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Food Chemistry 91 (2005) 269–274
Food Chemistry
www.elsevier.com/locate/foodchem

Inhibitory effects on mushroom tyrosinase by *p*-alkoxybenzoic acids

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Received 10 November 2003; received in revised form 28 January 2004; accepted 28 January 2004

Abstract

The inhibitory kinetics of the diphenolase of mushroom tyrosinase by seven *p*-alkoxybenzoic acids has been studied. The results show that these derivatives of benzoic acid behave as reversible inhibitors. Among them, *p*-hydroxybenzoic acid is competitive, while *p*-methoxybenzoic acid is non-competitive, *p*-ethoxybenzoic acid is mixed-II type, and the rest all behave as classical uncompetitive inhibitors. The inhibition constants of all of the seven compounds assayed, characterizing the inhibition, were evaluated. The models of the interactions between the enzyme and the inhibitors are compared.

Keywords: Mushroom tyrosinase; Diphenolase; *p*-alkoxybenzoic acid; Inhibitory mechanism

1. Introduction

Enzymatic browning in fruits and vegetables is predominantly catalyzed by a copper-containing enzyme, tyrosinase (EC.1.14.18.1), also called catecholase or diphenol oxidase (Mayer, 1995; Whitaker, 1995). This enzyme exists widely in nature and catalyzes both the hydroxylation of monophenols, such as tyrosine, to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones. Quinones polymerize to form melanins or react with amino acids and proteins, which are responsible for the loss of quality in many crops (Martínez & Whitaker, 1995; Prota, 1988). Therefore, the control of the tyrosinase is important in relation to browning control of fresh and hot-air-dried sliced apple 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 the browning of 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.

Many efforts have been addressed to the search for feasible and effective tyrosinase inhibitors. Although many naturally-occurring tyrosinase inhibitors have already been reported (Kubo & Kinst-Hori, 1998a, 1998b), their individual activity is either insufficient to be put into practical use or safety regulations of food additives limit their use in vivo. So, laboratory synthesis or extraction from plants (Kubo et al., 2000) have to resolve the problem. Benzoic acid was identified as a potent mushroom tyrosinase inhibitor by Liu, Huang, and Chen (2003). It was classified as a non-competitive inhibitor of diphenolase and monophenolase and the IC_{50} values were estimated to be 1.00 mM for diphenolase and 1.20 mM for monophenolase. Liu et al. (2003) also reported that benzoic acid could inhibit the monophenolase activity of tyrosinase. But there is little published about the inhibition mechanism of *p*-alkoxybenzoic acids on the enzyme. In the present investigation, several *p*-alkoxybenzoic acids were tested for tyrosinase inhibitory capacity. Despite their close structural similarity, these analogues showed many differences in their inhibition mechanisms against

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doi:10.1016/j.foodchem.2004.01.078

mushroom tyrosinase. The aim of this present experiment was, therefore, to carry out a kinetic study of the inhibition of *o*-diphenolase activity of mushroom tyrosinase by *p*-alkoxybenzoic acids and to evaluate the kinetic parameters and inhibition constants characterizing the system.

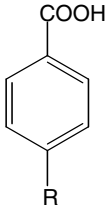
2. Materials and methods

2.1. Chemicals

p-Hydroxybenzoic acid (**a**, HO-BA) (see Fig. 1 for structures), *p*-methoxybenzoic acid (**b**, MeO-BA), *p*-ethoxybenzoic acid (**c**, EtO-BA), *p*-propoxybenzoic acid (**d**, PrO-BA), *p*-butoxybenzoic acid (**e,** BuO-BA), *p*-pentyloxybenzoic acid (**f**, PeO-BA), and *p*-hexyloxybenzoic acid (**g**, HeO-BA) were purchased 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 was 6680 U/mg. Dimethyl sulfoxide (DMSO) and L-3,4-dihydroxyphenyl- alanine (L-DOPA) were obtained from Aldrich Chemical Co. (USA). 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, Chen, Huang, Wang, and Zhang (2003). The *o*-diphenolase activity was monitored by dopachrome formation at 475 nm ($\varepsilon = 3700\ M^{-1}\ cm^{-1}$) (Jimenez, Chazarra, Escribano, Cabanes, & Garcia-Carmina, 2001), accompanying the oxidation of the substrate (L-DOPA). The reaction medium (3 ml) contained 0.5 mM L-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 a test tube. Then, 2.8 ml substrate system in sodium phosphate buffer, preincubated at 30 °C, were added and 0.1 ml of the aqueous solution of the mushroom tyrosinase (containing 20 μg) was added to the mixture. This solution was immediately monitored for 1 min after a lag period 5 s for the formation of dopachrome by measuring the linear increase in optical density at 475 nm. The reaction was carried out under a constant temperature of 30 °C. Absorption was recorded using a UV-6000 spectrophotometer. The inhibition mechanism was judged by the Lineweaver–Burk plots and the inhibition constants were obtained by the second plots of the kinetic parameters against the inhibitor concentration, as previously described (Chen, Lu, Zhu, Lin, & Zhou, 1998; Chen & Zhou, 1999).



(**a**): R = OH ; (**b**): R = OCH_3 ; (**c**): R = OCH_2CH_3 ;

(**d**): R = $OCH_2CH_2CH_3$; (**e**): R = $OCH_2CH_2CH_2CH_3$;

(**f**): R = $OCH_2CH_2CH_2CH_2CH_3$;

(**g**): R = $OCH_2CH_2CH_2CH_2CH_2CH_3$;

Fig. 1. Chemical structures of *p*-alkoxybenzoic acid derivatives.

3. Results

3.1. Effect of p-alkoxybenzoic acids on the activity of mushroom tyrosinase

The effects of the concentrations of various *p*-alkoxybenzoic acids on the oxidation of L-DOPA, by mushroom tyrosinase, were studied. The enzyme was inhibited by several *p*-alkoxybenzoic acids, as shown in Fig. 2. With increasing concentrations of *p*-alkoxybenzoic acids, the diphenolase activity of mushroom tyrosinase markedly decreased. Inhibition of the enzyme by *p*-alkoxybenzoic acid was concentration-dependent. From Fig. 2, it can be seen that, among these

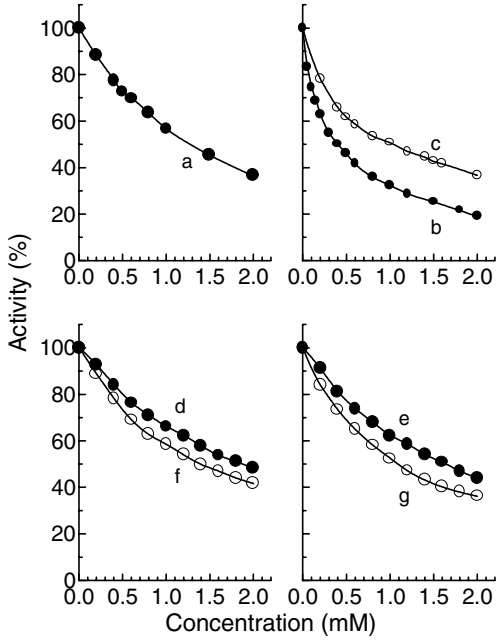


Fig. 2. Effects of *p*-alkoxybenzoic acids on the activity of mushroom tyrosinase.

Table 1
Inhibition effects and constants of some *p*-alkoxybenzoic acids on mushroom tyrosinase

Compounds	IC_{50} (mM)	Inhibition type	Inhibition constants (mM)	
a	1.30	Competitive	0.73	
b	0.42	Non-competitive	0.43	0.43
c	1.10	Mixed-type	1.46	0.84
d	1.85	Uncompetitive		0.80
e	1.65	Uncompetitive		0.71
f	1.40	Uncompetitive		0.60
g	1.15	Uncompetitive		0.49

p-alkoxybenzoic acids, MeO-BA (**b**) was the most potent inhibitor. When the concentration of these *p*-alkoxybenzoic acids were 2.0 mM, HO-BA (**a**), MeO-BA (**b**), EtO-BA (**c**), PrO-BA (**d**), BuO-BA (**e**), PeO-BA (**f**), and HeO-BA (**g**) caused the enzyme activity to decrease by 63.5%, 81.0%, 63.2%, 51.9%, 55.9%, 58.6% and 63.9%, respectively. The concentrations leading to 50% activity lost (IC_{50}) of all the tested inhibitors were estimated and are listed in Table 1.

3.2. The inhibition mechanism of several p-alkoxybenzoic acids against the activity of mushroom tyrosinase

Taking HO-BA (**a**), MeO-BA (**b**), EtO-BA (**c**), PrO-BA (**d**), BuO-BA (**e**), PeO-BA (**f**), and HeO-BA (**g**) as the effectors, respectively, we studied their inhibition mechanisms against the enzyme for oxidation of L-DOPA. The plots of the remaining enzyme activity versus the concentrations of enzyme at different effector concentrations gave a family of straight lines, which all passed through the origin. Increasing the effector concentration resulted in the descending of the slope of the line, indicating that the inhibition of these *p*-alkoxybenzoic acids on the enzyme was reversible. The presence of these effectors did not bring down the amount of the efficient enzyme, but just resulted in the inhibition and the descending of the activity of the enzyme. Fig. 3 shows the relationship of enzyme activity to its concentration in the presence of different concentrations of MeO-BA (**b**). Other effectors show the same behaviour. They are all reversible inhibitors of mushroom tyrosinase for oxidation of L-DOPA.

*3.3. Inhibition of HO-BA (**a**) on the enzyme activity, following the competitive mechanism*

The kinetic behaviour of mushroom tyrosinase during the oxidation of L-DOPA was studied. Under the conditions employed in the present investigation, the oxidation reaction of L-DOPA by mushroom tyrosinase follows Michaelis–Menten kinetics. In the presence of HO-BA (**a**), the kinetic studies of mushroom tyrosinase by the double-reciprocal Lineweaver–Burk plots are shown in Fig. 4. Fig. 4 shows that HO-BA was a competitive inhibitor, since increasing the HO-BA concentration resulted in a family of lines with a common intercept on the $1/v$ axis but with different slopes. The equilibrium constant for inhibitor binding with free

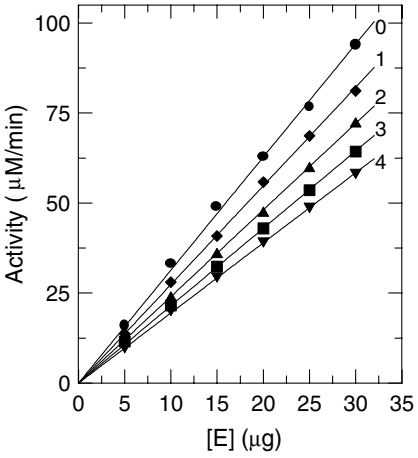


Fig. 3. Effects of concentrations of tyrosinase on its activity for the catalysis of L-DOPA at different concentrations of *p*-methoxybenzoic acid. Concentrations of *p*-methoxybenzoic acid for curves 0–4 were 0, 0.1, 0.2, 0.3, and 0.4 mM.

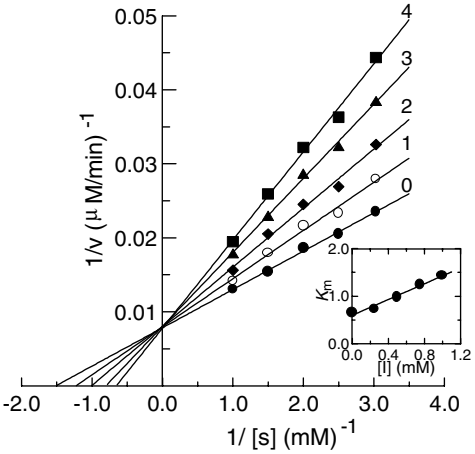


Fig. 4. Lineweaver–Burk plots, for inhibition of *p*-hydroxybenzoic acid, on the oxidation of L-DOPA by tyrosinase. Concentrations of *p*-hydroxybenzoic acid for curves 0–4 were 0, 0.25, 0.50, 0.75, and 1.00 mM, respectively.

enzyme, K_I, was obtained from a plot of the apparent Michaelis–Menten constant (K_m) versus the concentration of HO-BA, which is linear, as shown in the inset. The obtained constant is 0.73 mM, as summarized in Table 1.

3.4. Inhibition of MeO-BA (**b**) of the enzyme activity following a non-competitive mechanism

Inhibition of mushroom tyrosinase by MeO-BA (**b**) has been studied. The results illustrated in Fig. 5 show that double-reciprocal plots yield a family of straight lines with a common intercept on the 1/[*s*] axis but with different slopes, indicating that MeO-BA is a non-competitive inhibitor of the enzyme. It can decrease the apparent value of V_m with no effect on the K_m. The behaviour observed showed that the inhibitor can combine with both the free enzyme and the enzyme–substrate complex, and the equilibrium constants for inhibitor binding with free enzyme, K_I, and the enzyme–substrate complex, K_{IS}, were the same. The inhibition constant can obtained from a plot of the vertical intercept ($1/V_m$) versus the inhibitor concentration, which is a linear as showed in Fig. 5 inset. The obtained inhibition constant is 0.43 mM, also given in Table 1 for comparison.

3.5. Inhibition of EtO-BA (**c**) of the enzyme activity, following a mixed-type mechanism

Inhibition by EtO-BA (**c**) was of mixed-type, as shown by a Lineweaver–Burk plot (Fig. 6). The equilibrium constant for inhibitor binding with the free enzyme (E), K_I, was obtained from a plot of the slopes of the straight lines versus the EtO-BA concentrations. The equilibrium constant for inhibitor binding with the enzyme–substrate complex (ES), K_{IS}, was gained from a plot of the intercepts versus the inhibitor concentrations. The values obtained are also listed in Table 1.

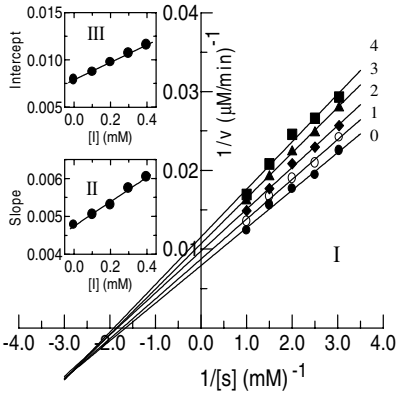


Fig. 6. Lineweaver–Burk plots for inhibition of *p*-ethoxybenzoic acid on the oxidation of L-DOPA by mushroom tyrosinase. Concentrations of *p*-ethoxybenzoic acid for curves 0–4 were 0, 0.1, 0.2, 0.3, and 0.4 mM, respectively.

3.6. Inhibition of the other four p-alkoxybenzoic acids of the enzyme activity, following uncompetitive mechanism

Inhibition of the diphenolase activity of mushroom tyrosinase by PrO-BA (**d**), BuO-BA (**e**), PeO-BA (**f**) and HeO-BA (**g**) were individually studied. Their inhibitory mechanisms were shown to be the same. All were shown to be of the uncompetitive type. Fig. 7 shows the double-reciprocal plots of the enzyme inhibited by BuO-BA (**e**). The results show that the plots of $1/v$ versus 1/[*S*] give a family of parallel straight lines with the same slopes. Accompanying the increase of the inhibitor concentration, the values of both K_m and V_m increased, but the ratio of K_m/V_m remained unchanged. The slopes were independent of the concentration of BuO-BA (**e**), which

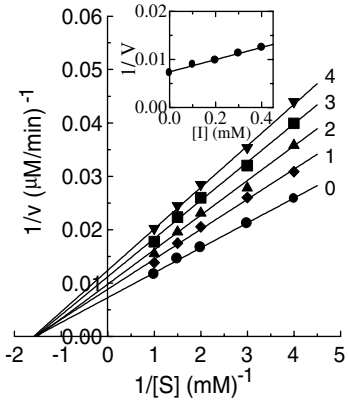


Fig. 5. Lineweaver–Burk plots for inhibition of *p*-methoxybenzoic acid on the oxidation of L-DOPA by tyrosinase. Concentrations of *p*-methoxybenzoic acid for curves 0–4 were 0, 0.1, 0.2, 0.3, and 0.4 mM, respectively.

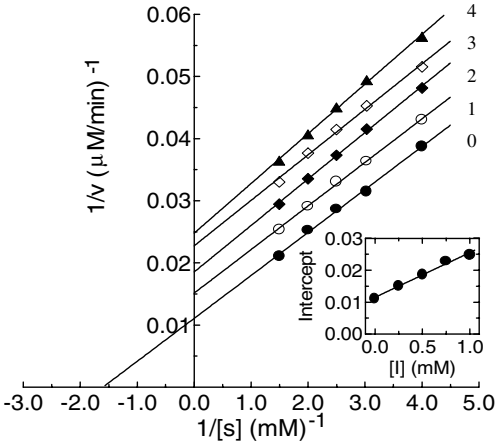


Fig. 7. Lineweaver–Burk plots for inhibition of *p*-propoxybenzoic acid on the oxidation of L-DOPA by mushroom tyrosinase. Concentrations of *p*-propoxybenzoic acid for curves 0–4 were 0, 0.25, 0.5, 0.75, and 1.0 mM, respectively.

indicated that BuO-BA (**e**) was an uncompetitive inhibitor of the enzyme. This behaviour indicated 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 inhibitor binding with enzyme–substrate complex (ES), K_{IS}, was obtained from a plot of the vertical intercept $(1/V_m)$ versus the concentration of BuO-BA (**e**), which is linear as shown in Fig. 7 inset. The obtained inhibition constant is also given in Table 1 for comparison. Similar results were obtained with PrO-BA (**d**), PeO-BA (**f**), and HeO-BA (**g**). The K_{IS} values were also obtained from secondary plots and are summarized in Table 1.

4. Discussion

As we know, tyrosinase has two distinct kinds of catalysis functions: the hydroxylation of monophenols and the oxidation of *o*-diphenols. In the process of catalysis, it has three existing forms, *met, oxy* and *deoxy*. Both the *met* form and *oxy* form cause oxidation of *o*-diphenols; the *met* form causes hydroxylation of monophenols; the *deoxy* form could bind with oxygen (Sanchez-Ferrer, Rodriguez-Lopez, Garcia-Canovas, & Garcia-Carmona, 1995). In the paper, we took L-DOPA as substrate to determine the *o*-diphenolase activity of the enzyme. The effects of some *p*-alkoxybenzoic acids on the oxidation of L-DOPA by mushroom tyrosinase were studied. The results showed that *p*-alkoxybenzoic acids can inhibit the diphenolase activity of mushroom tyrosinase, and the inhibition was displayed as reversible. Among these *p*-alkoxybenzoic acids, MeO-BA(**b**) was the most potent inhibitor. Kubo and Kinst-Hori (1998a, 1998b) reported that *p*-methoxybenzoic acid had inhibition effects on the diphenolase activity of mushroom tyrosinase, and the inhibitory type was classed as non-competitive. But they did not further discuss the mechanism or obtain the inhibition constant. Robit, Rouch, and Cadet (1997) reported the inhibition of palmito tyrosinase by benzoic acid and *p*-hydroxybenzoic acid. They found that the inhibitory character of aromatic acids was linked to the presence of the benzene ring. However, the effects of other *p*-alkoxybenzoic acids on the diphenolase activity of mushroom tyrosinase were little explored. In this paper, the inhibitory mechanism of a series of *p*-alkoxybenzoic acids on mushroom tyrosinase was investigated. The results obtained showed that *p*-hydroxybenzoic acid was a competitive inhibitor, *p*-methoxybenzoic acid was a non-competitive inhibitor, *p*-ethoxybenzoic acid was a mixed-type II inhibitor, which is more uncompetitive than competitive. When the carbon number of the hydrocarbon chain in *p*-alkoxybenzoic acids was three or more, the inhibition mechanism became uncompetitive.

Despite the structural similarities, the inhibition mechanisms of the seven compounds (and inhibitory effects) are to some extent different from each other. With the increasing length of hydrocarbon chain, the inhibitory type varied from competitive to uncompetitive, which may be because the longer hydrocarbon chain causes an effect of spatial block, making the inhibitors not able to approach the activity centre of enzyme, hence inhibiting the combination of inhibitor and free enzyme. For the uncompetitive inhibitors, the inhibition strength follows the order: HeO-BA (**g**)>PeO-BA (**f**)>BuO-BA (**e**)>PrO-BA (**d**). The values of K_I decrease from 0.49 to 0.84 mM, which may be explained by the lower electron-donating capacity of the substituents at the *para*-position. Walker and Wilson (1975) reported that tyrosinase had two sites of combination, one for the substrate and the other for the inhibitor. When the hydrocarbon chain of the *p*-alkoxybenzoic acid is long enough, the inhibitor cannot bind to free enzyme molecules and just combines with the enzyme–substrate complex. On the base of the conclusion and results obtained, possibly the combination site for the substrate is not just for substrate, but it can also combine with the inhibitor, but a different site may be only for the inhibitor (Walker & Wilson, 1975). When size of the inhibitor is moderate, it will be competitively coalesce with the site for the substrate. And if the inhibitor size is greater than the space, it might just coalesce with its own site and vacate the other site for the substrate. Since the substrate can combine with the enzyme to some extent, it may induce the enzyme conformation to change so that the hydrophobic pocket becomes bigger. From the results obtained, we can conclude that, just because of such a conformational change, the combination between inhibitors with longer hydrocarbon chain and enzyme–substrate complex is easier to engender and more tight, which means that the inhibitor could be better embraced by the hydrophobic pocket.

Acknowledgements

The present investigation was supported by Grant 2004N002 of the Science and Technology Foundation and by Grant B0410003 of the Natural Science Foundation of Fujian Province for Q.X. Chen.

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