzyme in post-harvest browning of fruit and vegetable. To control and inhibit its activity is the most effective method for delaying the browning and extend the shelf life. In this paper, the inhibitory kinetics of 4-hydroxy cinnamic acid on mushroom tyrosinase was investigated using the kinetics method of substrate reaction. The results showed that the inhibition of tyrosinase by 4-hydroxy cinnamic acid was a slow, reversible reaction with fractional remaining activity. The microscopic rate constants were determined for the reaction on 4-hydroxy cinnamic acid with tyrosinase. Furthermore, the molecular docking was used to simulate 4-hydroxy cinnamic acid dock with tyrosinase. The results showed that 4-hydroxy cinnamic acid interacted with the enzyme active site mainly through the hydroxy competed with the substrate hydroxy group. The cytotoxicity study of 4-hydroxy cinnamic acid indicated that it had no effects on the proliferation of normal liver cells. Moreover, the results of effects of 4-hydroxy cinnamic acid on the preservation of mushroom showed that it could delay the mushroom browning. These results provide a comprehensive underlying the inhibitory mechanisms of 4-hydroxy cinnamic acid and its delaying post-harvest browning, that is beneficial for the application of this compound.

1. Introduction

Tyrosinase (EC 1.14.18.1) is a copper containing mixed-function enzyme [1]. It has monophenolase and diphenolase activities which can catalyze both the hydroxylation of monophenol and the oxidation of *o*-diphenol [2]. The crystallographic structure of tyrosinase has been reported, and the active site of tyrosinase is composed of six conserved histidine residues coordinating two copper ions [3,4]. It is common in animals, plants, fungi and microorganisms. Moreover, the formation of the melanin of skin, hair, and eye are caused by the activity of tyrosinase [5]. Tyrosinase is the most influencing factor in the post-harvest browning of fruits and vegetables since tyrosinase and their polyphenolic substrates are mixed when cell strucutres are ruptured, resulting in the drop in its commercial value [6].

In mushroom crops, browning after harvest is a common phenomenon, and the loss of whiteness upon storage is particularly important [7]. Mushrooms are fungi which have a high water content and metabolic activity, therefore, naturally have a short shelf life [8]. Most research on the white buttom mushroom (*Agaricus bisporus*) has associated with tyrosinase. Cool storage could slow the rate of deterioration, but too low temperatures may cause chilling injury [9]. Therefore, the most effective method to delay the browning and extend the shelf life is to reduce or restrain tyrosinase activity. Meng et al. [10] reported that methyl jasmonate could inhibited the activities of polyphenoloxidase and lowered relative expression levels of three genes encoding polyphenol oxidase throughout the storage period. Jiang [11] reported that natamycin in combination with pure oxygen treatment could inhibited the activities of PPO stored at 4 °C for 16 days.

4-Hydroxy cinnamic acid also known as *p*-coumaric acid is one of cinnamic acid derivatives. Cinnamic acid derivatives are important compounds with a wide range of biological activities: antibacterial, antifungal, antioxidant, antiinflammatory and antitu-

Abbreviations: MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide; IC_{50}, the inhibitor concentrations leading to 50% activity lost; L-DOPA, L-3.4-dihydroxyphenylalanine; MOE, molecular operation environment; PBS, phosphate buffer solution; K_I, equilibrium constant of the inhibitor combining with the free enzyme; PPO, polyphenol oxidase; P_t, the tested amount of product at the time t; P_{calc}, the calculated amount of product at the time t; S, the concentration of substrate (l-DOPA).

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moral [12,13]. Thakkar et al. synthesized two carboxylated lignins based on 4-hydroxy cinnamic acid scaffold and found them not only inhibit HSV-1 entry into mammalian cells, but more petent than sulfated lignins [14]. Shi et al. reported that 4-hydroxy cinnamic acid could inhibit the diphenolase activity of mushroom tyrosinase with the IC_{50} value 0.50 mM, the inhibition mechanism was reversible and competitive [15].

The aim of this work was to determine the inhibitory kinetics model and parameter of 4-hydroxy cinnamic acid on mushroom tyrosianse. Molecular operation environment (MOE) was used to simulate 4-hydroxy cinnamic acid dock with tyrosinase. Moreover, the 4-hydroxy cinnamic acid used as an active agent to delay postharvest deteriotation in mushroom (*A. bisporus*) was also investigated. The results could provide theory and practice in delaying the browning of mushroom to extend the shelf life.

2. Materials and methods

2.1. Materials

Mushroom tyrosinase (EC 1.14.18.1) purchased from Sigma–Aldrich (St. Louis, MO). The activity of the enzyme is 1 MU. L-3,4-Dihydroxyphenylalanine (L-DOPA) was purchased from Sigma Chemical Co. (St. Louis, Mo, USA). 4-Hydroxy cinnamic acid was purchased from Sangon Biotech (Shanghai, China). All other reagents were of analytical grade. The water used was re-distillated and ion-free.

2.2. Methods

2.2.1. Enzyme activity

The progress-of-substrate-reaction method as previously described by Tsou [16] was used to study the inhibition kinetics of tyrosinase in reaction system containing 4-hydroxy cinnamic acid. In this method, 50 μL of tyrosinase (0.2 mg/mL) was added to 3.0 mL assay system containing different concentrations of L-DOPA in 0.2 M pH 6.8 PBS with different concentrations of 4-hydroxy cinnamic acid. The reaction was carried out at a constant temperature of 30 °C. The substrate reaction progress curve which was obtained using DU-800 spectrophotometer was analyzed to obtain the rate constants. The previous results showed that the inhibition of 4- hydroxy cinnamic acid was a competitive reaction. Hence, the kinetics model was just as Xie et al. [17] showed and the microscopic rate constant k_{-0} and k_{+0} could be obtained as follows:

Plots of $\ln([P]_{calc}-[P]_t)$ *versus* t give a series of straight lines at different concentrations of 4-hydroxy cinnamic acid ([I]) which slopes is $-(A[I]+B)$. A secondary plot of the slopes *versus* [I] gives a straight line which could obtain the apparent forward (A) and reverse rate constants (B). The value of B directly gives the microscopic rate constant k_{-0}. A plot of $1/A$ versus [S] gives a straight line with $1/k_{+0}$ as the intercept on ordinate axis, so the rate constant k_{+0} can be obtained.

2.2.2. Molecular docking study

Molecular operation environment 2010 software (MOE) was used for tyrosinase-inhibitor docking in this research. The structure of the deoxy tyrosinase from *A. bisporus* named PPO3 (pdb entry 2Y9W) [4] was used as the initial model for docking simulations after removal of the caddie protein, the exogenous ions and water molecules. And the 3D structures of the inhibitor was also prepared with MOE. Before docking, the structure models of tyrosinase and inhibitor were energy minimized using the energy minimization module of the MOE. For molecular docking, the refinement was set to forcefield and the retain of both the first and second scoring were set to 10. Docking poses were ranked by the MM/GBVI binding free energy scoring. More negative values reflect stronger interactions. Parameters not mentioned here were the default settings of the MOE software. The mode of binding analyzed was based on the docked conformation which had the highest score [18,19].

2.2.3. Cytotoxicity experiment

Normal liver cell L02 was used to determine the cytotoxicity of 4-hydroxy cinnamic acid. It was determined using the MTT assay. Cells were seeded in a 96-well plate at a density of 1×10^5/mL. After overnight growth, the cells were treated with various concentrations of 4-hydroxy cinnamic acid for 24 h and 48 h. After treated with 4-hydroxy cinnamic acid, 200 μL MTT (5 mg/mL) was added to each well, and the cells were cultured for another 4 h at 37 °C. The medium was then removed, and 150 μL DMSO was added to each well. The absorbance of each well was recorded at 490 nm [20].

2.2.4. Mushroom preservation

Mushroom (*A. bisporus*) were harvested from Factory of Nan Yuan in Zhangzhou of Fujian province, China, at commercial mature stage. Then, transported the mushroom under ambient conditions within 2 h to the laboratory. As 2796 is the tested mushroom strain. Any mechanical damaged mushroom was excluded and the uniformity of maturity and size of fruit was selected. The selected fruits were dipped in each solution for 1 min and air-dried, and then packed in 0.015 mm thick polyethylene bags (50 fruits per bag) and stored at (20 ± 1) °C and 85% ± 5% relative humidity. The fruits dipped in distilled water were used as control. The concentrations of 0, 5, 10, 50 and 100 μM of 4-hydroxy cinnamic acid were used in the preliminary study. And the results indicated that 10 μM 4-hydroxy cinnamic acid most effectively reduced mushroom browning. There were three replicates each per treatment.

2.2.5. Browning degree and surface color

The browning degree was used to determine the browning of the mushroom. 1 g pellicle from 10 mushrooms was grind with 2 mL 60% methanol, 0.2 g insoluble polyvinylpyrrolidone and a little silica sand. Then eluted in 8 mL 60% methanol. The supernatant was collected after centrifugated at 6000 r/min for 20 min, then diluted to 20 mL with 0.1 M pH 6.8 PBS. The supernatant was measured spectrophotometrically at 450 nm (Beckman DU-800), the numerical reading indicated the browning degree [21].

The surface color (L*) of mushroom caps were measured with an ADCI-60-C colorimeter (Beijing Chentaike Instrument Co. Ltd., Beijing, China). To analyze the L* value, each mushroom was measured at three points of the cap (n = 12 measurements per replicate). L* value of 0 and 100 represent black and white, respectively [22]. A white calibration plate was used for calibration (X = 82.15; Y = 86.86; Z = 90.50).

3. Results and discussion

3.1. Kinetics of the substrate reaction in the presence of different concentrations of 4-hydroxy cinnamic acid

The time course of L-DOPA catalyzed by tyrosinase in the presence of different 4-hydroxy cinnamic acid concentrations is shown in Fig. 1a. At the concentration of 4-hydroxy cinnamic acid, the reaction rate decreased with the increasing of time until approached a straight line, the slope of which decreased with an increase in the concentrations of 4-hydroxy cinnamic acid. The results showed that the formation of the enzyme-inhibitor complex was a slow and reversible reaction. Although, the enzyme and inhibitor binded into inert complex, since the inhibition was reversible, there was a section of enzyme not combined with the inhibitor until the reaction reached a final state, thus it showed some residual enzyme activity. Plots of $\ln([P]_{calc}-[P]_t)$ versus t give a family of straight lines at