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Inhibition of the activity of mushroom tyrosinase by alkylbenzoic acids

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Abstract

The inhibition kinetics of alkylbenzoic acids on the diphenolase activity of mushroom tyrosinase have been investigated. The results show that the alkylbenzoic acids assayed can lead to reversible inhibition of the enzyme; furthermore, *o*-toluic acid and *m*-toluic acid are mixed-type inhibitors and *p*-alkylbenzoic acids are uncompetitive inhibitors. The inhibition constants have been determined. For these *p*-alkylbenzoic acids, the inhibition strength follows the order: *p*-toluic acid < *p*-ethylbenzoic acid < *p*-propylbenzoic acid < *p*-isopropylbenzoic acid < *p*-*tert*-butylbenzoic acid < *p*-butylbenzoic acid < *p*-pentylbenzoic acid < *p*-hexylbenzoic acid < *p*-heptylbenzoic acid < *p*-octylbenzoic acid, indicating that the hydrophobic *p*-alkyl group played an important role in the inhibition of the enzyme. The inhibitory effects were potentiated with increasing lengths of the hydrocarbon chains. The inhibitory effects of *o*-toluic acid and *p*-isopropylbenzoic acid on the monophenolase activity have also been studied. The results show that both *o*-toluic acid and *p*-isopropylbenzoic acid can lengthen the lag time and decrease the steady-state activity of the enzyme.
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Keywords: Mushroom tyrosinase; Diphenolase activity; Monophenolase activity; Alkylbenzoic acids; Inhibition; Kinetics

1. Introduction

Enzymatic browning in fruits and vegetables is predominantly catalyzed by a copper-containing enzyme, tyrosinase (EC.1.14.18.1; also known as catecholase, diphenol oxidase) (Mayer, 1995; Whitaker, 1995). It exists widely in nature and catalyzes the oxidation of *o*-diphenols to *o*-quinones. Quinones chemically evolve to give rise to melanins or react with amino acids and proteins to enhance the colour products, which are brown, black, or red heterogeneous polymers (Martínez & Whitaker, 1995). The active site of tyrosinase consists of two copper atoms and the enzyme has three states: *oxy*tyrosinase (E_{oxy}), *met*tyrosinase (E_{met}) and *deoxy*tyrosinase (E_{deoxy}) (Espín, Varón, & Fenoll, 2000). Therefore, the control of the tyrosinase is important in relation to browning control of fresh and hot-air-dried apple slices, as well as potatoes, avocados and grape juices (McEvily, Iyengar, & Otwell, 1991). Additionally, tyrosinase inhibitors are becoming important constituents of cosmetic products in relation to hyperpigmentation (Maeda & Fukuda, 1991). Their activity is of importance in preventing the synthesis of melanin in plants and animals. For example, *p*-hexylresorcinol has been known to be effective in preventing shrimp and frozen crab melanosis, and it has been recognized as safe for use in browning control (McEvily et al., 1991).

Tyrosinase inhibitors have recently attracted concern (Chen, Song, Wang, & Huang, 2003) due to decreasing

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the hyperpigmentation resulting from enzyme action. Hence, the tyrosinase inhibitors should have broad applications in keeping fruit and vegetables fresh (Xie, Chen, Huang, Wang, & Zhang, 2003). It is well known that tyrosinase can be inhibited by aromatic aldehydes and aromatic acids (Robit, Rouch, & Cadet, 1997), tropolone (Espín & Wichers, 1999) and kojic acid (Kahn, Ben-Shalom, & Zakin, 1997). Recently, benzaldehydes (Huang, Liu, & Chen, 2003) and alkoxybenzoic acids (Chen et al., 2005) have been targeted for inhibition of the enzyme. They were noted to inhibit both diphenolase activity and monophenolase activity of mushroom tyrosinase. During this study, alkylbenzoic acids were found to inhibit the monophenolase and diphenolase activity of mushroom tyrosinase. In the present investigation, alkylbenzoic acids were tested for tyrosinase inhibitory capacity. Despite their close structural similarity, these analogues showed many differences in the inhibition mechanism on mushroom tyrosinase. The aim of this present series of experiments is, therefore, to carry out a kinetic study of the inhibition of the diphenolase activity of tyrosinase by alkylbenzoic acids and to evaluate the kinetic parameters and inhibition constants characterizing the system. The inhibition mechanism involved was also investigated.

2. Materials and methods

2.1. Chemicals

o-Toluic acid **(a)**; *m*-toluic acid **(b)**; *p*-toluic acid **(c)**; *p*-ethylbenzoic acid **(d)**; *p*-propylbenzoic acid **(e)**; *p*-isopropylbenzoic acid **(f)**; *p*-*tert*-butylbenzoic acid **(g)**; *p*-butylbenzoic acid **(h)**; *p*-pentylbenzoic acid **(i)**; *p*-hexylbenzoic acid **(j)**; *p*-heptylbenzoic acid **(k)**; *p*-octylbenzoic acid **(l)** (see Fig. 1 for structures) and L-3,4-dihydroxyphenylalanine (L-DOPA) were purchased from Aldrich (St. Louis, MO, USA). Dimethylsulfoxide (DMSO) was obtained from Sigma (St. Louis, MO, USA). Tyrosinase from mushroom was also the product of Sigma. All other reagents were of analytical grade. The water used was re-distilled and ion-free.

2.2. Assay of enzyme activity

The monophenolase activity and diphenolase activity assay was performed as reported by Chen et al. (2003). In this investigation, Tyr was used as the substrate for the monophenolase activity assay, and L-DOPA was used as the substrate for the diphenolase activity assay. The reaction media (3 ml) for activity assay contained 2.0 mM Tyr or 0.5 mM L-DOPA in 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8). The final concentration of mushroom tyrosinase was 33.3 μg/ml for the monophenolase activity and 6.67 μg/ml for the *o*-diphenolase

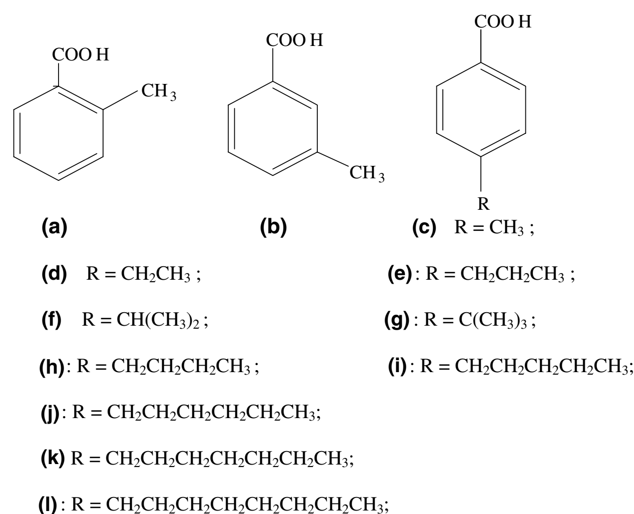

Fig. 1. Chemical structures of alkylbenzoic acids.

activity. The enzyme activity was determined by following the increasing absorbance at 475 nm accompanying the oxidation of the substrates with a molar absorption coefficient of 3700 (M^{-1}/cm) (Jiménez, Chazarra, Escribano, Cabanes, & Garcia-Carmina, 2001).

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 extent of inhibition by the addition of the sample was expressed as the percentage necessary for 50% inhibition (IC_{50}). The inhibition type was assayed by the Lineweaver–Burk plot, and the inhibition constant was determined by the second plots of the apparent K_m/V_m or $1/V_m$ versus the concentration of the inhibitor.

A Beckman UV-650 spectrophotometer was used for absorbance and kinetic measurements. All measurements were carried out at 30 °C.

3. Results

3.1. Effect of alkylbenzoic acids on the diphenolase activity of mushroom tyrosinase

Compounds **(a)**–**(l)** were used as the effectors. We probed the effects of these alkylbenzoic acids on the activity of mushroom tyrosinase for the oxidation of L-DOPA. All of them have inhibitory effects on the enzyme activity with dependence on the concentrations as shown in Fig. 2. With increasing concentrations of inhibitors, the enzyme activity markedly decreased. In order to compare the inhibitory potencies, the IC_{50} values, the concentrations leading to 50% activity lost, of all the tested inhibitors were determined in the same conditions. The IC_{50} values obtained from Fig. 2 are summarized in Table 1 for comparison. Among the three toluic acids (compounds **a, b** and **c**), the *para*-substituted toluic

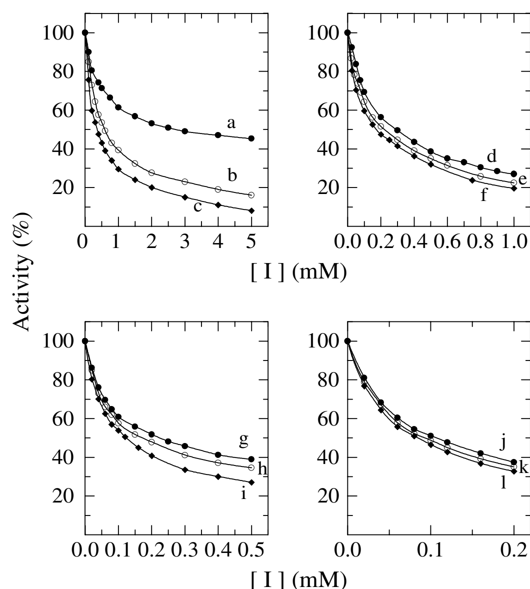

Fig. 2. Effects of alkylbenzoic acids on the diphenolase activity of mushroom tyrosinase.

Table 1
Inhibitory effects and constants of the diphenolase activity of mushroom tyrosinase by alkylbenzoic acids

Compounds	IC50 (mM)	Inhibition type	Inhibition constants (mM)	
			K_I	K_{IS}
a	2.56	Mixed	1.73	5.03
b	0.580	Mixed	0.267	0.824
c	0.350	Uncompetitive		0.150
d	0.290	Uncompetitive		0.125
e	0.235	Uncompetitive		0.102
f	0.225	Uncompetitive		0.095
g	0.215	Uncompetitive		0.090
h	0.165	Uncompetitive		0.072
i	0.120	Uncompetitive		0.052
j	0.110	Uncompetitive		0.047
k	0.095	Uncompetitive		0.040
l	0.082	Uncompetitive		0.035

acid (**c**) is the most potent inhibitor; the second is *meta*-substituted toluic acid (**b**); the *ortho*-substituted toluic acid (**a**) is the weakest one. *p*-Toluic acid is about two times as potent as *m*-toluic acid which is about four times as potent as *o*-toluic acid. For the *para*-substituted alkylbenzoic acids, the inhibition strength follows the order: **(c)** < **(d)** < **(e)** < **(f)** < **(g)** < **(h)** < **(i)** < **(j)** < **(k)** < **(l)**. The inhibitory effects were potentiated with increasing lengths of the hydrocarbon chains.

3.2. The inhibition mechanism of alkylbenzoic acids on the diphenolase activity

The inhibition mechanism on the enzyme by these alkylbenzoic acids for the oxidation of L-DOPA was studied. All of these inhibitors had the same behaviour. Fig. 3 shows the relationship of enzyme activity to the en-

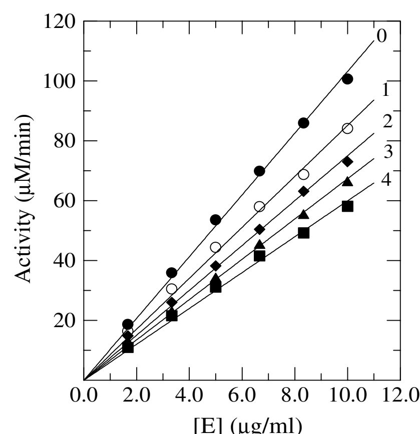

Fig. 3. The effects of concentrations of mushroom tyrosinase on its activity for the oxidation of L-DOPA at different concentrations of *p*-isopropylbenzoic acid **(f)**. The concentrations of **(f)** for curves 0–4 were 0, 10, 20, 30, and 40 μM, respectively.

zyme concentration in the presence of different concentrations of compound **(f)**. The plots of the remaining enzyme activity versus the concentrations of enzyme in the presence of different concentrations of **(f)** gave a family of straight lines, which all passed through the origin. Increasing the inhibitor concentration resulted in a descending slope of the line, indicating that the inhibition of **(f)** on the enzyme was reversible. The presence of inhibitor did not bring down the amount of effective enzyme, but just resulted in the inhibition and decreasing of activity of the enzyme. They are all reversible inhibitors of mushroom tyrosinase for oxidation of L-DOPA.

3.3. Inhibition by o-toluic acid (a) and m-toluic acid (b) in the diphenolase activity following a mixed-type mechanism

The kinetic behaviour of mushroom tyrosinase during the oxidation of L-DOPA has been studied. Under

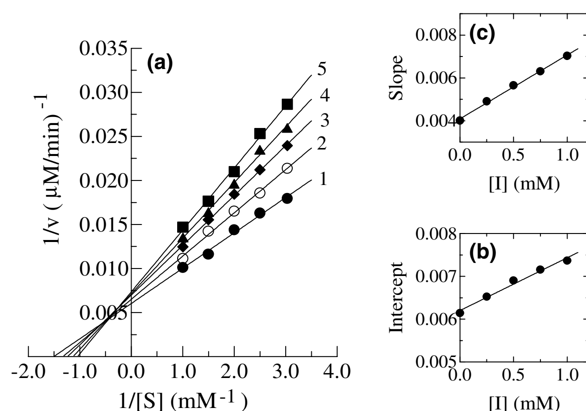

Fig. 4. Lineweaver–Burk plot: (a) for inhibition of *o*-toluic acid **(a)** on mushroom tyrosinase for the catalysis of L-DOPA at 30 °C, pH 6.8. Concentrations of **(a)** for curves 0–4 were 0, 0.25, 0.50, 0.75 and 1.0 mM, respectively; the enzyme concentration was 6.66 μg/ml. *Insets* (b) and (c) represent the secondary plot of the slope and the intercept of the straight lines versus concentration of **(a)**, respectively.

the conditions employed in the present investigation, the oxidation reaction of L-DOPA by mushroom tyrosinase follows Michaelis–Menten kinetics. In the presence of **(a)**, the kinetics of mushroom tyrosinase by Lineweaver–Burk the plots are shown in Fig. 4. The results show that compound **(a)** is a mix-typed inhibitor since increasing the concentration of **(a)** results in a family of lines with different slopes and intercepts, but they intersect one another in the second quadrant. This behaviour shows that compound **(a)** can bind, not only with free enzyme, but also with the enzyme–substrate complex, and the equilibrium constants are different. The inhibition 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 **(a)**, respectively, which are both linear (inset in Fig. 4). The results obtained are also given in Table 1 for comparison. Similar results were obtained with **(b)**. The K_I and K_{IS} values were also obtained from secondary plots and are summarized in Table 1.

3.4. Inhibition of para-substituted alkylbenzoic acids on the diphenolase activity following uncompetitive mechanism

Inhibitory mechanisms of mushroom tyrosinase by compounds of **(c)**–**(l)** have individually been studied. Fig. 5 shows the double-reciprocal plots of the enzyme inhibited by compound **(f)**. The results show that the plots of $1/v$ versus $1/[S]$ give 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 **(f)**, which indicates that compound **(f)** is an uncompetitive inhibitor of the enzyme. All of the other *para*-substituted alkylbenzoic acids show the same behaviour. They are all of the uncompetitive type. The results indicate that the inhibitor binds at a site distinct from the substrate and combines with the enzyme–substrate complex (ES) but not with the free enzyme (E). The equilibrium constant for binding with enzyme–substrate complex (ES), K_{IS}, is obtained from a plot of the vertical intercept ($1/V_m$) versus the inhibitor, which is linear, as shown in the inset of Fig. 5. The inhibition constants are also given in Table 1 for comparison.

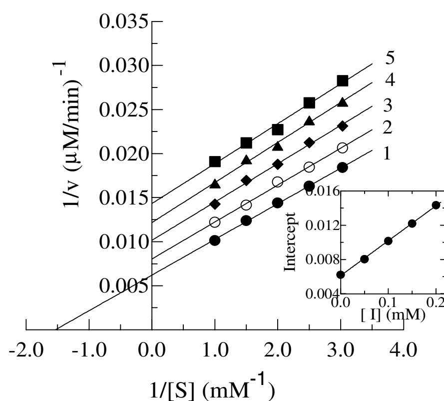

Fig. 5. Lineweaver–Burk plots for inhibition of *p*-isopropylbenzoic acid **(f)** on mushroom tyrosinase for the catalysis of L-DOPA. Concentrations of **(f)** for curves 1–5 were 0, 0.05, 0.10, 0.15 and 0.20 mM, respectively. The inset represents the plot of $1/V_{max}$ versus concentrations of **(f)** to determine the inhibition constant.

3.5. Effect of o-toluic acid (a) and 4-isopropylbenzoic acid (f) on the monophenolase activity of mushroom tyrosinase

The effects of the different concentrations of compounds **(a)** and **(f)** on the oxidation of Tyr by tyrosinase were studied. The kinetics of the oxidation of the substrate in the presence of different concentrations of **(a)** and **(f)** are shown in Figs. 6 and 7, respectively. The results show that both **(a)** and **(f)** have inhibitory effects on the monophenolase activity of the enzyme.

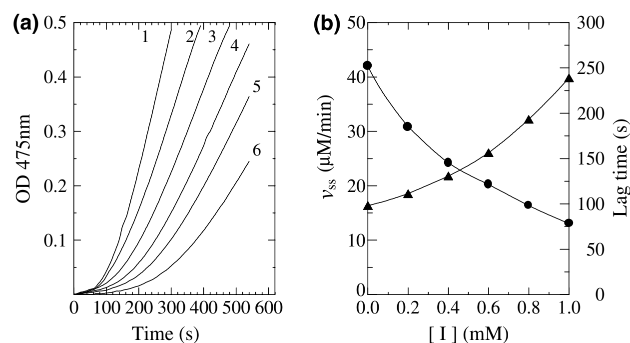

Fig. 6. Inhibition of *o*-toluic acid **(a)** on the monophenolase of mushroom tyrosinase: (a) course of the oxidation of Tyr by the enzyme in the presence of different concentrations of **(a)**. The concentrations of **(a)** for curves 1–6 were 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mM, respectively; (b) effect of **(a)** on the lag time (▲) and the steady-state rate of monophenolase activity (●) for the oxidation of Tyr.

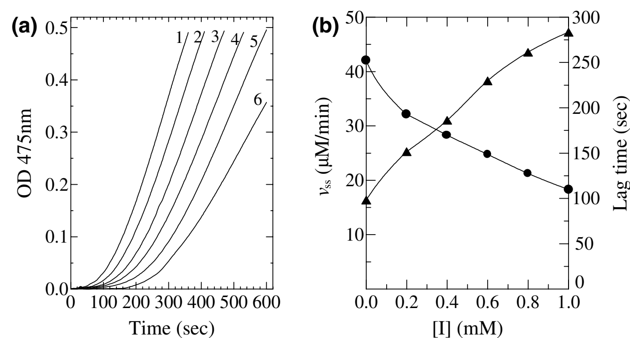

Fig. 7. Inhibition of *p*-isopropylbenzoic acid **(f)** on the monophenolase of mushroom tyrosinase: (a) course of the oxidation of L-tyrosine by the enzyme in the presence of different concentrations of **(f)**. The concentrations of **(f)** for curves 1–6 were 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mM, respectively; (b) effect of **(f)** on the lag time (▲) and the steady-state rate of monophenolase activity (●) for the oxidation of Tyr.

The lag time observably increased with increasing concentration of **(a)**, as shown in Fig. 6(b). The lag phase was estimated to be 98 s in the absence of **(a)**, and extended to 239 s in the presence of 1.0 mM concentration of **(a)**. The lag time lengthened by 144%. On the other hand, the steady-state rate (v_{ss}) decreased exponentially with increasing concentration of **(a)**, as shown in Fig. 6(b). When the concentration of **(a)** reached 1.0 mM, the remaining enzyme activity was determined to be 13.0 μM/min. The enzyme was inhibited by 69%. The inhibitor concentration (IC_{50}) leading to 50% activity lost was estimated to be 0.55 mM. Compound **(f)** also had an inhibitory effect on the monophenolase activity. The lag phase curve increased, as shown in Fig. 7(b). In the presence of 1.0 mM concentration of **(f)**, the lag time was extended from 98 to 283 s. The lag time lengthened by 189%. The steady-state rate (v_{ss}) was determined to be 18.3 μM/min. The monophenolase activity was inhibited by 56.4%, as shown in Fig. 7(b). The IC_{50} was estimated to be 0.81 mM. The results showed that compound **(a)** is more potent than **(f)** for the steady-state rate of monophenolase activity, but weaker in influencing the lag time of mushroom tyrosinase for the oxidation of Tyr, indicating that the inhibition mechanisms of these two compounds **(a, f)** on the monophenolase activity are different.

4. Discussion

In this paper, the effects of some alkylbenzoic acids on the oxidation of L-DOPA by mushroom tyrosinase have been investigated. The results show that all of the tested alkylbenzoic acids have inhibitory effects on the enzyme activity, and they were displayed as reversible inhibitors. The inhibitory mechanism of the *meta*- or *ortho*-substituted toluic acids on the diphenolase activity of the enzyme was shown to be a mix-typed inhibitory mechanism, but all of the *para*-substituted alkylbenzoic acids were displayed to be of the uncompetitive type, indicating that they have different molecular inhibitory mechanisms. In the process of catalysis, tyrosinase has three existing forms, E_{met}, E_{oxy} and E_{deoxy} (Espın et al., 2000). Both the E_{met} form and E_{oxy} form can catalyze the diphenol substrate (D); the E_{oxy} form can also catalyze the monophenol substrate (M) but the E_{met} form cannot. The E_{deoxy} form can combine with oxygen. In this paper, we used L-DOPA (diphenol, D) for the diphenolase activity of the enzyme. If an inhibitor binds only with the free enzyme molecule, E_{met} form and E_{oxy} form, to become $E_{met}I$ and $E_{oxy}I$, respectively, it will be a competitive inhibitor for the diphenolase. If an inhibitor binds only with the enzyme–substrate complex ($E_{met}D$ form and $E_{oxy}D$ form) to become $E_{met}DI$ and $E_{oxy}DI$, respectively, it will be an uncompetitive inhibitor for the diphenolase. From the results, it can be seen that compounds **(a)** and **(b)** can combine with both free enzymes (E_{met} and E_{oxy}) and enzyme–substrate complexes ($E_{met}D$ or $E_{oxy}D$), and they display a competitive and uncompetitive mixed type mechanism. The *para*-substituted alkylbenzoic acids, however, can only bind with enzyme–substrate complexes ($E_{met}D$ form and $E_{oxy}D$ form). All of *p*-alkylbenzoic acids acting on the diphenolase were shown to be uncompetitive inhibitors, which may be explained by the lower electron-donating capacity of the substituents at the *para*-position. Walker and Wilson (1975) reported that tyrosinase has two sites of combination, one for the substrate and the other for the inhibitor. Since the substrate can bind with the enzyme to some extent, it may induce the enzyme conformation to change so that the hydrophobic pocket becomes bigger. We can hypothesize that the combination of the substrate with the enzyme molecule will induce a new hydrophobic pocket in the enzyme–substrate complex, and the *para*-position hydrocarbon chain can just be inserted into the pocket. Among these *p*-alkylbenzoic acids tested, *p*-octylbenzoic acid **(l)** was the most potent inhibitor, suggesting that the hydrophobic pocket accepts the eight-hydrocarbon-chain well. From the results, we can conclude that because of the inducement, the combination between inhibitor (with *para*-position) hydrocarbon chain and enzyme–substrate complex is easier to engender and tighter, which means that the inhibitor could be embraced by the hydrophobic pocket.

The above conclusion was supported by another test using L-tyrosine as substrate. When the enzymatic reaction was started by the action of tyrosinase on Tyr, a marked lag time, characteristic of monophenolase activity, was observed, simultaneously with the appearance of the first stable product, dopachrome (Fenoll, Rodríguez-López, & García-Sevilla, 2001). The system reached a constant rate (the steady-state rate) after the lag time, which was estimated by the extrapolation curve to the abscissa. If the inhibitor combines with the free enzyme, it not only lengthens the lag period but also decreases the steady-state rate. If the inhibitor just combines with the enzyme–substrate complex, it can only decrease the steady-state rate but not lengthen the lag time. Compounds **(a)** and **(f)** not only lengthened the lag period but also decreased the steady-state rate. Compound **(a)** is more potent than **(f)** for the steady-state rate of monophenolase activity, but weaker in influencing the lag time of mushroom tyrosinase for the oxidation of Tyr, indicating that there is some difference between the inhibition mechanisms of these two compounds **(a, f)** on the monophenolase activity.

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