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Antityrosinase and antimicrobial activities of 2-phenylethanol, 2-phenylacetaldehyde and 2-phenylacetic acid

Yu-Jing Zhu [a,b,1], Han-Tao Zhou [b,1], Yong-Hua Hu [b], Jian-Yang Tang [a], Ming-Xing Su [a], Yun-Ji Guo [b], Qing-Xi Chen [b,c,**], Bo Liu [a,*]

[a] Agricultural Bio-resources Institute, Fujian Academy of Agricultural Sciences, Fuzhou, China
[b] Key Laboratory of the Ministry of Education for Coastal and Wetland Ecosystems, School of Life Sciences, Xiamen University, Xiamen 361005, China
[c] Key Laboratory for Chemical Biology of Fujian Province, Xiamen University, Xiamen, China

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ABSTRACT

The inhibition kinetics of 2-phenylethanol, 2-phenylacetaldehyde and 2-phenylacetic acid on the enzyme activity of mushroom tyrosinase have been investigated. The results showed that these aromatic compounds can lead to reversible inhibition of the enzyme; furthermore, 2-phenylacetaldehyde and 2-phenylacetic acid are uncompetitive inhibitors and 2-phenylethanol is a mixed-type inhibitor. The inhibition constants have been determined and the inhibiting ability was: 2-phenylacetaldehyde > 2-phenylacetic acid > 2-phenylethanol, indicating that the functional group on the benzene ring group played an important role in the inhibition of the enzyme. In addition, 2-phenylacetic acid and 2-phenylethanol were found to have effective antibacterial activities, and 2-phenylacetic acid was more effective against *Escherichia coli* and *Ralstonia solanacearum* than 2-phenylethanol, but 2-phenylacetaldehyde lacked of antibacterial activity.

1. Introduction

Enzymatic browning in fruits and vegetables is predominantly catalyzed by a copper-containing enzyme–tyrosinase (EC.1.14.18.1) (Baek et al., 2009; Chen & Kubo, 2002). The browning is an undesirable reaction that is responsible for a less attractive appearance and a loss in nutritional quality. It has become a major problem in the food industry and is one of the main causes of quality loss during post harvest handling and processing (Friedman, 1991). In addition, Tyrosinase is a key enzyme in melanin biosynthesis, involved in determining the colour of mammalian skin and hair (Parvez, Kang, Chung, & Bae, 2007). Its abnormal expression is responsible for the various dermatological disorders, such as melasama, age spots, and sites of actinic damage. It also contributes to neuromelanin formation in the human brain and the neurodegeneration associated with Parkinson's disease (Xu et al., 1997).

An important group of browning inhibitors is constituted by compounds structurally analogous to phenolic substrate. It is well known that tyrosinase can be inhibited by aromatic aldehydes (Huang et al., 2006a, 2006b), acids (Wang et al., 2004) , tropolone (Espin & Wichers, 1999) and kojic acid (Lee, Park, Kim, Seo, & Kim, 2006). These generally show competitive inhibition toward these substrates, although such inhibition may vary depending on the enzyme source and the substrate used (Voit et al., 1999). In the previous study, benzoic acid was found to inhibit the activity of mushroom tyrosinase, which was shown to be a reversible reaction, and to be a noncompetitive inhibitor for the oxidation of L-DOPA (Song, Chen, Wang, Qiu, & Huang, 2005). Recently, benzaldehydes (Chen, Song, Wang, & Huang, 2003) and alkoxybenzoic acids (Huang et al., 2006a, 2006b) have been targeted for inhibition of the enzyme.

2-Phenylethanol is a colourless liquid, it occurs widely in nature, being found in a variety of essential oils, including rose, camation, hyacinth, Aleppo pine and so on. It is used as an additive in cigarettes and a preservative in soaps. In biology it is of interest due to its antimicrobial properties. Lilly et al. reported that 2-phenylethanol exerts an inhibitory effect on the growth of gram-negative microorganisms (Lilly & Brewer, 1953). 2-Phenylacetaldehyde is an aliphatic compound found in buckwheat, chocolate and many other

Abbreviations: L-DOPA, L-3,4-dihydroxyphenylalanine; DMSO, dimethylsulfoxide; IC_{50}, the inhibitor concentrations leading to 50% activity lost; MIC, the minimum inhibitory concentration; MBC, the minimum bactericidal concentration; K_I, the equilibrium constant of the inhibitor combining with the free enzyme; K_{IS}, the equilibrium constant of the inhibitor combining with the enzyme–substrate complex.

* Corresponding author at: Agricultural Bio-resources Institute, Fujian Academy of Agricultural Sciences, Fuzhou 350003, China. Fax: +86 591 87864601.
** Corresponding author at: Key Laboratory for Chemical Biology of Fujian Province, Xiamen University, Xiamen 361005, China.
E-mail addresses: chenqx@xmu.edu.cn (Q.-X. Chen), laeptb@163.com (B. Liu).
[1] These authors contributed equally to this work.

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foods and flowers. It is responsible for the antibiotic activity of maggot therapy. 2-Phenylacetic acid is an organic compound containing a phenyl functional group and an acetic acid functional group. It is used in some perfumes and penicillin G production. 2-Phenylethanol, 2-phenylacetaldehyd and 2-phenylacetic acid have structural similarities, and these were investigated by carrying out a kinetic study of the inhibition on the activity of mushroom tyrosinase, with an evaluation of the kinetic parameters and constants characterizing the system. Furthermore, we studied the bacteriostatic activity of these compounds. All these data may provide a basis for developing novel tyrosinase inhibitors.

2. Materials and methods

2.1. Reagents

Mushroom tyrosinase (with specific activity 6680 U/mg), 2-phenylethanol 2-phenylacetaldehyde, 2-phenylacetic acid, L-3, 4-dihydroxyphenyl-alanine (L-DOPA) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). *Escherichia coli* and *Ralstonia solanacearum* were collected from a colony preserved at −80 °C in Fujian Academy of Agricultural Sciences. All other reagents were of analytical grade. The water used was re-distilled and ion-free.

2.2. Enzyme assay

The assay of the enzyme activity was performed as described by Xie et al. (2003). In this investigation, L-DOPA was used as the substrate for the enzyme activity assay. The reaction medium (3 ml) contained 0.5 mM L-DOPA in 50 mM sodium phosphate buffer (pH 6.8). The final concentration of mushroom tyrosinase was 6.67 μg/ml. The inhibitor was first dissolved in DMSO and used for the experiment at 30 times dilution. The final concentration of DMSO in the test solution was 3.3%. The enzyme activity was monitored by dopachrome formation at 475 nm ($\varepsilon = 3700\ M^{-1}\ cm^{-1}$) (Jimenez, Chazarra, Escribano, Cabanes, & Garcia–Carmona, 2001) accompanying the oxidation of the substrate. Absorption was recorded using a Beckman UV-650 spectrophotometer. The extent of inhibition by the addition of the sample was expressed as the percentage necessary for 50% inhibition (IC_{50}). Controls, without inhibitor but containing 3.3% DMSO, were routinely carried out. The inhibition type was determined by Lineweaver–Burk plots, and the inhibition constant was determined by secondary plots of the apparent K_m/V_{max} or $1/V_{max}$ versus the concentration of the inhibitor as described by Chen and Kubo, (2002). All measurements were carried out at 30 °C.

2.3. Antimicrobial assay

The antimicrobial activity of the three compounds tested was determined using the agar well diffusion method. Briefly, culture medium was inoculated with the given microorganism by spreading the bacterial inoculums in the media. Wells (7 mm diameter) were punched in the agar and filled with compounds with different concentrations. In this experiment, the antimicrobial assay was carried out on tryptone beef extract agar, at pH 7.2, with an inoculum of $1–2 \times 10^5$ cells/ml. Control wells, containing neat DMSO (negative control) and standard antibiotic streptomycin sulfate (100 U/ml) for the tested bacteria, were also run parallel in the same plate. Bacteria were incubated at 37 °C for 24 h. Antimicrobial activity was assessed by measuring the diameter of the zone of inhibition for the respective drug. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were tested by broth macrodilution methods according to Kubo, Fujita, Kubo, Nihei, and Ogura (2004).

3. Results

3.1. Concentration effects of 2-phenylethanol, 2-phenylacetaldehyde and 2-phenylacetic acid on the enzyme activity of mushroom tyrosinase

2-Phenylethanol, 2-phenylacetaldehyde and 2-phenylacetic acid (see Fig. 1 for structures) were tested for their effects on the oxidation of L-DOPA by mushroom tyrosinase. The inhibitory course is shown in Fig. 2. With increasing the concentrations of the compounds, the enzyme activity markedly decreased concentration-dependently. From Fig. 2, the values of IC_{50} of 2-phenylethanol, 2-phenylacetaldehyde and 2-phenylacetic acid were estimated to be 3.04, 0.39 and 2.38 mM, respectively.

3.2. The inhibition mechanism of 2-phenylethanol, 2-phenylacetaldehyde and 2-phenylacetic acid on the enzyme

Taking 2-phenylethanol, 2-phenylacetaldehyde and 2-phenylacetic acid as inhibitors, we studied their inhibition mechanism on the enzyme for the oxidation of DOPA. The plots of the remaining enzyme activity versus the concentrations of enzyme at different inhibitor concentrations gave a family of straight lines, which all passed through the origin. Increasing the inhibitor concentrations resulted in a decrease in the slope of the line, indicating that the inhibition of these three compounds on the enzyme was a reversible reaction course. The presence of these inhibitors did not bring down the amount of the efficient enzyme, but just resulted in the inhibition and a decrease in activity of the enzyme for oxidation of DOPA.

3.3. Inhibition type and inhibition constants of 2-phenylethanol, 2-phenylacetaldehyde and 2-phenylacetic acid on the enzyme

The inhibitory mechanism of 2-phenylethanol, 2-phenylacetaldehyde and 2-phenylacetic acid on mushroom tyrosinase has individually been studied. Fig. 3 showed the double-reciprocal plots of the enzyme inhibited by 2-phenylethanol (a), 2-phenylacetaldehyde (b) and 2-phenylacetic acid (c), respectively. When using 2-phenylethanol (a) as inhibitor, the plots of $1/v$ versus $1/[S]$ give a family of lines with different slope and intercept, which intersected in the second quadrant, indicating that 2-phenylethanol is a mixed-type inhibitor. 2-Phenylethanol (a) inhibited the free enzyme more potently than the enzyme–substrate complex did. The inhibitor constant (K_I) was obtained from the plots of the slope versus the concentration of 2-phenylethanol, and the

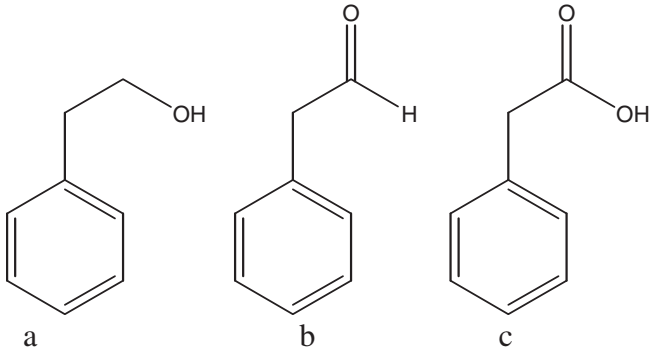

Fig. 1. Chemical structure of 2-phenylethanol (a), 2-phenylacetaldehyde (b) and 2-phenylacetic acid (c).

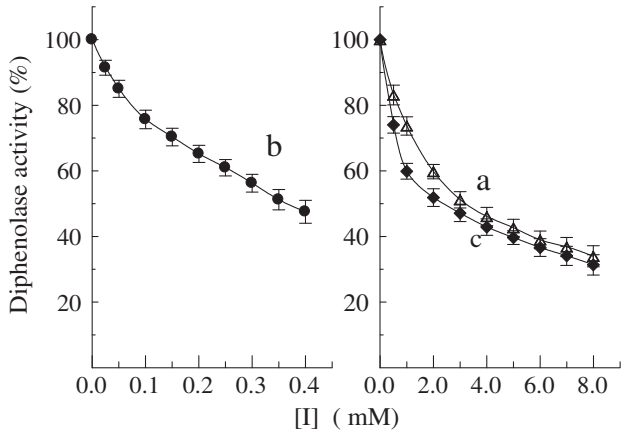

Fig. 2. The inhibition of 2-phenylethanol (a), 2-phenylacetaldehyde (b) and 2-phenylacetic acid (c) on the diphenolase activity of mushroom tyrosinase for the catalysis of L-DOPA at 30 °C. Assay conditions: 3.0 ml reaction systems contained 50 mM Na_2HPO_4-NaH_2PO_4 buffer pH 6.8, 0.5 mM L-DOPA, and 3.3% DMSO. The concentration of mushroom tyrosinase was 6.67 μg/ml.

enzyme–substrate complex (K_{IS}), was obtained from the vertical intercept versus the concentration of 2-phenylethanol. The values of K_I and K_{IS} were determined to be 0.63 mM and 4.83 mM, respectively. For compounds 2-phenylacetaldehyde (b) and 2-phenylacetic acid (c), the plots of $1/v$ versus $1/[S]$ gave a family of parallel straight lines with the same slopes. With increasing of the inhibitor concentration, the values of both K_m and V_{max} are enhanced, but the ratios of K_m/V_{max} are still unchanged. The slopes are independent of the concentration of the inhibitors, which indicated that 2-phenylacetaldehyde (b) and 2-phenylacetic acid (c) are uncompetitive inhibitors of the enzyme. The results indicated that 2-phenylacetaldehyde (b) and 2-phenylacetic acid (c) bound the enzyme molecule at a site distinct from the substrate and just combined with the enzyme–substrate complex (ES) but not with the free enzyme (E). The equilibrium constants for binding with enzyme–substrate complex (ES), K_{IS}, ware obtained from a plot of the vertical intercept ($1/V_m$) versus the inhibitor concentration, which is linear, as shown in the inset. The inhibition constants of 2-phenylacetaldehyde (b) and 2-phenylacetic acid (c) were determined to be 0.4 and 1.5 mM, respectively.

3.4. Antimicrobial activities of 2-phenylethanol, 2-phenylacetaldehyde and 2-phenylacetic acid

The antibacterial activity of 2-phenylethanol, 2-phenylacetaldehyde, and 2-phenylacetic acid on *E. coli* and *R. solanacearum* was investigated and the results were showed in Fig. 4 and Table 1. DMSO and 2-phenylacetaldehyde had no obvious inhibition on the proliferation of these two different kinds of bacteria. It was found that 2-phenylethanol and 2-phenylacetic acid could inhibit the growth of the two microbes to different extents. 2-Phenylacetic acid was more effective than 2-phenylethanol. The MIC values of 2-phenylacetic acid against *E. coli* and *R. solanacearum* were 150 μg/ml and 25 μg/ml respectively, but the MIC values of 2-phenylethanol against *E. coli* and *R. solanacearum* were 200 μg/ml and 200 μg/ml respectively. The MBC values of 2-phenylacetic acid against *E. coli* and *R. solanacearum* were 200 μg/ml and 100 μg/ml respectively, while the MBC values of 2-phenylethanol against *E. coli* and *R. solanacearum* were 400 μg/ml and 400 μg/ml respectively.

4. Discussion

Tyrosinase, a copper-containing multifunctional oxidase, is widely found in fungi, plants and animals, and catalyzes the hydroxylation of a monophenol and conversion of an *o*-diphenol to the corresponding *o*-quinone (Cho, Roh, Sun, Kim, & Park, 2006; Huang et al., 2009) 2-(4-hydroxyphenoxy)-tetrahydro-6-(hydroxylmethyl)-2*H*-pyran-3,4,5-triol (arbutin or arbutoside) (IC_{50} = 30 mM), is a tyrosinase inhibitor that has been used widely in the cosmetic industry (Hamed et al., 2006). While L-DOPA was taken as substrate for the diphenolase activity, 2-phenylethanol, 2-phenylacetaldehyde and 2-phenylacetic acid could inhibit the activity of mushroom tyrosinase and the inhibition was reversible. Compared to arbutin, our results demonstrated that the aromatic

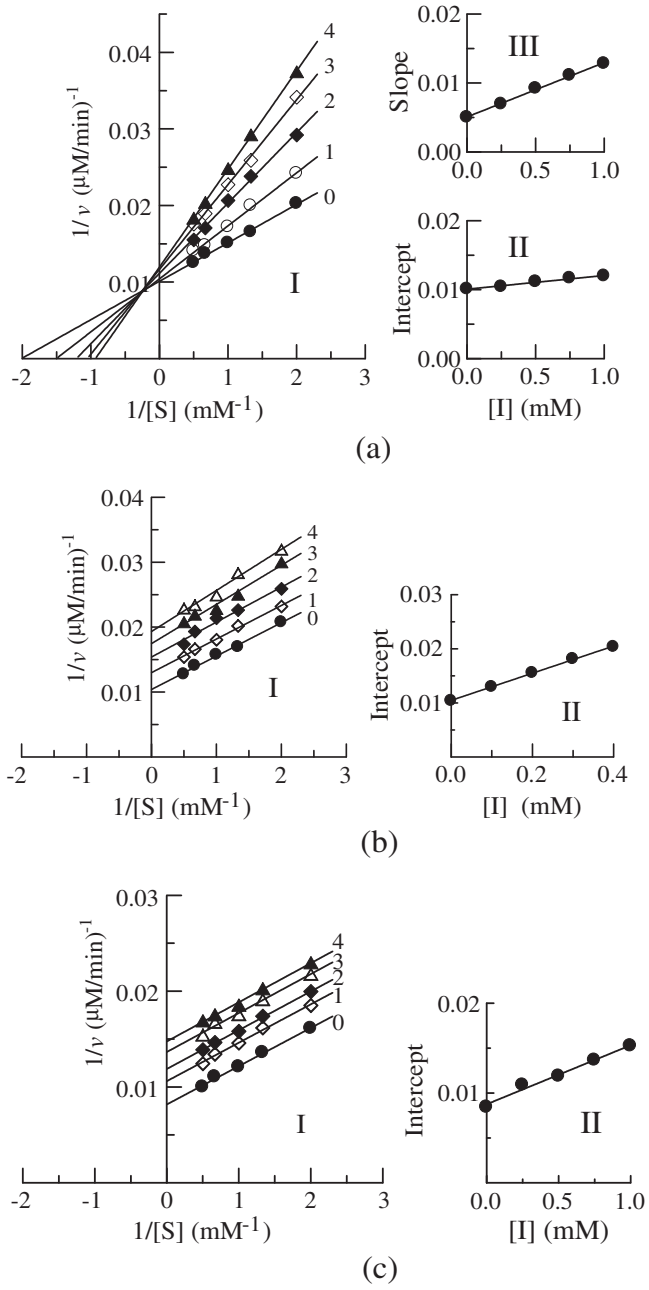

Fig. 3. Lineweaver–Burk plots (I) for inhibition of 2-phenylethanol (a), 2-phenylacetaldehyde (b) and 2-phenylacetic acid (c) on the activity of mushroom tyrosinase for the catalysis of L-DOPA. Concentrations of 2-phenylethanol (a) and 2-phenylacetic acid (c) for curves 0–4 were 0, 0.25, 0.50, 0.75 and 1.0 mM, respectively, and that of 2-phenylacetaldehyde (b) for curves 0–4 were 0, 0.1, 0.2, 0.3 and 0.4 mM, respectively. The inset represents the second plot of the intercept (II) and the slope (III) versus the concentrations of inhibitor to determine the inhibition constant. The line is drawn using linear least squares fit.

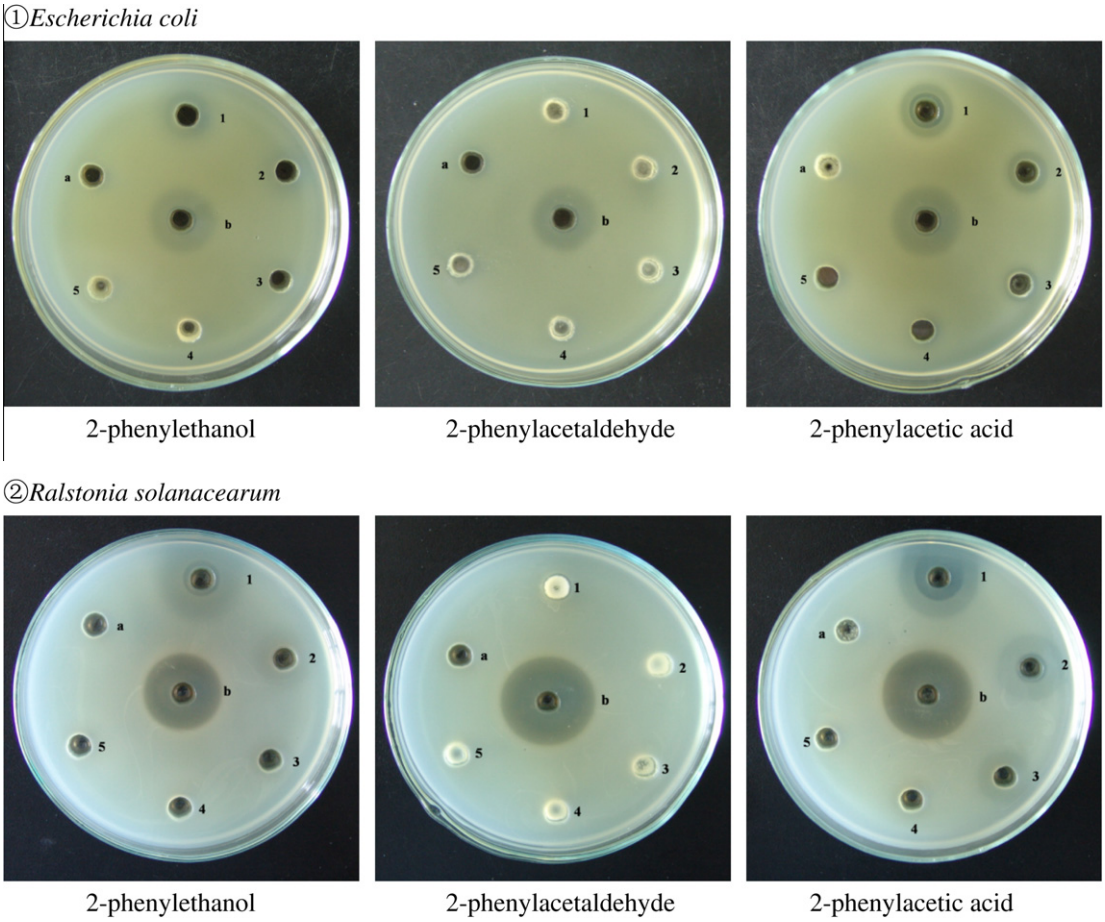

Fig. 4. The antimicrobial activity of 2-phenylethanol, 2-phenylacetaldehyde and 2-phenylacetic acid and at different concentrations. The concentrations of compounds for dishes 1–5 were 50, 25, 12.5, 6.25 and 3.125 mg/ml, respectively. (a) Negative control with DMSO. (b) Positive control with 100 U/ml of streptomycin sulfate for bacterium.

Table 1
Antimicrobial activities of 2-phenylethanol, 2-phenylacetaldehyde and 2-phenylacetic acid.

Bacteria	Concentration (mg/ml)							MIC (μg/ml)	MBC (μg/ml)
	a[a]	b[b]	3.125	6.25	12.5	25	50		
Escherichia coli									
2-Phenylethanol	–[f]	++[d]	–	–	–	–	+[e]	200	400
2-Phenylacetaldehyde	–	++	–	–	–	–	–	>500	Not tested
2-Phenylacetic acid	–	++	–	–	+	+	+	150	200
Ralstonia solanacearum									
2-Phenylethanol	–	+++[c]	–	–	+	+	+++	200	400
2-Phenylacetaldehyde	–	+++	–	–	–	–	–	>500	Not tested
2-Phenylacetic acid	–	+++	–	++	+	++	+++	25	100

[a] Negative control with DMSO.
[b] Positive control with 100 U/ml of streptomycin sulfate for bacterium.
[c] +++, antimicrobial zone is above 20 mm in diameter.
[d] ++, antimicrobial zone is between 16 and 20 mm.
[e] +, antimicrobial zone is less than 15 mm.
[f] No inhibition.

compounds in this study were more effective inhibitors on mushroom tyrosinase with IC_{50} as 3.04, 0.39 and 2.38 mM for 2-phenylethanol, 2-phenylacetaldehyde and 2-phenylacetic acid, respectively.

However, the inhibition types of 2-phenylacetaldehyde and 2-phenylacetic acid were determined to be uncompetitive, whereas 2-phenylethanol displayed a competitive and uncompetitive mixed-type mechanism. The results indicated that they have different molecular inhibitory mechanisms. In the process of catalysis, tyrosinase has three existing forms, E_{met}, E_{oxy} and E_{deoxy}; both the E_{met} form and E_{oxy} form can catalyze the diphenol substrate (D) (Espin et al., 2000). If an inhibitor binds only with the free enzyme molecule, it will be a competitive inhibitor for the diphenolase, whereas it will be an uncompetitive inhibitor if an inhibitor binds only with the enzyme–substrate complex. From the results, it can be seen that 2-phenylacetaldehyde and 2-phenylacetic acid can bind with the enzyme–substrate complexes ($E_{met}D$ or $E_{oxy}D$) but cannot bind with the free enzyme forms (E_{met} and E_{oxy}). On the other hand, 2-phenylethanol can combine with both free enzymes (E_{met} and E_{oxy}) and enzyme–substrate complexes

($E_{met}D$ or $E_{oxy}D$); the value of K_{IS} was about sevenfold that of K_I, indicating that the affinity of the inhibitor for free enzyme complexes was stronger than for the enzyme–substrate.

Mushroom tyrosinase is composed of four subunits and contains two binuclear coppers in its active sites per tetramer. Walker and Wilson (1975) reported that tyrosinase has two sites of combination, one for the substrate and the other for the inhibitor. The result of the inhibitory type leads us to suppose the 2-phenylethanol might compete for attachment on the active site of the enzyme with L-DOPA due to its –OH group. On the other hand, the presence of a –COH group on 2-phenylacetaldehyde and a –COOH group on 2-phenylacetic acid had no effect on the competition with the substrate. Since the substrate can bind with the enzyme to some extent, it may induce the enzyme conformation to change so that the hydrophobic pocket surrounding the binuclear copper active site in the enzyme becomes bigger. Therefore, it is concluded that 2-phenylacetaldehyde and 2-phenylacetic acid could not attach to the enzyme but could be embraced by the hydrophobic pocket.

The aldehyde group is known to react with biologically important nucleophilic groups, e.g. sulfhydryl, amino and hydroxyl groups. Their antityrosinase activities presumably come from their ability to form a Schiff base with a primary amino group in the enzyme (Song et al., 2005). Most of the aldehyde compounds were reported to be noncompetitive or mixed-type, such as benzaldehyde, anisaldehyde and cuminaldehyde (Chen et al., 2003). In this paper, 2-phenylacetaldehyde exhibited a significant antityrosinase mechanism. It might not form a stable Schiff base but still show some inhibitory activity, which could be explained by their hydrophobic interaction with the enzyme, leading to disturbing the tertiary structure of enzyme.

2-Phenylethanol had been shown to be inhibitory to Gram-negative (Lilly & Brewer, 1953) and Gram-positive (Berrah & Konetzka, 1962) bacteria. 2-Phenylethanol acted as an inhibitor of the bacteria, Berrah and Konetzka reported that 2-phenylethanol blocked deoxyribonucleic acid synthesis (Berrah & Konetzka, 1962). But Sliver and Wendt found that 2-phenylethanol affected the permeability (Silver & Wendt, 1967). In our study, 2-phenylethanol and 2-phenylacetic acid were both found to have effective antibacterial activities against *E. coli* and *R. solanacearum*. *R. solanacearum* is a soil-borne bacterium that caused bacterial wilt disease in diverse and important food crops such as tomato, potato, banana and ginger (Hayward, 1991). But 2-phenylacetaldehyde was not found to have antibacterial activity. 2-Phenyaldehyde is highly reactive due to its formyl group, and it may therefore be degraded before blocking essential enzymes, which could be a reason why we did not find any antibacterial activity. In conclusion, all of these data may provide the basis for developing novel tyrosinase inhibitors and searching for new potent food preservatives or insecticides.

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