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

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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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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.

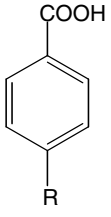
2. Materials and methods

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

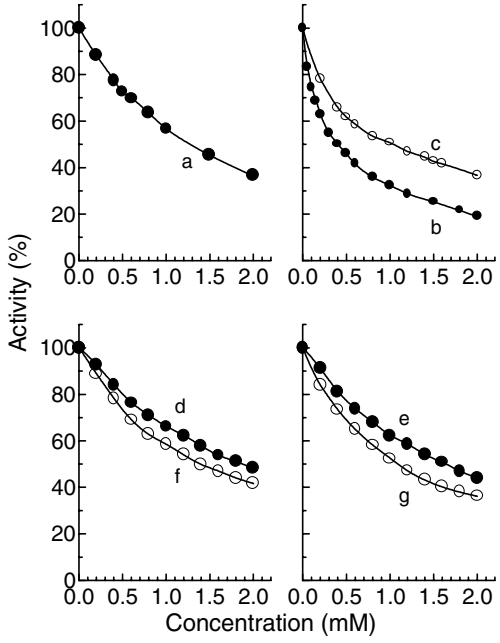


Fig. 2. Effects of *p*-alkoxybenzoic acids on the activity of mushroom tyrosinase.