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Inhibitory effects of substrate analogues on enzyme activity and substrate specificities of mushroom tyrosinase

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Abstract

Tyrosinase can catalyze the oxidation of *o*-diphenols to *o*-quinones. In this paper, some *o*-diphenols were used as mushroom tyrosinase substrates to study the catalyzed specificity of the enzyme. The enzyme kinetic analysis of substrate specificities and the substrate analogues towards mushroom tyrosinase has been investigated. Taking L-3,4-dihydroxyphenylalanine (**I**), 3,4-dihydroxyhydrocinnamic acid (**II**), 3,4-dihydroxycinnamic acid (**III**) and 1,2,4-benzenetriol (**IV**) as substrates, the results of specificity studies showed that the oxidation reaction of tested *o*-diphenols by mushroom tyrosinase followed Michaelis–Menten kinetics. The Michaelis–Menten constants for these four substrates were determined to be 0.615, 1.238, 0.331 and 1.886 mM, respectively. The values of V_m/K_m, which denotes the affinity of the enzyme to the substrate, were determined and compared, and the results showed that the affinity of the enzyme to these substrates followed the order: compound **IV** > **III** > **I** > **II**. Furthermore, mushroom tyrosinase cannot catalyze the oxidation of 3,4-dihydroxybenzonitrile (**a**), 3,4-dihydroxybenzaldehyde (**b**), 3,4-dihydroxybenzoic acid (**c**) and 2,3-dihydroxybenzoic acid (**d**). On the contrary, compounds **a**, **b** and **c** can inhibit the activity of tyrosinase for the oxidation of DOPA, while compound **d** had no effects on enzyme activity. The results show that compounds **a** and **b** are reversible non-competitive inhibitors.

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1. Introduction

Tyrosinase (EC 1.14.18.1) is a polyphenolase with broad selectivity. It displays two enzymatic activities which are located on the same active sites: cresolase activity, which is thought to be selective towards monophenols (the hydroxylation of L-tyrosine), and catechol oxidase activity, which is determined to be selective towards diphenols (the oxidation of diphenols into corresponding quinones) (Espín, Jolivet, Overeem, & Wichers, 1999). The active site of tyrosinase consists of two copper atoms and three states: *met, deoxy* and *oxy* (Espín, Varón, & Fenoll, 2000; Jiménez & García-Carmona, 2000). Study of the active site of tyrosinase has been the subject of numerous studies. Different methods such as IR and NMR have been employed for this purpose (Bubacco, Salgado, Tepper, Vijgenboom, & Canters, 1999; Gentschev, Lüken, Möller, Rompel, & Krebs, 2001). Structural models for the active site of these three forms of tyrosinase have been proposed (Fenoll et al., 2001), but the crystal structure of tyrosinase has not been obtained. The reaction kinetic assay based on a spectrophotometric technique for the study of the activity of tyrosinase has attracted attention, mainly because it is convenient, sensitive and inexpensive.

Specificity studies can not only help to unravel the reaction pathway or catalytic mechanism, but also guide

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substrate structure-based inhibitors development and drug design. We are involved in a systematic study of the specificity of mushroom tyrosinase towards different substrates, aimed at the understanding of the factors that govern the interaction between the enzyme and the substrate or some substrate analogues.

Due to the decrease in pigmentation caused by the enzyme action, tyrosinase inhibitors are supposed to have broad applications in medicinal and cosmetic whitening agents (Fenoll et al., 2001; Friedman, 1996; Likhitwitayawuid & Sritularak, 2001). Many efforts have been spent in the search for feasible and effective tyrosinase inhibitors. Substrate analogues, such as cinnamic acid derivatives and 4-substituted resorcinols, could be potent enzyme inhibitors (Jiménez & García-Carmona, 1997; Shi, Chen, Wang, Song, & Qiu, 2005).

In this paper, four kinds of substrate, L-3,4-dihydroxyphenylalanine (L-DOPA) (**I**); 3,4-dihydroxyhydrocinnamic acid (**II**); 3,4-dihydroxycinnamic acid (**III**) and 1,2,4- benzenetriol (**IV**), were tested and compared. The effects of substrate analogues, 3,4-dihydroxybenzonitrile (**a**); 3,4-dihydroxybenzaldehyde (**b**); 3,4-dihydroxybenzoic acid (**c**) and 2,3-dihydroxybenzoic acid (**d**) were also tested for tyrosinase inhibitory ability (Fig. 1). Despite their close structural similarity, these analogues showed many differences in inhibition towards mushroom tyrosinase. The aim of this present experiment is, therefore, to carry out a kinetic study on the inhibition of tyrosinase by these *o*-diphenol derivatives and to evaluate the kinetic parameters and inhibition constants characterizing the system, as well as investigate the inhibition mechanism involved.

2. Materials and methods

2.1. Reagents

Mushroom tyrosinase (EC 1.14.18.1), L-3,4-dihydroxyphenylalanine (L-DOPA), 3,4-dihydroxyhydrocinnamic acid, 3,4-dihydroxycinnamic acid and 1,2,4-benzenetriol were purchased from Sigma. 3,4-Dihydroxybenzonitrile, 3,4-dihydroxybenzaldehyde, 3,4-dihydroxybenzoic acid and 2,3-dihydroxybenzoic acid were the products of Aldrich (St. Louis, MO, USA).

2.2. Enzyme activity assay

Tyrosinase catalyzes the reaction between two substrates, a phenolic compound and oxygen, so the assay was carried out in air-saturated solutions. The diphenolase activity of mushroom tyrosinase was measured spectrophotometrically at 30 °C, by following the oxidation of substrates as previously reported (Chen et al., 2005). The enzyme activity was monitored by dopachrome formation at 475 nm ($\varepsilon = 3700\ M^{-1}\ cm^{-1}$) (Jiménez, Chazarra, Escribano, Cabanes, & García-Carmona, 2001). The reaction media (3 ml) for *o*-diphenolase activity contained 0.5 mM of substrate in 50 mM sodium phosphate buffer (pH 6.8). The final concentration of mushroom tyrosinase was 6.67 μg/ml. This solution was immediately monitored for 1 min, after a lag period of 5 s, for the formation of dopachrome, by measuring the linear increase in optical density at 475 nm. Absorption was recorded using a UV-6000 spectrophotometer.

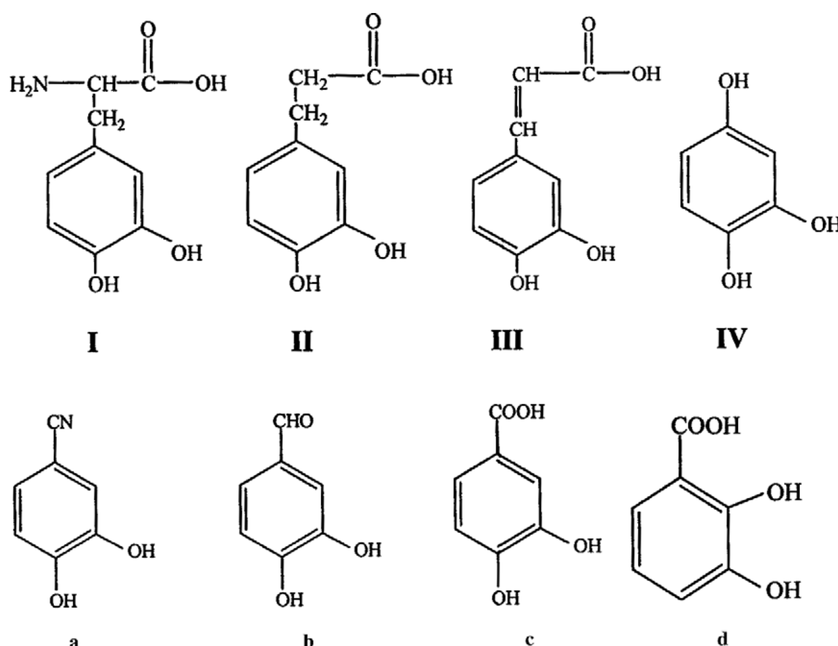

Fig. 1. Chemical structures of L-3,4-dihydroxyphenylalanine (**I**), 3,4-dihydroxyhydrocinnamic acid (**II**), 3,4-dihydroxycinnamic acid (**III**), 1,2,4-benzenetriol (**IV**) as substrates and 3,4-dihydroxybenzonitrile (**a**), 3,4-dihydroxybenzaldehyde (**b**), 3,4-dihydroxybenzoic acid (**c**), 2,3-dihydroxybenzoic acid (**d**) as substrate analogues.

2.3. The assay of specificity of tyrosinase substrates

In the activity assay system, we changed the substrate's concentrations, to determine the enzymatic oxidation initial velocity. According to Michaelis–Menten kinetics, plots of Lineweaver–Burk were made, to estimate the kinetic parameters (K_m and V_m). Then, the value of the specificity constant (V_m/K_m) could be calculated and compared.

2.4. Effect of substrate analogues on enzyme activity

In this method, 0.1 ml of different concentrations of effectors were first mixed with 2.8 ml of reaction solution (consisting of 0.5 mM L-DOPA in 50 mM sodium phosphate buffer, pH 6.8). Then, a portion of 100 μl of enzyme solution (20 μg) was added into this mixture and we determined the residual activity at 30 °C. The extent of inhibition by the compounds was expressed as the inhibitor concentration leading to 50% decrease in enzyme activity (IC_{50}). The inhibition type was assayed by Lineweaver–Burk plots and the inhibition constants were determined by the second plots of the kinetic parameters *versus* the inhibitor concentration, as previously described (Xie et al., 2003).

3. Results

3.1. Determination of kinetic parameters of tyrosinase for the oxidation of different substrates

Under the conditions employed in the present study, the enzymatic reaction follows Michaelis–Menten kinetics. Kinetic parameters of the enzyme for the oxidation of the tested substrates were determined by Lineweaver–Burk plots as shown in Fig. 2. The results are summarized in Table 1.

3.2. Effects of substrate analogues on diphenolase activity of mushroom tyrosinase

Taking 3,4-dihydroxybenzonitrile (**a**); 3,4-dihydroxybenzaldehyde (**b**); 3,4-dihydroxybenzoic acid (**c**) and 2,3-dihydroxybenzoic acid (**d**) (Fig. 1) as substrate analogues, we studied their effects on the oxidation of L-DOPA by mushroom tyrosinase. The results showed that compounds **a**, **b** and **c** had inhibitory effects on enzyme activity, while compound **d** had no effect on the enzyme, as shown in Fig. 3. With increasing concentrations of compounds **a**

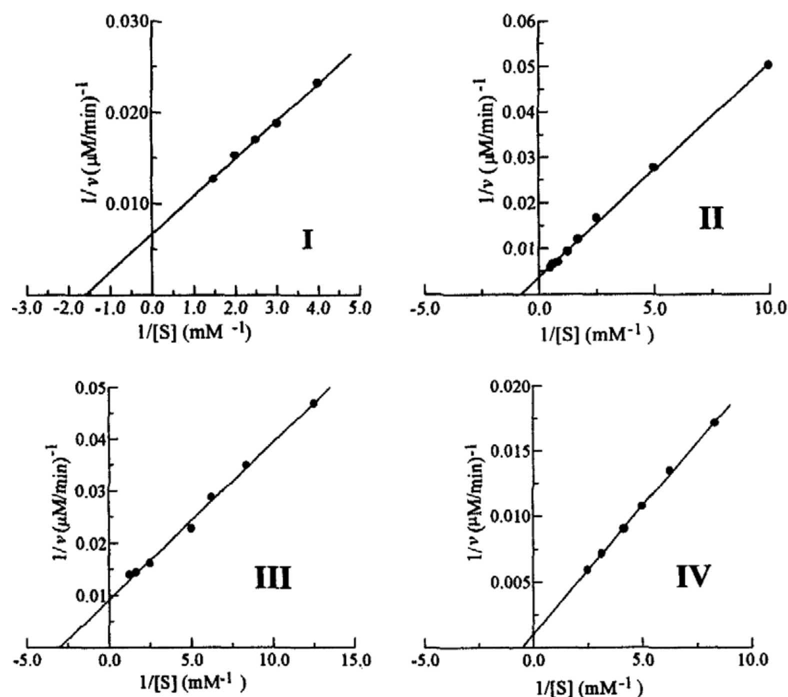

Fig. 2. Plots of Lineweaver–Burk for the determination of kinetic constants (K_m and V_m) for mushroom tyrosinase in the oxidation of L-3,4-dihydroxyphenylalanine (**I**), 3,4-dihydroxyhydrocinnamic acid (**II**), 3,4-dihydroxycinnamic acid (**III**) and 1,2,4-benzenetriol (**IV**).

Table 1
The kinetic parameters of mushroom tyrosinase for the oxidation of several *o*-diphenol substrates

Substrates	K_m (mM)	V_m (μM/min)	V_m/K_m (10^{-3} min^{-1})
3,4-Dihydroxyphenylalanine (**I**)	0.615 ± 0.010	150 ± 2.50	243 ± 2.50
3,4-Dihydroxyphenylalanine acid (**II**)	1.24 ± 0.022	265 ± 3.00	214 ± 3.00
3,4-Dihydroxycinnamic acid (**III**)	0.331 ± 0.008	110 ± 1.60	331 ± 3.50
1,2,4-Benzenetriol (**IV**)	1.89 ± 0.025	971 ± 3.50	515 ± 5.00

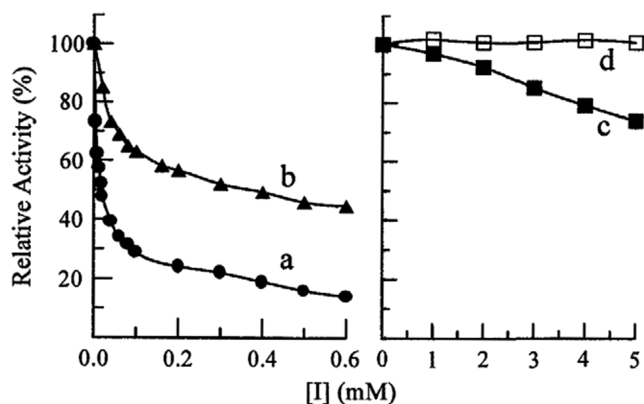

Fig. 3. Effects of the substrate analogues 3,4-dihydroxybenzonitrile (**a**), 3,4-dihydroxybenzaldehyde (**b**), 3,4-dihydroxybenzoic acid (**c**), 2,3-dihydroxybenzoic acid (**d**) on the activity of mushroom tyrosinase for the oxidation of L-DOPA.

and **b**, the diphenolase activity of mushroom tyrosinase markedly decreased. From Fig. 4, for the values of IC_{50} for compounds **a** and **b** were estimated to be 13.5 μM and 232.9 μM, respectively. In comparison, compound **c** had a much smaller inhibitory effect. At 5.0 mM, enzyme activity decreased by 15.2%.

Plots of the remaining enzyme activity *versus* the concentration of enzyme at different inhibitor concentrations for **a** and **b**, gave a series of straight lines, which all passed through the origin (Fig. 4). Increasing the inhibitor concentrations resulted in a decrease in the slope of the line, indicating that the inhibition of **a** and **b** on the enzyme was reversible.

Under the conditions employed in the present investigation, the oxidation reaction of L-DOPA by mushroom tyrosinase followed the Michaelis–Menten equation by Lineweaver–Burk plots (Fig. 5), showed that 3,4-dihydroxybenzonitrile was a non-competitive inhibitor, since increasing its concentration, resulted in a family of lines with a common intercept on the $1/[S]$ axis but with different slopes. The equilibrium constant (K_I) for inhibitor binding with free enzyme or enzyme–substrate complex was determined ($K_I = 13.3$ μM) from a plot of the intercept on the Y-axis *versus* the inhibitor concentration, which was linear as shown in the inset.

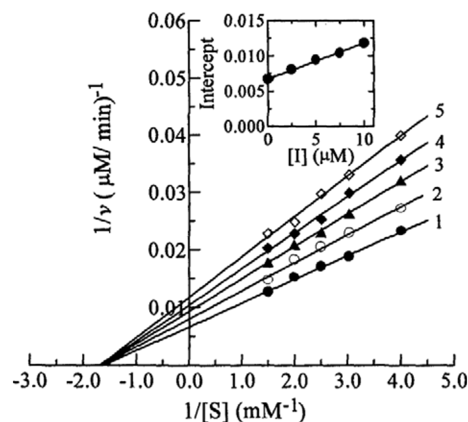

Fig. 5. Lineweaver–Burk plots for the inhibition of 3,4-dihydroxybenzonitrile (**a**) on the oxidation of L-DOPA by mushroom tyrosinase. Concentrations of **a** for curves 1–5 were 0, 2.5, 5.0, 7.5 and 10 μM, respectively. The inset shows the plot of $1/V_{max}$ *versus* the concentration of **a**, to determine the inhibition constant.

Lineweaver–Burk plots (Fig. 6) showed that 3,4-dihydroxybenzaldehyde (**b**) was also a non-competitive inhibitor. The K_m value did not change with increasing 3,4-dihydroxybenzaldehyde concentration, while V_m decreased. The equilibrium constant (K_I) for compound **b** binding with free enzyme or enzyme–substrate complex was determined ($K_I = 253.3$ μM), from a plot of the intercept on the Y-axis *versus* the inhibitor concentration, which was linear, as shown in the inset.

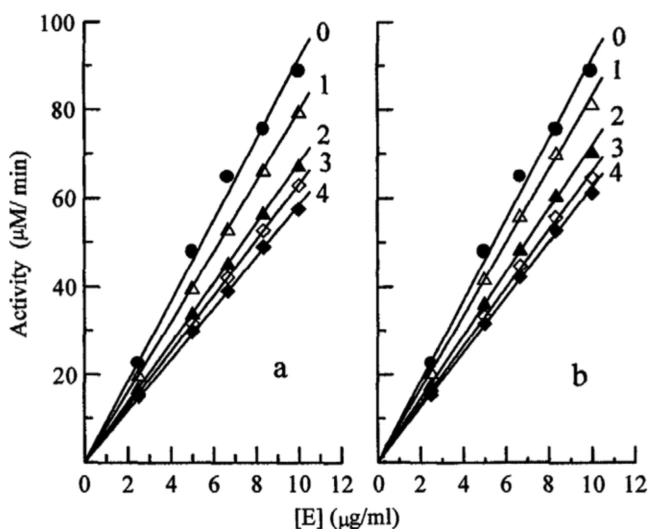

Fig. 4. The inhibition mechanism of 3,4-dihydroxybenzonitrile (**a**), 3,4-dihydroxybenzaldehyde (**b**) on mushroom tyrosinase. The concentrations of **a** for curves 0–4 were 0, 2.5, 5.0, 7.5 and 10 μM, respectively. The concentrations of **b** for curves 0–4 were 0, 25, 50, 75 and 100 μM, respectively.

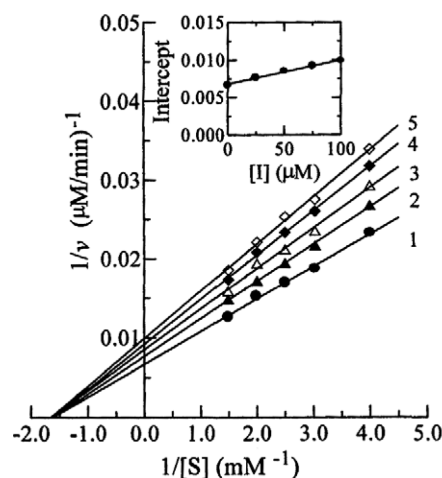

Fig. 6. Lineweaver–Burk plots for the inhibition of 3,4-dihydroxybenzaldehyde (**b**) on the oxidation of L-DOPA by mushroom tyrosinase. Concentrations of **b** for curves 1–5 were 0, 25, 50,75 and 100 μM, respectively. The inset shows the plot of $1/V_{max}$ *versus* the concentration of **b**, to determine the inhibition constant.

4. Discussion

The order of K_m values for the four *o*-diphenols substrates studied were as follows: compound **IV** > compound **II** > compound **I** > compound **III**, and the maximum value for compound **IV** (1.886 mM) was about six times greater than the minimum for compound **III** (0.331 mM). In addition V_m values indicate that compound **IV** and compound **III** are catalyzed by the enzyme at the maximum and minimum rates, respectively. The calculated values of V_m/K_m show the specificity of substrates toward mushroom tyrosinase. The order of the V_m/K_m values for the four substrates was as follows: compound **IV** > compound **III** > compound **I** > compound **II**. Therefore, compound **IV** was the most suitable substrate for mushroom tyrosinase, although the affinity of the enzyme toward this substrate was rather low. Compound **I** is a usual substrate for the diphenolase activity assay of mushroom tyrosinase because it is the oxidation product of tyrosine. 1,2,4-Benzenetriol is an active metabolite of the human leukaemogen benzene (Zhang, Brian, & Davison, 1996) and is a suitable substrate of tyrosinase. Thus, tyrosinase might be responsible for the detoxification of benzene *in vivo*. As 1,2,4-benzenetriol is unstable and easy to oxidise *in vitro*, it is unsuitable to use as a substrate of tyrosinase for the assay of the enzyme activity. In this of investigation, we chose 3,4-dihydroxybenzonitrile (**a**), 3,4-dihydroxybenzaldehyde (**b**), 3,4-dihydroxybenzoic acid (**c**) and 2,3-dihydroxybenzoic acid (**d**) as inhibitors of the mushroom tyrosinase. 3,4-Dihydroxybenzonitrile (**a**) was a more potent inhibitor than 3,4-dihydroxybenzaldehyde (**b**), which was a more potent inhibitor than 3,4-dihydroxybenzoic acid (**c**), while 2,3-dihydroxybenzoic acid (**d**) had no effects on the enzyme. The order of electron-absorbing capacity of the three functional groups present is –CN > –CHO > –COOH. Hence, it can be proposed that the bigger the electron-absorbing capacity of the group in the *para*-position of an *o*-diphenolic compound is, the more effectively will that compound inhibit mushroom tyrosinase. Furthermore, the –CN and –CHO groups in compounds **a** and **b** might bind at an amino acid residue near the catalytic pocket, so that the active site is blocked for binding of substrate. This supposition would explain why compounds **a** and **b** are non-competitive inhibitors of mushroom tyrosinase.

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References

Bubacco, L., Salgado, J., Tepper, A. W. J. W., Vijgenboom, E., & Canters, G. W. (1999). ^{1}H NMR spectroscopy of the binuclear Cu(II) active site of *Streptomyces antibioticus* tyrosinase. *FEBS Letters, 442*(2–3), 215–220.

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.

Espín, J. C., Jolivet, S., Overeem, A., & Wichers, H. J. (1999). Agaritine from *Agaricus bisporus* is capable of preventing melanin formation. *Phytochemistry, 50*(4), 555–563.

Espín, J. C., Varón, R., & Fenoll, L. G. (2000). Kinetic characterization of the substrate specificity and mechanism of mushroom tyrosinase. *European Journal of Biochemistry, 267*, 1270–1279.

Fenoll, L. G., Rodríguez-López, J. N., García-Sevilla, F., García-Ruiz, P. A., Varón, R., García-Canovas, F., et al. (2001). Analysis and interpretation of the action mechanism of mushroom tyrosinase on monophenols and diphenols generating highly unstable *o*-quinones. *Biochimica et Biophysica Acta (BBA)/Protein Structure and Molecular Enzymology, 1548*(1), 1–22.

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

Gentschev, P., Lüken, M., Möller, N., Rompel, A., & Krebs, B. (2001). Synthesis of a novel μ-acetate bridged dinuclear Cu(II) complex as model compound for the active site of tyrosinase: crystal structure, magnetic properties and catecholase activity. *Inorganic Chemistry Communications, 4*(12), 753–756.

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

Jiménez, M., & García-Carmona, F. (2000). Hydroxylating activity of tyrosinase and its dependence on hydrogen peroxide. *Archives of Biochemistry and Biophysics, 373*(1), 255–260.

Jiménez, M., & García-Carmona, F. (1997). 4-Substituted resorcinols (sulfite alternatives) as slow-binding inhibitors of tyrosinase catecholase activity. *Journal of Agricultural and Food Chemistry, 45*, 2061–2065.

Likhitwitayawuid, K., & Sritularak, B. (2001). A new dimeric stilbene with tyrosinase inhibitory activity from *Artocarpus gomezianus*. *Journal of Natural Products, 64*, 1457–1459.

Shi, Y., Chen, Q. X., Wang, Q., Song, K. K., & Qiu, L. (2005). Inhibitory effects of cinnamic acid and its derivatives on the diphenolase activity of mushroom (*Agaricus bisporus*) tyrosinase. *Food Chemistry, 92*(4), 707–712.

Xie, L. P., Chen, Q. X., Huang, H., Liu, X. D., Chen, H. T., & Zhang, R. Q. (2003). Inhibitory effects of cupferron on the monophenolase and diphenolase activity of mushroom tyrosinase. *The International Journal of Biochemistry and Cell Biology, 35*(12), 1658–1666.

Zhang, L. P., Brian, B., & Davison, A. J. (1996). Effects of metals, ligands and antioxidants on the reaction of oxygen with 1,2,4-benzenetriol. *Free Radical Biology and Medicine, 20*(4), 495–505.