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Different inhibition mechanisms of gentisic acid and cyaniding-3-O-glucoside on polyphenoloxidase

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ABSTRACT

Gentisic acid and cyanidin-3-O-glucoside are important bioactive polyphenols which are widely distributed in many fruits and cereals. In this work, kinetic study, spectral analysis and computational simulation were used to compare the inhibitory effects and inhibition mechanisms of gentisic acid and cyanidin-3-O-glucoside on mushroom polyphenoloxidase (PPO). The inhibitory effect of cyanidin-3-O-glucoside on PPO was much stronger than that of gentisic acid. Gentisic acid inhibited PPO in a reversible mixed-type manner while cyanidin-3-O-glucoside was an irreversible inhibitor. Gentisic acid and cyanidin-3-O-glucoside made the thermal inactivation of PPO easier, and induced apparent conformational changes of PPO. Compared with gentisic acid, cyanidin-3-O-glucoside had stronger effects on the thermal inactivation and conformation of PPO. Molecular docking results revealed gentisic acid bound to the active site of PPO by hydrogen bonding, π–π stacking and van der Waals forces. However, cyanidin-3-O-glucoside might irreversibly interact with the Met or Cys in PPO by covalent bonds.

1. Introduction

Polyphenoloxidase (PPO) is a group of copper-containing enzymes, which widely exists in animals, plants, fungi and bacteria (Mayer, 2006). PPO is responsible for enzymatic browning in fruits and vegetables by catalyzing the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones (Espín, Jolivet, & Wichers, 1998). Enzymatic browning significantly deteriorates the colour and flavour. These deteriorative changes can lead to a limited shelf-life of fruits and vegetables (Lin et al., 2011). In addition to the undesirable colour and flavour, quinones may react with amino and sulfhydryl groups of protein. These reactions result in a loss of nutritional quality and decrease the bioavailability of essential amino acids (Kim & Uyama, 2005). Therefore, inhibition of PPO activity is important for the control of enzymatic browning in fruits and vegetables. Recently, a lot of PPO inhibitors have been reported in many studies, including organic acids (Liu et al., 2013; Zhou, Liu, Xiong, Zou, Chen, et al., 2016), flavonoid (Dong et al., 2016; Wang, Zhang, Yan, & Gong, 2014; Xiong, Liu, Zhou, Zou, & Chen, 2016) and sodium sulphite (Palma-Orozco, Ortiz-Moreno, Dorantes-Alvarez, Sampedro, & Najera, 2011). However, a few of them have been applied in food industry because of the efficacy and safety requirements. Thus, it is important to search for high effective inhibitors from natural sources.

Gentisic acid is a phenol acid which can be found in many kinds of foods such as cereal grains (Naczk & Shahidi, 2006) and red wine (del Alamo Sanza, Nevares Domínguez, Cárcel Cárcel, & Navas Gracia, 2004). Recent studies showed that gentisic acid was a potential inhibitor of free radicals (Girish, Pratape, & Prasada Rao, 2012) and α-glucosidase (Girish et al., 2012). Cyanidin-3-O-glucoside is a typical representative of anthocyanins (Tang, Li, Bi, & Gao, 2016), which widely exists in edible plant materials such as wild berries (Feng et al., 2016), pomegranate (Legua, Forner-Giner, Nuncio-Jáuregui, & Hernández, 2016), tart cherry (Homoki et al., 2016) and black rice (Shao, Xu, Sun, Bao, & Beta, 2014). Many studies have reported the beneficial effects of cyanidin-3-O-glucoside and its inhibitory effect on enzymes. For example, cyanidin-3-O-glucoside possesses anti-inflammatory (Wang et al., 1999), free radical scavenging activity (Kähkönen & Heinonen, 2003) and α-amylase inhibitor activity (Homoki et al., 2016). A number of studies found that some polyphenols bound with PPO and showed inhibitory effects with different inhibition types. Recently, some studies have tried to investigate the interaction between PPO and some reversible inhibitors. For example, apigenin reversibly inhibited PPO activity in a mixed-type manner and computational docking results showed that apigenin inserted into the hydrophobic cavity (Xiong et al., 2016). Morin induced a reversible competitive inhibition on PPO, and it bound to PPO at a single

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binding site mainly by hydrogen bonds and van der Walls force (Wang et al., 2014). A few studies also found some irreversible inhibitors of PPO by kinetic assays (Liu et al., 2012; Zhuang et al., 2010). However, to our knowledge, there is less information about the inhibitory effect and reversibility of gentisic acid and cyanidin-3-O-glucoside on PPO, especially the different inhibition mechanisms of reversible and irreversible inhibitors. The lack of understanding in different inhibition mechanisms at the molecular level has seriously affected the discovery and application of inhibitors.

In order to investigate the different inhibition mechanisms of reversible and irreversible inhibitors, gentisic acid and cyanidin-3-O-glucoside were selected as PPO inhibitors. The inhibitory effects of gentisic acid and cyanidin-3-O-glucoside on PPO were studied by UV–vis spectrophotometry. The inhibition mechanisms of gentisic acid and cyanidin-3-O-glucoside were analyzed through reversibility, inhibition types, thermodynamic changes, conformational changes and intermolecular interactions. The reversibility and inhibition types were determined by kinetic analysis. The conformational changes of PPO were analyzed by Far-UV circular dichroism (CD) and fluorescence emission spectra. Interactions between PPO and inhibitor were predicted by the computational simulation. These contents not only compared the different inhibition mechanisms of gentisic acid and cyanidin-3-O-glucoside on PPO, but also provided useful information for application of natural polyphenols as PPO inhibitors.

2. Materials and methods

2.1. Materials

Mushroom (*Agaricus bisporus*) polyphenoloxidase (LS003789-25 ku, 1070 u/mg) was obtained from Worthington Bio-chemical Co. Gentisic acid and reaction substrate catechol were of analytical grade and purchased from Aladdin Chemicals Co. (Shanghai, China). Cyanidin-3-O-glucoside was purchased from Baoji Herbest Bio-Tech Co. (Baoji, China). All solutions were prepared in double-distilled water and stored at 4 °C.

2.2. Preparation of gentisic acid and Cyanidin-3-O-glucoside solution

Gentisic acid was dissolved in sodium phosphate buffer (0.05 mol l^{-1}, pH 6.8) and the concentration of stock solution was 1.5×10^{-2} mol l^{-1}. Gentisic acid (0, 0.30, 0.60, 1.20, 2.40, 4.80, 6.00 and 9.00 ml) was added into test tubes, then sodium phosphate buffer (0.05 mol l^{-1}, pH 6.8) was added to obtain the final volume of 10 ml. The final concentrations of gentisic acid were 0, 4.5×10^{-4}, 9.0×10^{-4}, 1.8×10^{-3}, 3.6×10^{-3}, 7.2×10^{-3}, 9.0×10^{-3}, 1.35×10^{-2} mol l^{-1}, respectively. The final concentration of PPO used in the activity assay was kept at 3.9×10^{-8} mol/l and the molar ratios between gentisic acid and PPO were 0, 1.15×10^{4}, 2.31×10^{4}, 4.62×10^{4}, 9.23×10^{4}, 1.85×10^{5}, 2.31×10^{5} and 3.46×10^{5}: 1, respectively. Cyanidin-3-O-glucoside was prepared as described above and the final concentrations were 0, 4.5×10^{-6}, 9.0×10^{-6}, 1.8×10^{-5}, 3.6×10^{-5}, 5.4×10^{-5}, 7.2×10^{-5}, 9.0×10^{-5}, and 1.08×10^{-4} mol l^{-1}, respectively. The molar ratios between Cyanidin-3-O-glucoside and PPO were 0, 1.15×10^{2}, 2.31×10^{2}, 4.62×10^{2}, 9.23×10^{2}, 1.38×10^{3}, 1.85×10^{3}, 2.31×10^{3} and 3.46×10^{3}: 1, respectively.

2.3. Activity assay

PPO activity was measured by a UV–vis spectrophotometer (MAPADA, Shanghai, China) (Zhou, Liu, Xiong, Zou, Liu, et al., 2016). PPO was dissolved in sodium phosphate buffer (0.05 mol l^{-1}, pH 6.8) at a concentration of 0.15 mg/ml. Then 0.1 ml PPO solution was mixed with 2.7 ml gentisic acid or cyanidin-3-O-glucoside solution and the incubation was performed at room temperature for 30 min. The reaction was initiated by adding 0.2 ml substrate catechol. The oxidation of catechol was monitored immediately at 420 nm for 1 min. The specific activity of PPO was calculated from the slope of a linear segment:

Specific activity = $A_{420\ nm}$/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 of polyphenoloxidase inhibition

Kinetic analysis was applied to estimate the inhibition types for gentisic acid or cyanidin-3-O-glucoside. The plots of v versus [PPO] at different concentrations of inhibitors were constructed to determine the reversibility. 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

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

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

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

2.5. Thermodynamic parameters analysis

Thermodynamic parameters analysis of PPO treated with gentisic acid or cyanidin-3-O-glucoside were conducted according to Gouzi, Depagne, and Coradin (2012) and Liu et al. (2013) with a slight modification. The thermal inactivation of PPO was studied from 45 to 60 °C (318–333 K) at atmospheric. 1 ml PPO sample were mixed with 27 ml preheated inhibitor solution, which was incubated in water at the required temperature. At predetermined time intervals, 2.8 ml mixture solution was pipetted into a test tube and instantaneously immersed in an ice bath to stop thermal inactivation. After the mixture solution had been brought to room temperature, 0.2 ml substrate catechol was added to initiate the reaction. PPO treated with 0, 9.0×10^{-4}, 1.8×10^{-3} and 3.6×10^{-3} mol l^{-1} gentisic acid was used to thermodynamic parameters analysis and represented as PG_0, PG_9, PG_{18} and PG_{36}, respectively. PPO treated with 0, 9.0×10^{-6}, 1.8×10^{-5} and 3.6×10^{-5} mol l^{-1} cyanidin-3-O-glucoside was represented as PC_0, PC_9, PC_{18} and PC_{36}, respectively. The untreated PPO sample was used as blank (A_0). Residual activity (A_t) was determined as described above. Generally, the first-order reaction (Eq. (5)) was used to describe the kinetic analysis of thermal inactivation of enzymes:

$$\frac{dA_t}{t} = -k \cdot A_t \quad (5)$$

where A_t is the remaining activity of PPO at time t, and k is the inactivation rate constant (min^{-1}) at the temperature studied.