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Different modes of inhibition for organic acids on polyphenoloxidase

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ABSTRACT

It is still unclear whether the inhibitory effect of organic acid on polyphenoloxidase (PPO) is due to the reversible inhibition or decrease of pH. In this study, cinnamic acid, citric acid and malic acid inhibited PPO in different modes. Results showed that the inhibition by cinnamic acid resulted from reversible inhibition, while the decrease of pH was the main cause for citric acid and malic acid. The kinetic results showed that cinnamic acid reversibly inhibited PPO in a mixed-type manner. Fluorescence emission spectra indicated that cinnamic acid might interact with PPO and quench its intrinsic fluorescence, while the decrease of the fluorescence intensity induced by citric acid or malic acid was due to the acid-pH. Cinnamic acid bound to PPO and induced the rearrangement of secondary structure. Molecular docking result revealed cinnamic acid inserted into the hydrophobic cavity of PPO by forming π–π stacking.

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1. Introduction

Polyphenoloxidase (PPO) is a group of copper-containing enzymes, which are widely distributed in animals, plants, fungi and bacteria (Mayer, 2006). PPO is responsible for the browning of damaged fruits and vegetables by catalyzing two different reactions: the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones, which polymerize to brown pigments subsequently (Espín, Jolivet, & Wichers, 1998; Virador et al., 2009). Besides the color changes, enzymatic browning deteriorates the nutritional quality of fruits and vegetables and cause a reduction of market value. Therefore, the inactivation of PPO is necessary for controlling browning. Over the years, many methods have been used to adjust the PPO activity. For example, heating (Gouzi, Depagne, & Coradin, 2012), high hydrostatic pressure (Yi et al., 2012), and addition of chemical reagents such as sodium sulfite (Palma-Orozco, Ortiz-Moreno, Dorantes-Álvarez, Sampedro, & Nájera, 2011) and organic acids. Organic acids are widely used to inhibit PPO activity. Many studies have been conducted on the inhibitory effect of various organic acids on PPO in recent years. The commonly used organic acids are citric acid (Liu et al., 2013), ascorbic acid (Landi, Degl'Innocenti, Guglielminetti, & Guidi, 2013), oxalic acid (Yoruk & Marshall, 2003a) and cinnamic acid (Shi, Chen, Wang, Song, & Qiu, 2005).

Cinnamic acid is an organic acid occurring naturally in a number of plants that has low toxicity and a broad spectrum of biological activities (Sova, 2012). Cinnamic acid and its derivatives are widely used in food industry (Hu et al., 2014). Citric acid and malic acid naturally present in some fruits, and they are widely used in food processing to diminish browning and had been shown to inhibit PPO (Yoruk & Marshall, 2003b). Recently, Shi et al. (2005) and Hu et al. (2014) reported that cinnamic acid and its derivatives strongly inhibited PPO activity. Altunkaya and Gökmen (2008) and Queiroz, da Silva, Lopes, Fialho, and Valente-Mesquita (2011) found that citric acid showed effective inhibition of PPO activity. Nevertheless, to our knowledge, only enzymatic activity assay was conducted in these studies. It is still unclear whether the inhibitory effect of organic acid on PPO is due to the reversible inhibition or decrease of pH. Few reports have mentioned the mode of inhibition for organic acids on PPO, especially the difference among these commonly used organic acids. The lack of understanding in inhibitory mode of organic acids on PPO has seriously affected the application of organic acids on PPO.

In our previous study, it was found that as the concentration of citric acid increased, the activity of PPO decreased gradually (Liu et al., 2013). However, the mode of inhibition for citric acid on PPO was not clear. Consequently, in this study, different inhibitory effects of organic acids on PPO were evaluated. The inhibition kinetics and binding constant were determined. Furthermore, conformational changes and the interaction between cinnamic acid and PPO were provided. The objective of the study was to distinguish between the mode of action as reversible inhibition and decrease of pH, and to provide more information for application of organic acids as PPO inhibitors.

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2. Materials and methods

2.1. Materials

Mushroom (*Agaricus bisporus*) polyphenoloxidase (T3824-50 Ku, 3130 μ/mg) was purchased from Sigma Chemical Co. (St. Louis, MO). Cinnamic acid, citric acid, malic acid, and reaction substrates (L-DOPA and catechol) were of analytical grade, purchased from Aladdin Chemicals Co. (Shanghai, China). All other chemicals were of analytical grade, solutions were prepared in double-distilled water.

2.2. Preparation of cinnamic acid, citric acid and malic acid solution

Preparation of cinnamic acid, citric acid and malic acid solution was carried out according to Liu et al. (2013) and Qiu et al. (2009) with a slight modification. Cinnamic acid was first dissolved in DMSO, then phosphate buffer (50 mM, pH 6.8) was used for a 30-fold dilution. The final concentration of DMSO was 3.33% and the concentration of cinnamic acid was 20 mM. Cinnamic acid (0.5, 1.5, 2.5, 5.0, 7.5 and 10.0 mL) was added into test tubes, then phosphate buffer (50 mM, pH 6.8) was added to obtain the final volume (10 mL). Citric acid and malic acid (1, 3, 5, 10, 15, 20 mM) were prepared as described above without DMSO.

To obtain pH 6.8, pH 6.5, pH 6.0, pH 5.5, pH 5.0 reaction system, the corresponding concentrations of cinnamic acid (0, 8.52, 18.67, 23.53, 28.92 mM), citric acid (0, 2.52, 6.63, 9.71, 12.35 mM) and malic acid (0, 3.08, 8.82, 11.95, 14.58 mM) were prepared with phosphate buffer (50 mM, pH 6.8). Phosphate buffer (pH 6.8, pH 6.5, pH 6.0) and acetate buffer (pH 5.5, pH 5.0) acted as controls.

In order to maintain the pH of the reaction system at any organic acids concentration, 25 mL stock organic acid solutions (0, 2, 6, 10, 20, 30 mM) were prepared with phosphate buffer (50 mM, pH 6.8), stock solutions were adjusted to pH 6.80 ± 0.02 with NaOH. The final volume of each solution was brought to 50 mL with phosphate buffer (50 mM, pH 6.8) to obtain the final organic acid concentrations of 0, 1, 3, 5, 10, 15 mM. To maintain pH 6.0 and pH 5.0 at any organic acids concentration, organic acid, phosphate buffer (50 mM, pH 6.0) and acetate buffer (50 mM, pH 5.0) were used as described above (Yoruk & Marshall, 2003a).

2.3. Activity assay

PPO activity was assayed using a UV–vis spectrophotometer (MAPADA, Shanghai, China) (Liu, Liu, Xie, et al., 2009). Firstly, PPO was dissolved in phosphate buffer (50 mM, pH 6.0) to a concentration of 40 μg/mL. Incubation was performed by mixing 1 mL PPO with 49 mL buffer or organic acid solutions (prepared in Section 2.2). Samples were incubated at room temperature for 30 min. The reaction mixture included 2.8 mL incubated sample, then 0.2 mL of 20 mM substrate (L-DOPA/catechol) was added to the reaction mixture to initiate the enzyme reaction. The mixture solution was allowed to stand for 1 min at 37 °C. The oxidation of L-DOPA and catechol was determined at room temperature (25 ± 1 °C) by monitoring the absorbance at 475 and 420 nm, respectively.

Specific activity = A/1 min/0.1 mL of enzyme solution.

$$\text{Relative activity} = \frac{\text{Activity of treated PPO}}{\text{Activity of untreated PPO}} \times 100\% \quad (1)$$

2.4. Kinetic analysis for the mixed-type inhibition

The inhibition type was assayed by the Lineweaver–Burk plot. The mixed-type inhibition mechanism can be described by the Lineweaver–Burk equation in double-reciprocal form:

$$\frac{1}{v} = \frac{K_m}{V_{\max}}\left(1 + \frac{[I]}{K_i}\right)\frac{1}{[S]} + \frac{1}{V_{\max}}\left(1 + \frac{[I]}{\alpha K_i}\right) \quad (2)$$

Secondary plots can be constructed from

$$Slope = \frac{K_m}{V_{\max}} + \frac{K_m[I]}{V_{\max}K_i} \quad (3)$$

$$Y\text{-}intercept = \frac{1}{V_{\max}^{app}} = \frac{1}{V_{\max}} + \frac{1}{\alpha K_i V_{\max}}[I] \quad (4)$$

where v is the PPO reaction rate. K_i and K_m are the inhibition constant and Michaelis–Menten constant, respectively. $[I]$ and $[S]$ are the concentrations of inhibitor and substrate, respectively. The K_i, α, K_m and $V_{\max}$ values can be derived from the equations above. The secondary replot of *Slope* or *Y-intercept* vs. $[I]$ is linearly fitted, assuming a single inhibition site or a single class of inhibition site (Hu et al., 2012).

2.5. Fluorescence emission spectra analysis

Fluorescence emission spectra measurement was conducted according to the method used by Liu, Liu, Liu, et al. (2009) with a slight modification. Samples were scanned at room temperature (25 ± 1 °C) using a FS F-4500 Spectrophotometer (Hitachi, Tokyo, Japan), excitation and emission slits were 5 nm. PPO solutions were excited at its maximum excitation wavelength (λ_{ex} = 280 nm) and emission spectra was scanned from 450 to 290 nm. All spectra were corrected with baseline spectrum by sample without protein. Binding constants and the number of binding sites were determined as Si et al. (2011). When small molecules were bound to equivalent sites on PPO, the following equation was used to describe the equilibrium between free and bound molecules.

$$\frac{F_0}{F_0 - F} = \frac{1}{n} + \frac{1}{K}\frac{1}{[Q]} \quad (5)$$

The F_0 and F denote the fluorescence intensity of PPO in the absence and presence of cinnamic acid, respectively. $[Q]$ is the concentration of quencher (cinnamic acid). The binding constant (K) and number of binding sites (n) can be calculated by Eq. (5).

2.6. Circular dichroism (CD) analysis

CD measurement was conducted according to Yi et al. (2012) and Liu, Liu, Liu, et al. (2009) with a slight modification. CD spectra were recorded by a MOS-450 spectropolarimeter (French Bio-Logic SAS, Claix, France), using a quartz cuvette of 1 mm optical path length at room temperature (25 ± 1 °C), scanning the CD spectra at the far UV range (250–190 nm) at 50 nm/min. The step resolution was 1 nm, band width was 0.5 nm. PPO was incubated with cinnamic acid at molar ratios ([cinnamic acid]/[PPO]) of 0:1, 1:4 and 1:2, the concentration of PPO was kept at 3.0×10^{-6} mol/L. The secondary structures elements were estimated from the spectra using the Dichro Web Online SELCON 3 algorithms with the reference set No. 4 (http:// dichroweb.cryst.bbk.ac.uk/html/home.shtml). The CD data was expressed in terms of mean residual ellipticity (θ), in mdeg cm^2/dmol, using a Mean Residual Weight (MRW) of 113.7. The four replicates were averaged and Origin 8.0 was used to smooth the spectra.

2.7. Molecular docking of PPO and cinnamic acid

The molecular docking program AutoDock Vina was applied to explore the probable interaction between cinnamic acid and PPO. Crystal structure of *A. bisporus* mushroom PPO (PDB ID: 2Y9X) from the RCSB Protein Data Bank (http:// www.rcsb.org/pdb) was used to model the PPO structure, 3D structure of cinnamic acid

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