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Inhibitory effects of salicylic acid family compounds on the diphenolase activity of mushroom tyrosinase

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Abstract

Mushroom tyrosinase (EC 1.14.18.1) catalyzes both the hydroxylation of tyrosine into *o*-diphenols and the oxidation of *o*-diphenols into *o*-quinones that form brown or black pigments. In the present paper, inhibitory effects on the diphenolase activity of 10 compounds of the salicylic acid-family on the diphenolase activity of mushroom tyrosinase have been studied. The results show that some of these compounds behave as reversible inhibitors. Salicylic acid is a competitive inhibitor while 4-methoxysalicylic acid is non-competitive, 5-methoxysalicylic acid is mixed-I type and 4-methylsalicylic acid and 5-methylsalicylic acid are mixed-II type. The inhibition constants of these five compounds were evaluated. The inhibition strength follows the order: 4-methylsalicylic acid > 5-methylsalicylic acid > 4-methoxysalicylic acid > salicylic acid > 5-methoxysalicylic acid. Models of the interaction between the enzyme and the inhibitors are further discussed and compared.

Keywords: Mushroom tyrosinase; Diphenolase activity; Salicylic acid-family; Inhibition mechanism; Kinetics

1. Introduction

Tyrosinase (EC 1.14.18.1), a copper-containing mixed-function oxidase, is widely distributed in microorganisms, animals and plants. The enzyme catalyzes both the hydroxylation of monophenols and the oxidation of *o*-diphenols into *o*-quinones which polymerize to form brown or black pigments (Prota, 1988). In some vegetables and fruits, tyrosinase is responsible for browning and is considered to be deleterious to the colour quality of plant-derived foods and beverages (Martynez & Whitaker, 1995). This unfavourable darkening from enzymatic oxidation generally results in loss of nutritional and market value. Therefore, the browning is a major problem in the food industry and the control of the tyrosinase activity is important in preventing the synthesis of melanin in the browning of vegetables and fruits.

Tyrosinase inhibitors have recently attracted concern due to their decreasing the hyper pigmentation resulting from the enzyme action (Friedman, 1996), and become increasingly important in medicinal (Mosher, Pathak, & Fitzpatrick, 1983) and cosmetic products (Maeda & Fukuda, 1991). Although a large number of tyrosinase inhibitors have already been reported, some of their individual activities are not potent enough to be put into practical use or they are not compatible with safety regulations for food additives. So we still have to rely on laboratory synthesis or extraction from plants to resolve

Abbreviations: DMSO, dimethyl sulfoxide; DOPA, L-3 4-dihydroxyphenylalanine; IC_{50}, the inhibitor concentrations leading to 50% activity lost.

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the problems. It is well known that mushroom tyrosinase can be inhibited by aromatic aldehydes (Jimenez, Chazarra, Escribano, Cabanes, & Garcia-Carmona, 2001), aromatic acids (Robit, Rouch, & Cadet, 1997), tropolone (Valero, Garcia-Moreno, Varon, & Garcia-Carmona, 1991) and kojic acid (Cananes, Chazarra, & García-Carmona, 1994). In the present investigation, some salicylic acid-family compounds were tested for tyrosinase inhibitory capacity. The aim of this present experiment is, therefore, to carry out a kinetic study of the inhibition of the diphenolase activity of mushroom tyrosinase and to evaluate the kinetic parameters in order to help the laboratory to synthesize and alter some tyrosinase inhibitors.

2. Materials and methods

2.1. Reagents

Salicylic acid (**a**), 3-hydroxysalicylic acid (**b**), 4-hydroxysalicylic acid (**c**), 5-hydroxysalicylic acid (**d**), 3-methoxysalicylic acid (**e**), 4-methoxysalicylic acid (**f**), 5-methoxysalicylic acid (**g**), 3-methylsalicylic acid (**h**), 4-methylsalicylic acid (**i**), 5-methylsalicylic acid (**j**) (Fig. 1 for structures) and L-3,4-dihydroxyphenylalanine (DOPA) were purchased from Aldrich Chemical Co. Dimethyl sulfoxide (DMSO) was obtained from Sigma Chemical Co. Tyrosinase (EC 1.14.18.1), from mushroom, was also the product of Sigma Chemical Co. The specific activity of the enzyme is 6680 U/mg. All other reagents were home-made and of analytical grade. The water used was re-distilled and ion-free.

2.2. Enzyme assay

Diphenolase activity in the mushroom tyrosinase assay was determined as previously reported (Chen, Liu, & Huang, 2003). The reaction media (3 ml) contained 0.5 mM DOPA in 50 mM sodium phosphate buffer (pH 6.8), the indicated concentration of inhibitor and 3.3% DMSO. The final concentration of mushroom tyrosinase was 6.67 μg/ml. In this method, 0.1 ml of different concentrations of effector dissolved in DMSO solution was placed in test tubes. Then, 2.8 ml of substrate system in sodium phosphate buffer preincubated at 30 °C was mixed in and 0.1 ml of the aqueous solution of the mushroom tyrosinase (containing 20 μg) was added to the mixture. The enzyme activity was determined by following the increase in optical density at 475 nm accompanying the oxidation of DOPA to dopachrome with the molar absorption coefficient of $3.70 \times 10^3\ M^{-1}\ cm^{-1}$ (Jimenez et al., 2001). The reaction was carried out at a constant temperature of 30 °C. Controls, without inhibitor but containing 3.3% DMSO, were routinely carried out. A Beckman UV-650 spectrophotometer was used for absorbance and kinetic measurements.

The inhibitory extent of the compounds was expressed as the inhibitor concentrations leading to 50% of the enzyme activity lost (IC_{50}). The inhibition type of some salicylic acid-family compounds on the enzyme was assayed by a Lineweaver–Burk plot. The inhibition constant was determined by plots of the apparent $1/V_m$ or K_m/V_m versus the concentration of the inhibitor, as described by Chen et al. (2003).

3. Results

3.1. Effect of salicylic acid-family compounds on the diphenolase activity of mushroom tyrosinase

Taking (**a**)–(**j**) as the effectors, we probed the effects of these 10 salicylic acid-family compounds on the activity of mushroom tyrosinase for the oxidation of DOPA. The results (Fig. 2) show that compounds of (**a**), (**f**), (**g**), (**i**) and (**j**) have prominent inhibition effects on the enzyme activity while (**d**) and (**h**) have weak inhibition effects and (**b**), (**c**) and (**e**) have hardly any inhibition effects. With regard to compounds (**a**), (**f**), (**g**), (**i**) and (**j**), the IC_{50}, the inhibitor concentration leading to 50% activity lost, can be determined from Fig. 2, and the results are listed in Table 1 for comparison. The inhibi-

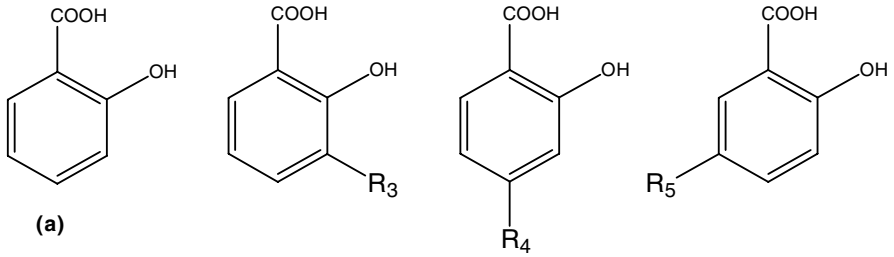

Fig. 1. Chemical structures of salicylic acid-family compounds.

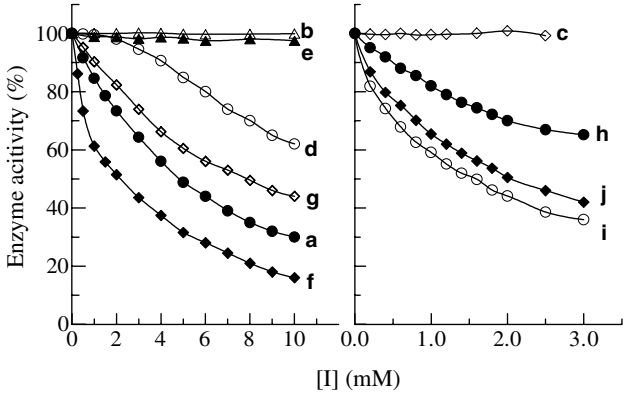

Fig. 2. Effects of salicylic acid-family compounds on the diphenolase activity of mushroom tyrosinase.

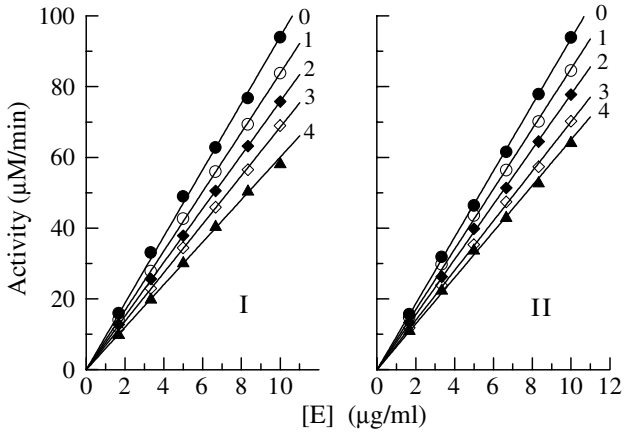

Fig. 3. The effect of concentrations of tyrosinase on its activity for the catalysis of DOPA at different concentrations of 4-methoxysalicylic acid (**f**) (Fig. 1) and 4-methylsalicylic acid (**i**) (Fig. 2). The concentrations of (**f**) for curves 0–4 are 0, 0.5, 1.0, 1.5 and 2.0 mM, respectively. The concentrations of (**i**) for curves 0–4 are 0, 0.25, 0.50, 0.75 and 1.0 mM, respectively.

tion strength follows the order: (**i**) > (**j**) > (**f**) > (**a**) > (**g**) > (**h**) > (**d**). 4-methylsalicylic acid is the most potent inhibitor of the diphenolase of mushroom tyrosinase among these compounds.

3.2. The inhibition mechanism of salicylic acid-family compounds on the enzyme

The inhibition mechanism on the enzyme by compounds (**a**), (**f**), (**g**), (**i**) and (**j**), for the oxidation of DOPA, was studied. Fig. 3 shows the relationship between enzyme activity and concentration in the presence of different concentrations of compounds (**f**) and (**i**), respectively. The plots of the remaining enzyme activity versus the concentrations of enzyme in the presence of different concentrations of (**f**) and (**i**) gave a family of straight lines, which all passed through the origin. Increasing the inhibitor concentration resulted in decreasing slope of the line, the presence of (**f**) and (**i**) did not decrease the amount of effective enzyme, but just resulted in the inhibition and decrease of enzyme activity, indicating that the inhibition of (**f**) and (**i**) on the enzyme was reversible. Other inhibitors tested (**a**, **g** and **j**) showed the same behaviour. They are all reversible inhibitors of mushroom tyrosinase for oxidation of DOPA.

Table 1
Inhibition effects and constants of salicylic acid-family compounds on mushroom tyrosinase

Compounds	Relative activity (%)	IC_{50} (mM)	Inhibition type	Inhibition constants (mM)	
				K_I	K_{IS}
Control	100				
A	30.5[a]	4.30	Competitive	2.49	
B	100[a]				
C	100[a]				
D	60.5[a]				
E	99.8[a]				
F	15.2[a]	2.28	Non-competitive	2.30	2.30
G	42.1[a]	7.90	Mixed type I	5.45	17.6
H	65.3[b]				
I	35.3[b]	1.65	Mixed type II	2.49	1.19
J	43.2[b]	2.15	Mixed type II	3.39	1.40

[a] Inhibitor concentration is 10 mM.
[b] Inhibitor concentration is 3 mM.

*3.3. Inhibition by salicylic acid (**a**) of the diphenolase activity of mushroom tyrosinase, following competitive mechanism*

The kinetic behaviour of mushroom tyrosinase during the oxidation of DOPA was studied. Under the conditions employed in the present investigation, the oxidation reaction of DOPA by mushroom tyrosinase follows Michaelis–Menten kinetics. In the presence of (**a**), the kinetics of the enzyme, using the double-reciprocal Lineweaver–Burk plots, were studied. The results (Fig. 4) show that the plots of $1/v$ versus $1/[S]$ give a family of straight lines with different slopes that intersected one another in the Y-axis. The value of V_m remains the same and the value of K_m increases on increasing the concentrations of the inhibitor, which indicates that salicylic acid is a competitive inhibitor of the enzyme. It only binds free enzyme and not enzyme–substrate complex. The inhibition constant for the inhibitor binding to the free enzyme, K_I, was obtained from the secondary plot (inset in Fig. 4) and is summarized in Table 1.

*3.4. Inhibition of 4-methoxysalicylic acid (**f**), following noncompetitive mechanism*

The kinetic behaviour of 4-methoxysalicylic acid (**f**) for the inhibition of the enzyme was studied and the

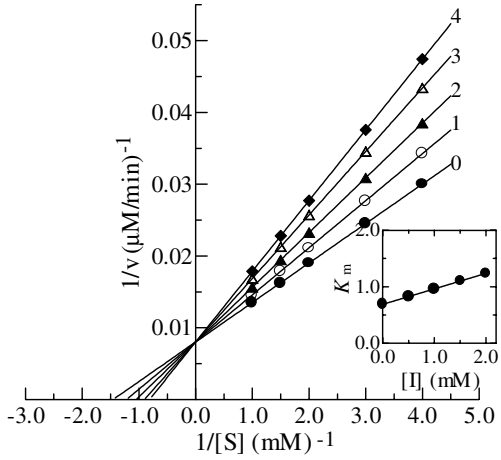

Fig. 4. Lineweaver–Burk plots for inhibition of salicylic acid (**a**) on the oxidation of DOPA by tyrosinase. Concentrations of (**a**) for curves 0–4 are 0, 0.5, 1.0, 1.5 and 2.0 mM, respectively. The enzyme concentration is 6.66 μg/ml. The inset represents the plot of K_m versus concentrations of (**a**) to determine the inhibition constant.

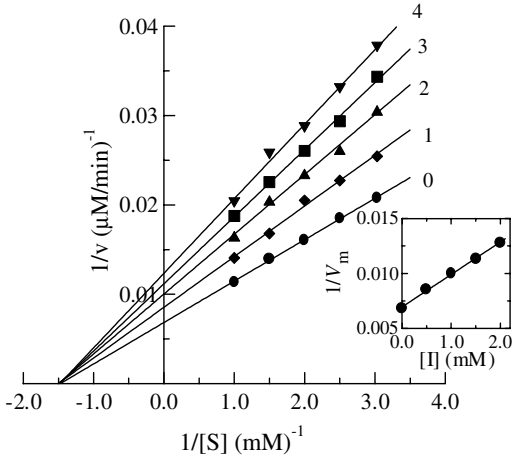

Fig. 5. Lineweaver–Burk plots for inhibition of 4-methoxysalicylic acid (**f**) on the oxidation of DOPA by tyrosinase. Concentrations of (**f**) for curves 0–4 are 0, 0.5, 1.0, 1.5 and 2.0 mM, respectively. The inset represents the plot of $1/V_m$ versus concentrations of (**f**) to determine the inhibition constant.

results show that inhibition behaviour of (**f**) is different from that of (**a**). The Lineweaver–Burk plots in the presence of (**f**) yield a family of straight lines with different slopes and with a common intercept on the *X*-axis. The results are shown in Fig. 5, indicating that (**f**) can decrease the apparent value of V_m with no effect on K_m. So, it is a noncompetitive inhibitor of the enzyme which can bind with both the free enzyme and the enzyme–substrate complex, and the equilibrium constants are the same. The inhibition constant can obtained from a plot of the vertical intercept ($1/V_m$) versus the inhibitor concentration, which is linear as shown by the inset of Fig. 5.

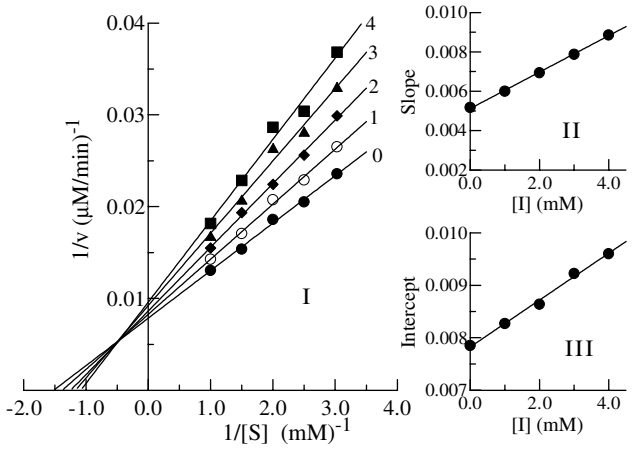

Fig. 6. Inhibition kinetics of 5-methoxysalicylic acid (**g**) on the enzyme by Lineweaver–Burk plots. Concentrations of (**g**) for curves 0–4 are 0, 1.0, 2.0, 3.0 and 4.0 mM, respectively. Insets (II) and (III) represent the secondary plot of the slope and the intercept of the straight lines versus concentration of (**g**), respectively.

*3.5. Inhibition kinetics of 5-methoxysalicylic acid (**g**) on the enzyme activity*

The inhibition kinetics of 5-methoxysalicylic acid (**g**) on the enzyme were studied by Lineweaver–Burk plots. The results are shown in Fig. 6. The double-reciprocal plots yield a family lines with different slopes and different intercepts, and they intersect one another in the second quadrant. This behaviour indicates that (**g**) can bind, not only with free enzyme, but also with the enzyme–substrate complex, and their equilibrium constants are different; (**g**) is a mixed-I type inhibitor of the enzyme. The equilibrium constants for its binding with free enzyme, K_I, and with enzyme–substrate complex, K_{IS}, were obtained from the second plots of the K_m/V_m and $1/V_m$ versus concentration of (**g**), respectively. The value of K_{IS} is about three times as great as K_I, listed in Table 1, indicating that the affinity of inhibitor for free enzyme is stronger than that of inhibitor for the enzyme–substrate complex.

*3.6. Inhibition type and inhibition constants of 4-methylsalicylic acid (**i**) and 5-methylsalicylic acid (**j**) on the diphenolase activity*

The inhibition kinetics of 4-methylsalicylic acid (**i**) and 5-methylsalicylic acid (**j**) on the enzyme were determined by Lineweaver–Burk plots. The inhibition behaviour of (**i**) is the same as that of (**j**). Fig. 7 shows the Lineweaver–Burk plots of the enzyme in the presence of (**i**). The results show that (**i**) is a mixed-II type inhibitor. The equilibrium constants for the inhibitor binding with free enzyme (*E*), K_I, and with enzyme–substrate (ES) complex, K_{IS}, are obtained from the second plots of the K_m/V_m and $1/V_m$ versus concentration of (**i**),

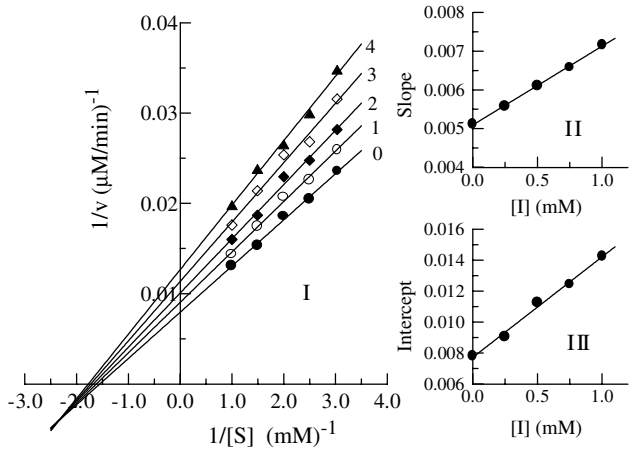

Fig. 7. Inhibition kinetics of 4-methylsalicylic acid (**i**) on the enzyme by Lineweaver–Burk plots. Concentration of (**i**) for curves 0–4 was 0, 0.25, 0.50, 0.75 and 1.0 mM, respectively.

respectively, which are linear (inset in Fig. 7). The value of K_I is larger than that of K_{IS}, listed in Table 1, indicating that the affinity of inhibitor for free enzyme is weaker than that for the enzyme–substrate complex. Similar results were obtained with (**j**). The K_I and K_{IS} values were also obtained from secondary plots and are summarized in Table 1.

4. Discussion

Tyrosinase has two distinct kinds of catalysis functions: the hydroxylation of monophenols and the oxidation of *o*-diphenols (Kubo & Kinst-Hori, 1999). In this paper, we report the effects of salicylic acid and its derivatives on the diphenolase activity of mushroom tyrosinase for the oxidation of DOPA. The results show that, like benzoic acid, some salicylic acid-family compounds (**a**, **f**, **g**, **i**, **j**) are well documented as reversible tyrosinase inhibitors, while (**b**), (**c**) and (**e**) have no inhibitory effect on the activity of the enzyme; the reason is probably that (**b**) and (**e**) have 3-hydroxy and 3-methoxy group, respectively, which can impede their binding to the enzyme molecule by steric hindrance. Interestingly, 4-hydroxysalicylic acid (**c**) has no inhibitory effect on the mushroom tyrosinase. It is dramatically different from certain hexylresorcinols reported, which are very potent inhibitors of mushroom tyrosinase (Chen, Ke, Song, Huang, & Liu, 2004). From the results obtained, we can conclude that the inhibition of salicylic acid (**a**) on diphenolase activity of the enzyme is depressed when the 3rd or 5th position of salicylic acid is substituted by hydroxy or methoxy groups. When the 4th position of salicylic acid is substituted by methyl or methoxy groups, the inhibitory capability of salicylic acid increases. Among these compounds with potent inhibitory ability, salicylic acid (**a**) is a competitive inhibitor while 4-methoxysalicylic acid (**f**) is a non-competitive inhibitor; 5-methoxysalicylic acid (**g**) is mixed-I type; 4-methylsalicylic acid (**i**) and 5-methylsalicylic acid (**j**) are mixed-II type.

Liu, Huang, and Chen (2003) reported that benzoic acid had inhibition effects on the diphenolase activity of mushroom tyrosinase, and Chen et al. (2005) reported that *p*-alkoxybenzoic acids had inhibitory effects on the diphenolase activity of mushroom tyrosinase. Huang, Liu, and Chen (2003) reported that benzaldehyde-family compounds had inhibitory effects on the diphenolase activity of mushroom tyrosinase. These results suggested that the inhibitory effects of these compounds were contributed, not only by the phenyl group but also by other substituted groups. In the present paper, the results showed that the salicylic acid-family compounds substituted in the ring position *para* to the carboxyl bound better than those without substituents. Moreover, more hydrophobic electron donor groups at the *para* position in salicylic acid-family compounds, such as in compounds (**i**), (**j**) and (**f**), make a more potent inhibitor.

Walker and Wilson (1975) suggested the existence of two distinct sites on the enzyme: one site for the binding of the substrate and another site, adjacent, for binding the inhibitor. The inhibitory mechanism of compounds (**f**, **g**, **i**, **j**) led us to hypothesize that these inhibitors can bind not only with the free enzyme but also with the enzyme–substrate complex. They can bind to the coupled binuclear copper active site with the carboxylic group and can be classified as a HA-type inhibitors. For the tested salicylic acid-family compounds, the inhibition strength follows the order: **(i) > (j) > (f) > (a) > (g)**. 4-Methylsalicylic acid is the most potent inhibitor of the diphenolase of mushroom tyrosinase among these compounds.

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References

Cananes, J., Chazarra, S., & García-Carmona, F. (1994). Kojic acid, a cosmetic skin whitening agent, is a slow-binding inhibitor of catecholase activity of tyrosinase. *Journal of Pharmacy and Pharmacology, 46*(12), 982–985.

Chen, Q. X., Liu, X. D., & Huang, H. (2003). Inactivation kinetics of mushroom tyrosinase in the dimethyl sulfoxide solution. *Biochemistry (Moscow), 68*(6), 644–649.

Chen, Q. X., Ke, L. N., Song, K. K., Huang, H., & Liu, X. D. (2004). Inhibitory effects of hexylresorcinol and dodecylresorcinol on mushroom (*Agaricus bisporus*) tyrosinase. *The Protein Journal, 23*(2), 135–141.

Chen, Q. X., Song, K. K., Qiu, L., Liu, X. D., Huang, H., & Guo, H. Y. (2005). Inhibitory effects on mushroom tyrosinase by *p*-alkoxybenzoic acids. *Food Chemistry, 91*(2), 269–274.

Friedman, M. (1996). Food browning and its prevention: an overview. *Journal of Agricultural and Food Chemistry, 44*(3), 631–653.

Huang, H., Liu, X. D., & Chen, Q. X. (2003). Studies on whitening effect of kojic acid as cosmetic additive. *Journal of Xiamen University (Natural Science), 42*(5), 97–101.

Jimenez, M., Chazarra, S., Escribano, J., Cabanes, J., & Garcia-Carmona, F. (2001). Competitive inhibition of mushroom tyrosinase by 4-substituted benzaldehydes. *Journal of Agricultural and Food Chemistry, 49*(8), 4060–4063.

Kubo, I., & Kinst-Hori, I. (1999). Flavonols from saffron flower: tyrosinase inhibitory activity and inhibition mechanism. *Journal of Agricultural and Food Chemistry, 47*, 4121–4125.

Liu, X. D., Huang, H., & Chen, Q. X. (2003). Studies on inhibitory effects of benzoic acid on mushroom tyrosinase. *Journal of Xiamen University (Natural Science), 42*(1), 102–106.

Maeda, K., & Fukuda, M. (1991). In vitro effectiveness of several whiterning cosmetic components in human melanocytes. *Journal of the Society of Cosmetic Chemists, 42*(6), 361–368.

Martynez, M. V., & Whitaker, J. R. (1995). The biochemistry and control of enzymatic browning. *Trends in Food Science and Technology, 6*(6), 195–200.

Mosher, D. B., Pathak, M. A., & Fitzpatrick, T. B. (1983). In T. B. Fitzpatrick, A. Z. Eisen, K. Wolff, I. M. Freedberg, & K. F. Austen (Eds.), *Update: Dermatology in general medicine* (pp. 205–225). New York: McGraw-Hill.

Prota, G. (1988). Progress in the chemistry of melanins and related metabolites. *Medicinal Research Reviews, 8*(4), 525–556.

Robit, C., Rouch, C., & Cadet, F. (1997). Inhibition of palmito (Acanthophoenix rubra) polyphenol oxidase by carboxylic acids. *Food Chemistry, 59*(3), 355–360.

Valero, E., Garcia-Moreno, M., Varon, R., & Garcia-Carmona, F. (1991). Time-dependent inhibition of grape polyphenol oxidase by tropolone. *Journal of Agricultural and Food Chemistry, 39*(6), 1043–1046.

Walker, J. R. L., & Wilson, E. L. (1975). Studies on the enzymatic browning of apples. Inhibition of apple o-diphenol oxidase by phenplics acid. *Journal of the Science of Food and Agriculture, 26*, 1825–1831.