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Inhibitory Effects on Mushroom Tyrosinase by Some Alkylbenzaldehydes

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The inhibition kinetics on the diphenolase activity of mushroom tyrosinase by some alkylbenzaldehydes has been investigated. The results show that the alkylbenzaldehydes assayed can lead to reversible inhibition to the enzyme; *o*-tolualdehyde and *m*-tolualdehyde are mixed-type inhibitors and *p*-alkylbenzaldehydes are uncompetitive inhibitors. For the *p*-alkylbenzaldehydes, the inhibition potency follows the order: *p*-tolualdehyde < *p*-ethylbenzaldehyde < *p*-propylbenzaldehyde = *p*-Isopropylbenzaldehyde < *p*-tert-butylbenzaldehyde = *p*-butylbenzaldehyde < *p*-pentylbenzaldehyde < *p*-hexylbenzaldehyde > *p*-heptylbenzaldehyde > *p*-octylbenzaldehyde, indicating the hydrophobic *p*-alkyl group played an important role in inhibition to the enzyme. The inhibitory effects of alkylbenzaldehydes on the monophenolase activity have also been studied. The results show that *o*-tolualdehyde and *m*-tolualdehyde can lengthen the lag time and decrease the steady-state activity of the enzyme, but *p*-alkylbenzaldehydes only decrease the steady-state activity and do not lengthen the lag time, indicating that their inhibitory mechanisms are different.

Keywords: Inhibition; Mushroom tyrosinase; Monophenolase activity; Diphenolase activity; Alkylbenzaldehydes

INTRODUCTION

Tyrosinase (EC.1.14.18.1) widely exists in nature and catalyzes both the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones. Quinones can chemically give rise to melanins or react with amino acids and proteins to produce colored products, which are brown, black, or red heterogeneous polymers responsible for the loss of quality in many crops.[1,2] The active site of tyrosinase consists of two copper atoms and the enzyme has three states: E_{met}, E_{deoxy} and E_{oxy}.[3] Structural models for the active site of these three forms of tyrosinase have been proposed.[4] The enzyme catalyzing reaction can be summarized as in Scheme 1.

Here M and D represent two different kinds of substrate, monophenol (L-tyrosine) and diphenol (L-DOPA). Q is the product–Quinone and E_{oxy} (*oxy*tyrosinase), E_{met} (*met*tyrosinase) and E_d (*deoxy* tyrosinase) represent the three forms of the enzyme respectively. E_{oxy} M, E_{met}M, E_{oxy}D, E_{met}D are the enzyme-substrate complexes, whereas k_+ and k_- are the rate constants for forward and reverse reaction of each step. The E_{oxy} form starts the turnover by acting on the monophenol (M), which is hydroxylated to the *met*tyrosinase-diphenol intermediate (E_{met}D). At this point, the enzyme can either oxidize D to *o*-quinone (Q), produce the *deoxy*tyrosinase (E_d), or release D to form the E_{met}, which binds with M to produce the inactive form, E_{met}M. D is recycled in the medium through intramolecular cyclization and further redox steps. This would involve the transformation of the E_{met} form of the enzyme (which is inactive towards M) into the E_{oxy} form (which is active towards M) giving rise to the lag time, which is a characteristic of this activity.[5] In such cases the system would reach the steady state.

Tyrosinase inhibitors have attracted concern recently[6] due to decreasing the hyper pigmentation resulting from the enzyme action.

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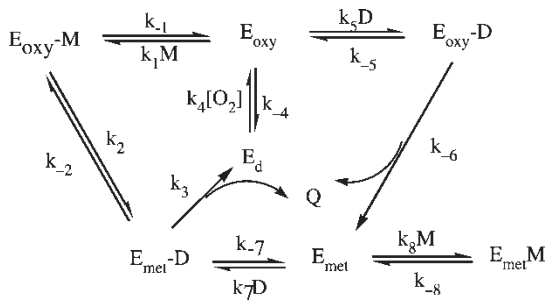
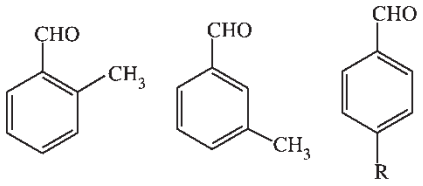


SCHEME 1



(a) **(b)** **(c)** R=CH_3;

(d) R = CH_2CH_3; **(e)**: R = $CH_2CH_2CH_3$;

(f) R = $CH(CH_3)_2$; **(g)** : R = $C(CH_3)_3$;

(h) : R = $CH_2CH_2CH_2CH_3$; **(i)** :R = $CH_2CH_2CH_2CH_2CH_3$;

(j) : R = $CH_2CH_2CH_2CH_2CH_2CH_3$;

(k) R = $CH_2CH_2CH_2CH_2CH_2CH_2CH_3$;

(l) : R = $CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_3$;

FIGURE 1 Chemical structures of alkylbenzaldehydes.

Hence, the tyrosinase inhibitors should have broad applications in medicinal[7] and cosmetic[8] products. Many efforts have been spent in the search for feasible and effective tyrosinase inhibitors. Although a large number of naturally occurring tyrosinase inhibitors have already been reported,[9] their individual activity is not sufficiently potent to be of practical use and the safety regulations of food additives limit their in vivo use, so laboratory synthesis or extraction from plants[10] has been relied upon to resolve the problem. It is well known that mushroom tyrosinase can be inhibited by aromatic aldehydes,[11] aromatic acids,[12] tropolone[13] and kojic acid.[14] Recently, copper chelators have also been targeted for inhibition of the enzyme. The characterization of quercetin was reported as a principal tyrosinase inhibitor[15] and this common flavonoid was also noted to inhibit the diphenolase activity of mushroom tyrosinase.[16] In the present investigation, alkylbenzaldehydes were tested for tyrosinase inhibitory ability. Despite their close structural similarity, these analogues showed many differences in their inhibition mechanism towards mushroom tyrosinase. The aim of this present experiment is, therefore, to carry out a kinetic study on the inhibition of the diphenolase and monophenolase activity of tyrosinase by alkylbenzaldehydes and to evaluate the kinetic parameters and inhibition constants characterizing the system as well as investigate the inhibition mechanism involved.

MATERIALS AND METHODS

o-Tolualdehyde **(a)**, *m*-tolualdehyde **(b)**, *p*-tolualdehyde **(c)**, *p*-ethylbenzaldehyde **(d)**, *p*-propylbenzaldehyde **(e)**, *p*-Isopropylbenzaldehyde **(f)**, *p*-tert-butylbenzaldehyde **(g)**, *p*-butyl-benzaldehyde **(h)**, *p*-pentylbenzaldehyde **(i)**, *p*-hexylbenzaldehyde **(j)**, *p*-heptylbenzaldehyde **(k)**, *p*-octylbenzaldehyde **(l)** (see Figure 1 for structures) and L-3,4-dihydroxyphenylalanine (L-DOPA) were purchased from Aldrich Chemical Co. L-Tyrosine, dimethylsulfoxide (DMSO) and tyrosinase (EC 1.14.18.1 specific activity 6680 U/mg), from mushroom were obtained from Sigma Chemical Co. All other reagents were local and of analytical grade. Re-distillated and ion-free water was used.

Tyrosinase catalyzes a reaction between two substrates, a phenolic compound and oxygen, and the assay was carried out in air-saturated aqueous solutions. Therefore, K_m and V_m values determined under these conditions were only apparent, and the effect of oxygen concentration on these parameters is unknown. The enzyme activity was monitored by dopachrome formation at λ475 nm ($\varepsilon = 3700\,M^{-1}\,cm^{-1}$)[11]accompanying the oxidation of L-DOPA. The diphenolase activity assay was performed as previously reported.[15] The reaction was carried out at a constant temperature of 30°C. The monophenolase assay was performed with L-tyrosine as substrate at 30°C. To the 3 ml assay system containing 50 mM phosphate sodium buffer (pH 6.8), 0.5 mM L-tyrosine, different concentrations of inhibitor, and 0.1 ml of the enzyme (containing 100 μg) was added and the absorption of solution was immediately monitored at λ475 nm for 10 min. Absorption was recorded using a Beckman UV-650 spectrophotometer.

The extent of inhibition by the compounds was expressed as the inhibitor concentration leading to 50 percentage decrease in enzyme activity (IC_{50}). The inhibition type by alkylbenzaldehydes on the enzyme was determined by the 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.[17]

RESULTS

Effect of Alkylbenzaldehydes on the Diphenolase Activity of Mushroom Tyrosinase

Using the alkylbenzaldehydes **(a)–(k)** and **(l)** as inhibitors, we probed the effects of these alkylbenzaldehydes on the activity of mushroom tyrosinase for the oxidation of L-DOPA. The activity of mushroom tyrosinase was inhibited by these alkylbenzaldehydes and was concentration-dependent as shown in Figure 2; With increasing concentrations of alkylbenzaldehydes, the diphenolase activity of mushroom tyrosinase markedly decreased. The IC_{50} values obtained, for the tested inhibitors are given in Table I for comparison. Among the three tolualdehydes (compounds of **a**, **b** and **c)**, the para-substituted isomer is the most potent inhibitor, the second is the meta-substituted isomer and the ortho-substituted isomer is the weakest. *p*-Tolualdehyde is about 4-fold more potent than the *m*-isomer which is about 5-fold more potent than *o*-tolualdehyde. For the para-substituted alkylbenzaldehydes, the inhibition potenoy follows the order: (**c**) < (**d**) < (**e**) = (**f**) < (**g**) = (**h**) < (**i**) < (**j**) > (**k**) > (**l**). *p*-Hexylbenzaldehyde is the most potent inhibitor for the diphenolase action of mushroom tyrosinase.

The Inhibition Mechanism for Alkylbenzaldehydes on the Enzyme

The inhibition mechanism on the enzyme by these alkylbenzaldehydes for the oxidation of L-DOPA was studied. Figure 3 shows the relationship between enzyme activity and its concentration in the presence

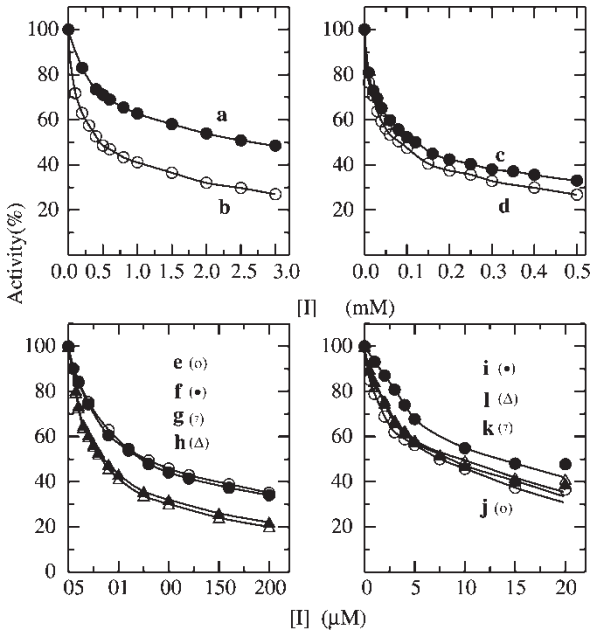


FIGURE 2 Effects of alkylbenzaldehydes on the diphenolase activity of mushroom tyrosinase.

TABLE I Inhibitory effects and constants for of the activity of mushroom tyrosinase with alkylbenzaldehydes

Compounds	IC_{50} (μM)	Inhibition type	Inhibition constants (μM) K_I	K_{IS}
a	2600	Mixed	1853	4775
b	450	Mixed	290	880
c	120	Uncompetitive		51.5
d	95.0	Uncompetitive		40.8
e	75.0	Uncompetitive		32.2
f	75.0	Uncompetitive		32.2
g	38.0	Uncompetitive		16.3
h	38.0	Uncompetitive		16.3
i	13.5	Uncompetitive		5.8
j	8.0	Uncompetitive		3.4
k	8.8	Uncompetitive		3.8
l	10.0	Uncompetitive		4.3

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 decrease in the slope of the line, indicating that the inhibition by **(f)** was of the reversible type. In the presence of **(f)** titration of the enzyme as for irreversible inhibition did not occur, but just linear inhibition with a descent in the activity of the enzyme. Other inhibitors had the same behaviors they are all reversible inhibitors of mushroom tyrosinase for the oxidation of L-DOPA.

Inhibition by *o*-tolualdehyde (a) and *m*-tolualdehyde (b) Followed a (Mixed)-type Mechanism

The kinetic behavior of mushroom tyrosinase during the oxidation of L-DOPA has been studied. Under the conditions employed in the present investigation, the oxidation of L-DOPA by mushroom tyrosinase followed Michaelis-Menten kinetics. In the presence of **(a)**, kinetic studies on mushroom tyrosinase with inhibitors using Lineweaver-Burk plots (Figure 4) showed that **(a)** was a mixed-type inhibitor since increasing the concentration of **(a)** resulted in a family of lines with different slope and intercept, which intersected in the second quadrant. This behaviour showed that **(a)** can bind not only with free enzyme, but also with the enzyme-substrate complex, and their equilibrium constants are different. The equilibrium constants for the inhibitor binding with free enzyme (E), K_I, and with enzyme-substrate (ES) complex, K_{IS}, were obtained from the linear secondary, plots of K_m / V_m and $1/V_m$ versus concentration of **(a)**, respectively (inset in Figure 4). The results obtained are given in Table I for

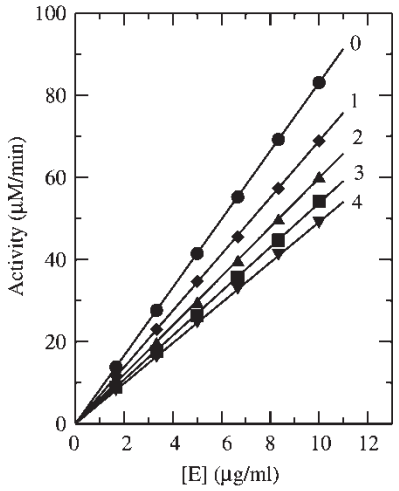


FIGURE 3 The effect of concentrations of mushroom tyrosinase on its activity for the oxidation of L-DOPA at different concentrations of *p*-isopropylbenzaldehyde **(f)**. The concentrations of **(f)** for curves 0–4 were 0, 10, 20, 30, and 40 μM, respectively.

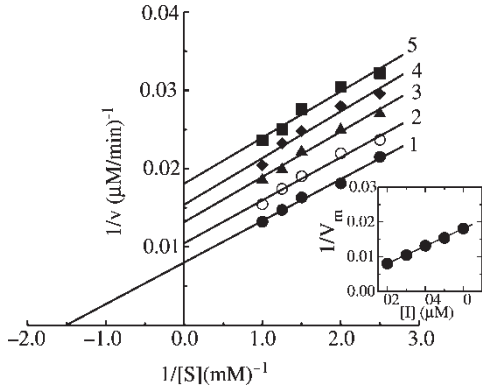


FIGURE 5 Lineweaver-Burk plots for inhibition of *p*-isopropylbenzaldehyde **(f)** on mushroom tyrosinase for the catalysis of L-DOPA. Concentration of **(f)** for curves 1–5 was 0, 10, 20, 30 and 40 μM, respectively. The inset represents the secondary plot of $1/V_{max}$ versus concentrations of **(f)** to determine the inhibition constant.

comparison. Similar results were obtained with **(b)** (Table I).

Inhibition by Para-substitued Alkylbenzaldehydes Followed an Uncompetitive Mechanism

Inhibition of the diphenolase activity of mushroom tyrosinase by compounds **(c)**–**(l)** was individually studied and their inhibitory mechanism was shown to be of the uncompetitive type. Figure 5 showed the double-reciprocal plots of the enzyme inhibited by **(f)**. The results showed that the plots of $1/v$ *versus* $1/[S]$ give a family of parallel straight lines with the same slope. Accompanying an increase in inhibitor concentration, the values of both K_m^{app} and V_{max}^{app} aggrandized, but the ratio of K_m^{app}/V_{max}^{app} remained unchanged, The slope was independent of the concentration of **(f)**, indicating that **(f)** is an uncompetitive inhibitor to the enzyme. This observed behavior shows 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 **(f)** binding with enzyme-substrate complex (ES), K_{IS} was obtained from the linear plot of the vertical intercept ($1/V_m$) *versus* the concentration of **(f)**, as shown in the inset to Figure 5. The inhibition constant is given in Table I for comparison. Similar results were obtained with all other para-substitued alkylbenzaldehydes (Table I).

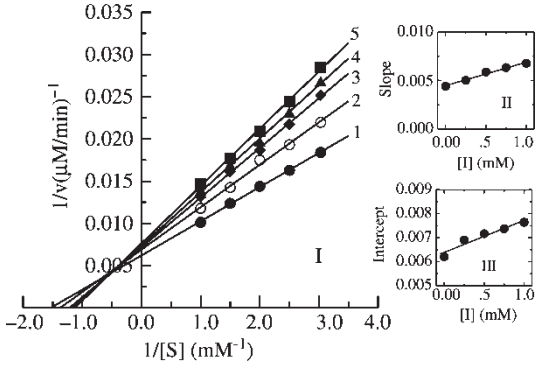


FIGURE 4 Lineweaver-Burk plot (I) for inhibition of *o*-tolualdehyde **(a)** on mushroom tyrosinase for the catalysis of L-DOPA at 30 °C, pH 6.8. Concentration of **(a)** for curves 1–5 was 0, 0.25, 0.50, 0.75 and 1.0 mM, respectively; the enzyme concentration was 6.66 μg/ml. Inset (II) and (III) represent a secondary plot of the slope and the intercept of the straight lines versus concentration of **(a)**, respectively.

Concentration Effects of *m*-tolualdehyde (a) and *o*-tolualdehyde (b) on the Monophenolase Activity of Tyrosinase

Using compounds **(a)** and **(b)** as inhibitors, the inhibitory effect of different concentrations of inhibitors on the oxidation of L-tyrosine by tyrosinase has individually been studied. Figure 6 showed the kinetics course of the oxidation of the substrate in the presence of different concentrations of **(a)**. The lag time increased exponentially with increasing the concentration of **(a)** as shown in Figure 6b. The lag time was estimated to be 110 sec in the absence of **(a)**, and extended to 360 sec in the presence of 1.6 mM of **(a)** i.e. the lag time increased by 3.3-fold. Alternatively, the steady-state rate decreased with increasing the concentration of **(a)** as shown in Figure 6b. Compound **(b)** showed the same behavior.

Concentration Effects of *p*-alkylbenzaldehydes on the Monophenolase Activity of Tyrosinase

Using compounds **(c)**–**(f)** as inhibitors the inhibitory effect of different concentrations of inhibitor on monophenolase activity was individually studied.

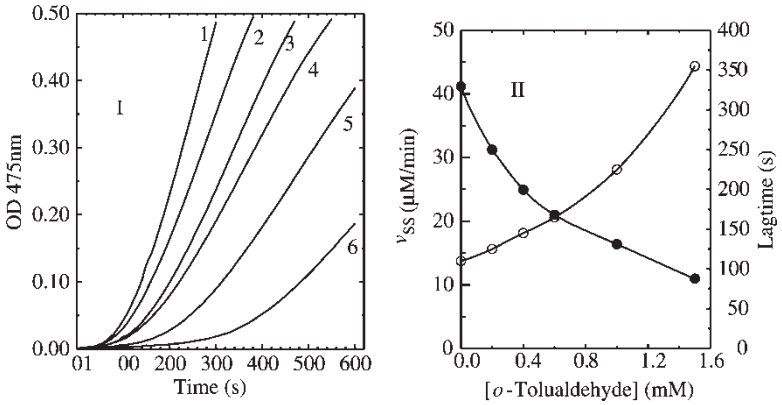


FIGURE 6 Inhibition of *o*-tolualdehyde **(a)** on the monophenolase of mushroom tyrosinase. (I) Course of the oxidation of L-tyrosine by the enzyme in the presence of different concentrations of **(a)**. The concentration of **(a)** for curves 1–6 was 0, 0.2, 0.4, 0.6, 1.0, and 1.5 mM, respectively. (II): Effect of **(a)** on the lag time (o) and the steady-state rate of monophenolase activity (•) for the oxidation of L-tyrosine.

All of them showed the same behavior as illustrated in Figure 7 for the kinetics course of the oxidation of L-tyrosine in the presence of different concentrations of compound **(f)**. The steady-state rate decreased with increasing the concentration of **(f)** but the lag time remained almost unchanged, as shown in Figure 7b. The results indicated that *p*-alkylbenzaldehydes inhibit the monophenolase activity by decreasing the steady-state rate.

DISCUSSION

Tyrosinase has two distinct catalytic functions: the hydroxylation of monophenol and the oxidation of *o*-diphenol. This paper reports the effects of some alkylbenzaldehydes on the oxidation of L-DOPA by mushroom tyrosinase. The results show that the alkylbenzaldehydes have inhibitory effects on enzyme activity, and they were reversible inhibitiors. The inhibitory mechanism of the para-substitued alkylbenzaldehydes on the diphenolase activity of the enzyme was shown to be of the uncompetitive type, but the meta- or ortho-substitued tolualdehydes displayed a mixed-type of inhibitory mechanism, indicating that they have different molecular inhibitory mechanism. In the processing of catalysis, tyrosinase has three existing forms, E_{met}, E_{oxy} and E^3_{deoxy}. Both 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, L-DOPA (diphenol, D) was used for the diphenolase activity of the enzyme. If inhibitor only combines with the free enzyme molecule, E_{met} form and E_{oxy} form, to become $E_{met}I$ and $E_{oxy}I$, respectively, it would be a competitive inhibitor for diphenolase. If inhibitor only combines with the enzyme-substrate complex, $E_{met}D$ form and $E_{oxy}D$ form, it would be an uncompetitive inhibitor for diphenolase. From Scheme I, 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 displayed competitive and uncompetitive

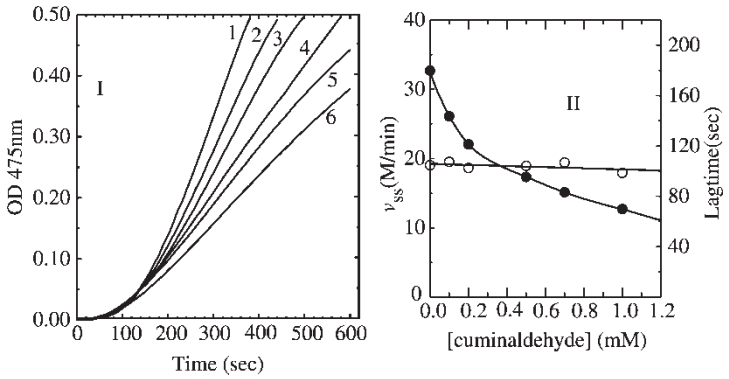


FIGURE 7 Inhibition of *p*-isopropylbenzaldehyde **(f)** on the monophenolase of mushroom tyrosinase. (I) Course of the oxidation of L-tyrosine by the enzyme in the presence of different concentrations of **(f)**. The concentration of **(f)** for curves 1–6 was 0, 0.1, 0.2, 0.5, 0.7, and 1.0 mM, respectively. (II): Effect of **(f)** on the lag time (o) and the steady-state rate of monophenolase activity (•) for the oxidation of L-tyrosine.

mixed-type. The para-substitued alkylbenzaldehydes, however, can only combine with enzyme-substrate complexes ($E_{met}D$ form and $E_{oxy}D$ form) and are uncompetitive types. 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 L-tyrosine, a marked lag time, characteristic of monophenolase activity, was observed simultaneously with the appearance of the first stable product, dopachrome[4]. The system reached a constant rate (the steady-state rate) after the lag period, which was estimated by an extrapolation curve to the abscissa (Figure 6 curve 1). If the inhibitor combines with the free enzyme, it not only lengthens the lag peroid 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 **(b)** lengthened the lag time, but para-substitued alkylbenzaldehydes did not so, they have a different inhibitory mechanism towards the enzyme.

In this paper, we investigate the inhibitory mechanism of a series of *p*-alkylbenzaldehydes on the diphenolase and all of them were showed to be uncompetitive inhibitors, which may be explained by the lower electron-donating capacity of the substituents at the *para*-position. Walker and Wilson [18] reported that tyrosinase has two sites of combination, one for the substrate and the other for the inhibitor. Since the substrate can combine with the enzyme to some extent, it perhaps induced the enzyme conformation to change so that the hydrophobic pocket became larger. We can hypothesize that the combination of the substrate with the enzyme molecule induces a new hydrophobic pocket in the enzyme-substrate complex, and the *para*-position hydrocarbon chain in the inhibitor can just be inserted into the pocket. Among the *p*-alkylbenzaldehydes tested, *p*-hexylbenzaldehyde **(j)** was the most potent inhibitor, suggesting that the hydrophobic pocket can accept a six carbon hydrocarbon-chain well. From the results, it can be concluded that because of the inducement, the combination between inhibitors with a *para*-position hydrocarbon chain and the enzyme-substrate complex is easier to engender and will bind more tightly, which means that the inhibitor could be better embraced by the hydrophobic pocket.

Acknowledgements

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