1 **4-Hydroxy cinnamic acid as mushroom preservation:**

2 **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 provides 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.

Keywords: 4-hydroxy cinnamic acid; inhibitory kinetics; mushroom preservation

1. Introduction

Tyrosinase (EC 1.14.18.1) is a copper containing mixed-function enzyme which also called polyphenol oxidase [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 structures 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 button 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 antitumoral [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 potent 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 tyrosinase. 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 deterioration in mushroom (*Agaricus 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 μ L 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 $^{\circ}$ 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 $\ln([P]_{\text{calc}}-[P]_t)$ 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 strusture of the deoxy tyrosinase from *Agaricus 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 strusture 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 $1 \times 10^5/\text{mL}$. 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 (*Agaricus 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 were excluded and the uniformity of maturity and size of fruit were 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 $(20 \pm 1) ^\circ\text{C}$ and $85\% \pm 5\%$ 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 was 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 was showed some residual enzyme activity. Plots of $\ln([P]_{\text{calc}}-[P]_t)$ versus t give a family of straight lines at different concentrations of 4-hydroxy cinnamic acid with slopes of $-(A[I]+B)$, which was showed in Fig. 1b.

(Insert Fig. 1)

3.2. Kinetics of the reaction at different substrate concentration in the presence of 4-hydroxy cinnamic acid

The kinetic courses of the reaction at different L-DOPA concentrations in the presence of 0.5 mM 4-hydroxy cinnamic acid was showed in Fig. 2. When the reaction time (t) is sufficiently long, both the initial rate and the slope of the asymptote increased with the increasing of substrate concentrations (Fig. 2a). Similarly, plots of $\ln([P]_{\text{calc}}-[P]_t)$ versus t give a family of straight lines at different concentrations of the substrate with slopes of $-(A[I]+B)$, which was showed in Fig. 2b. The apparent forward (A) and reverse rate constants (B) can be obtained through the lines.

(Insert Fig. 2)

3.3. Determination of the microscopic rate constants of inhibition of the enzyme by 4-hydroxy cinnamic acid

A plot of the slopes of the straight lines in Fig. 1b against the concentration of 4-hydroxy cinnamic acid gave the straight line, curve 1 in Fig. 3a. Similarly, the datas in the other straight lines in Fig. 3a were collected from other concentrations of substrate (figures did not show). All the straight lines have a common intercept on the ordinate. The value of A was obtained from the slope of the straight line, which was showed in Table 1. And the ordinate intercept was the apparent reverse rate constant B .

The value of B is equal to the microscopic rate constant k_{-0} . The intercept on the ordinate from the straight line of $1/A$ versus $[S]$ is equal to the $1/k_{+0}$, so microscopic rate constant k_{+0} could be obtained (Fig. 3b). Since K_m and V_{max} were known from the substrate reaction in the absence of 4-hydroxy cinnamic acid. The plot of A/v versus $1/[S]$ gave a straight line which passes through the origin (Fig. 3c). The slope of the straight line gave the value of $(K_m \cdot k_{+0})/V_{max}$. The microscopic rate constant k_{+0} was then also obtained from the slope, k_{+0} was $1.26 \times 10^3 \text{ mM}^{-1} \cdot \text{s}^{-1}$, k_{-0} was $7.9 \times 10^{-4} \text{ s}^{-1}$, and K_I was 0.67 mM.

(Insert Fig. 3)

(Insert Table 1)

3.4. Molecular docking analysis

The docking mode of 4-hydroxy cinnamic acid was examined in the catalytic site of the tyrosinase to understand the inhibitory mechanism of 4-hydroxy cinnamic acid. Fig. 4 showed that the docked conformation of 4-hydroxy cinnamic acid in the tyrosinase catalytic center. The docking pose of 4-hydroxy cinnamic acid in the active site of tyrosinase was shown in Fig. 4a. The docked conformation revealed that the oxygen ion located in para-hydroxy of 4-hydroxy cinnamic acid could form metal interaction with Cu^+ of the enzyme. The hydroxy in the carboxyl of 4-hydroxy cinnamic acid could form the side chain with tyrosinase residue Asn₂₆₀. Moreover, 4-hydroxy cinnamic acid could also form interactions with tyrosinase residues: His₂₆₃, Val₂₈₃, His₂₅₉, Phe₂₆₄, Asn₂₆₀, Glu₂₅₆, His₈₅, His₂₄₄, His₆₁ and Phe₂₉₂ (Fig. 4c).

(Insert Fig. 4)

3.5. Effect of 4-hydroxy cinnamic acid on proliferation of normal liver cells L02

To evaluate the cytotoxicity of 4-hydroxy cinnamic acid on the proliferation of normal liver cells, L02 cells were treated with 0, 25, 50, 75, 100, 200 and 400 mM 4-hydroxy cinnamic acid for 24 h and 48 h, respectively, which were showed in Fig. 5. After treated for 24 h, the survival rates were 91.64% to 100.23%. While after treated for 48 h, the survival rates were 97.15% to 102.23%. It indicated that there is no effect on the proliferation of L02 with the treated different concentrations of 4-hydroxy cinnamic acid. Therefore, 4-hydroxy cinnamic acid is no cytotoxicity and is safe.

(Insert Fig. 5)

3.6. Effect of 4-hydroxy cinnamic acid on the preservation of mushroom (Agaricus bisporus) stored at 20 °C

The effect of 4-hydroxy cinnamic acid on the preservation of mushroom was showed in Fig. 6. As it was showed in Figure 6a~6d, after stored at 20 °C for 4 d, the untreated mushroom became brown and rotted (Fig. 6a), the cap and veil of mushroom was detached which became open cap (Fig. 6c), and the quality of the mushroom was too bad to accept. On the contrary, the mushroom treated with 4-hydroxy cinnamic acid maintained the inherent characteristics with the white and unopen caps (Fig. 6b and 6d). From the apparence of the mushroom, it could be indicated that 4-hydroxy cinnamic acid could delay the browning and open cap of mushroom.

Browning degree is an indicator to describe the mushroom epidermal browning. The browning degree increased with the storage time increasing which was showed in Fig. 6e. Compared with the treated mushroom, the control mushroom increased more rapidly during the whole storage. L^* value is another browning index. From Fig. 6f, L^* value decreased with the storage time increasing. It decreased rapidly from 2 d both treated and control mushroom, while treated mushroom decreased more slight

than control mushroom. The L^* value in the control samples sharply decreased to 69.42 in 4th day. However, the L^* value in treated samples was 71.90 in 4th day. Mushroom with L^* values <69 were considered as unacceptable from a whiteness point of view at wholesale or consumer levels respectively [10]. Hence, 4-hydroxy cinnamic acid could be considered as mushroom color protector.

(Insert Fig. 6)

Since tyrosinase become more important in the fields of cosmetic, agaricultural and medicinal, the tyrosinase inhibitors with higher bioactivities and lower side effects is required. This paper showed the inhibitory kinetics model of 4-hydroxy cinnamic acid and its simulating the docking with tyrosinase. The results indicated that 4-hydroxy cinnamic acid was a competitive inhibitor with K_I value 0.67 mM. 4-hydroxy cinnamic acid interacted with the enzyme active site mainly through the hydroxy competed with the substrate hydroxy group, since it was an analogue of L-DOPA, just as the results of MOE docking showed that the hydroxy of 4-hydroxy cinnamic acid could form metal interaction with Cu^+ of the enzyme. This result was also observed by Park et al. [23] in the simulation docking of 4-(6,7-dihydro-5*H*-indeno[5,6-*d*]thiazol-2-yl)benzene-1,3-diol with tyrosinase. From the result of docking, the hydroxy in the carboxyl of 4-hydroxy cinnamic acid could form the side chain with tyrosinase residue Asn₂₆₀. However, Vontzalidou et al. [24] synthesised dihydrostilbene derivatives and studied the molecular simulation as tyrosinase inhibitors, the results showed that a hydrogen bond is formed between OH group in position 2 of the phenyl ring and the side chain of Asn₂₆₀ which is placed near the entrance of the binding pocket. And the interaction of the compounds with Asn₂₆₀ emphasizes the importance of this residue located close to the entrance of the receptor. Therefore, the results of

inhibitory kinetics model and the molecular docking are conform. This will provide a theory to screen the tyrosinase inhibitors.

The cytotoxicity study of 4-hydroxy cinnamic acid indicated it had no effects on the proliferation of normal liver cells. So, the preservation of mushroom (*Agaricus bisporus*) by 4-hydroxy cinnamic acid was investigated. The appearances and browning degree of mushroom were studied in this manuscript to evaluate the effect of 4-hydroxy cinnamic acid on mushroom preservation. Nowadays, there are more and more researches on the fruit and vegetable preservation. Lin et al. [25] reported that propyl gallate could potently inhibit diphenolase activity of mushroom tyrosinase with a reversible and mixed type inhibitor. Furthermore, propyl gallate could control pericarp browning by inhibiting PPO activities in pericarp of harvested longan fruits to extend the shelf life. Gao et al. [26] reported that essential oil fumigation treatment could inhibit the senescence of mushroom and the most effective compounds was cinnamaldehyde. Jiang [27] investigated that alginate coating under a high oxygen modified atmosphere could inhibit the activity of PPO throughout storage to extend the mushroom postharvest life to 16 d at 4 °C. The results of browning degree and L* value showed 4-hydroxy cinnamic acid could delay the browning.

4. Conclusion

4-hydroxy cinnamic acid exhibited competitive inhibition on mushroom tyrosinase, the inhibitory kinetics model and the parameters were investigated. Molecular docking technique predicted that 4-hydroxy cinnamic acid binding sites were located within the catalytic pocket of the enzyme. Cytotoxicity study showed 4-hydroxy cinnamic acid is no cytotoxicity and is safe. Furthermore, the effect of 4-hydroxy cinnamic acid on the preservation of mushroom showed that it could delay the browning and open cap in order to extend the shelf life. Therefore, it is suggested that

application of 4-hydroxy cinnamic acid may be a promising method for inhibiting tyrosinase and controlling mushroom browning.

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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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Table 1. Inhibitory parameters for tyrosinase by 4-hydroxy cinnamic acid

[S] (mM)	0.50	0.75	1.00	2.00
$1/[S]$	2.00	1.33	1.00	0.67
$A (\times 10^{-4} \text{ s}^{-1})$	7.24	5.97	5.38	3.68
$k_0 (\times 10^{-3} \text{ s}^{-1})$	1.92	1.92	1.91	1.91
$1/A$	1382	1676	1858	2714
$A/v (\times 10^{-4})$	10.24	6.84	5.44	3.23

Figure caption

Fig. 1. Course of substrate reaction in the presence of different concentrations of 4-hydroxy cinnamic acid. The enzyme final concentration was 3.33 $\mu\text{g/mL}$. The substrate concentration of L-DOPA was 0.5 mM. (a) Substrate reaction course. The concentrations of 4-hydroxy cinnamic acid for curves 0-4 were 0, 0.5, 1.0, 1.5 and 2.0 mM. (b) Semilogarithmic plots of $\ln([P]_{\text{calc}} - [P]_t)$ against time.

Fig. 2. Course of inhibition at different substrate concentrations in the presence of 0.5 mM 4-hydroxy cinnamic acid. (a) Curves 1-4 are progress curves with 0.5, 0.75, 1.0 and 2.0 mM L-DOPA, respectively. (b) Semilogarithmic plot of $\ln([P]_{\text{calc}} - [P]_t)$ against time.

Fig. 3. (a) Second plots of slope versus the concentrations of 4-hydroxy cinnamic acid. The substrate concentrations for lines 1-4 were 0.5, 0.75, 1.0 and 2.0 mM. (b) Plot of $1/A$ versus $[S]$. The A were obtained from the slopes of the straight lines in (a). (c) Plots of A/v versus $1/[S]$.

Fig. 4. Docking pose of 4-hydroxy cinnamic acid in the binding site of tyrosinase. (a) Tertiary structure for the interaction of 4-hydroxy cinnamic acid with tyrosinase residues. (b) The interaction of 4-hydroxy cinnamic acid with tyrosinase residues in the active site. (c) 2D structure for the interaction of 4-hydroxy cinnamic acid with tyrosinase residues. 4-hydroxy cinnamic acid was depicted as purple stick.

Fig. 5. Growth inhibitory effects of 4-hydroxy cinnamic acid on normal liver cells L02. Cell growth inhibition was analyzed by the MTT assay.

Fig. 6. Effect of 4-hydroxy cinnamic acid on the preservation of mushroom (*Agaricus*

bisporus) stored at 20 °C for 4 d. (a) Changes of the mushroom caps treated with distilled water (control); (b) Changes of the mushroom caps treated with 4-hydroxy cinnamic acid; (c) Changes of the mushroom bottom treated with distilled water (control); (d) Changes of the mushroom bottom treated with 4-hydroxy cinnamic acid; (e) Changes in browning degree of mushroom; (f) Changes in L* value of mushroom. Each data point is the mean of three replicate samples. Vertical bars represent standard deviation of means.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6