Inhibitory Kinetics of 2'-Hydroxy-4'-methoxyacetophenone on Tyrosinase-catalyzing Reaction

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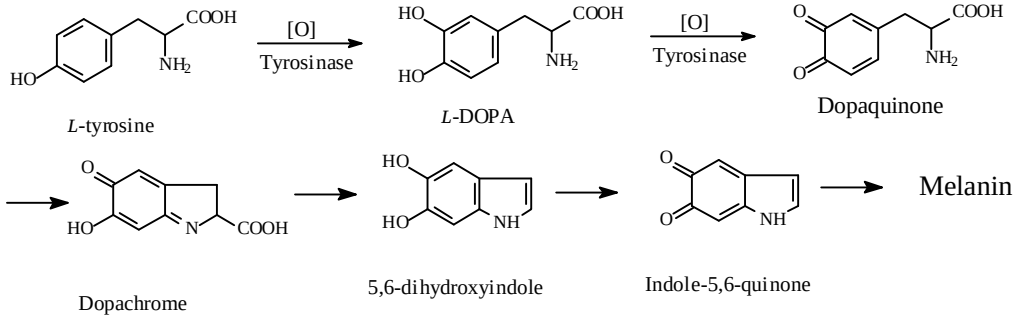
Abstract：The inhibitory effects of 2'-hydroxy-4'-methoxyacetophenone on the activity diphenolase contained in tyrosinase was studied using enzymological kinetic method. 2'-hydroxy-4'-methoxyacetophenone was found to inhibit diphenolase activity of tyrosinase. The 2'-hydroxy-4'-methoxyacetophenone concentration leading to 50 % activity lost (IC50) was 0.60 mmol/L for diphenolase activity. The inhibition kinetics analyzed by Lineweaver-Burk plots found 2'-hydroxy-4'-methoxyacetophenone to be a mixed inhibitor for the oxidation of L-DOPA, the equilibrium constants for inhibitor binding with free enzyme (E), KI, and with enzyme-substract (ES) complex, KIS, were 0.08mmol·L - 1 and 0.12 mmol·L - 1 respectively.

Introduction

Tyrosinase, also known as polyphenol oxidase, is a copper-containing mixed-function oxidase widely distributed in microorganisms, animals, and plants[1,2]. It is recognized as a pivotal enzyme in the process of melanin biosynthesis and a catalyst in the following reactions:



Tyrosinase catalyzes two critical reactions in melanin synthesis, namely i) hydroxylation of monophenol to o-diphenol (monophenolase activity) and ii) conversion of o-diphenol to the corresponding o-quinone (diphenolase activity). The resulting quinone is subsequently subjected to a series of oxidation/polymerization processes to form dark pigments, also known as “melanine”.

It is evident that tyrosinase is a key enzyme controlling the melanization process of skin, eye, inner ear and hair, as well as the enzymatic browning process in fruits and vegetables [3,4,5]. Meanwhile, the application of tyrosinase inhibitors has attracted more and more attentions in the fields of cosmetic, food and pharmaceutical industry, primarily because of their high efficacy in mitigating hyperpigmentation [6]. Many efforts have been made in searching for feasible and effective tyrosinase inhibitors. For instance, based on systematic studies on the inhibitory effect of quercetin, dodecyl gallate and thymol on mushroom tyrosinase, Kubo et al. proposed a kinetic model of the inhibition process and pointed out some favorable features in molecular structure for a potential effective inhibitor[6,7]. Nerya et al. analyzed a series of inhibitors extracted from the root of Licorice [8]. Gong et al. has previously reported some potent tyrosinase inhibitors, such as ferulic acid and isoferulic Acid [9,10].

2'-hydroxy-4'-methoxyacetophenone is a main active ingredient of Paeonia moutan Sim, Pycnostelma paniculatum (Bunge) K. schum. and Betula platyphylla Suk. Var japonica (Sieb.) Hara. Reported in

this paper is a kinetic study on the inhibitory effect of 2'-hydroxy-4'- methoxyacetophenone on mushroom tyrosinase. By investigating the in vitro inhibitory effect of 2'-hydroxy-4'-methoxyacetophenone on diphenolase activities of mushroom tyrosinase, a mixed inhibition model was established and the kinetic parameters were calculated. The current results provide experimental supports on the potential application of 2'-hydroxy-4'-methoxyacetophenone as a high-efficacy anti-pigment ingredient in industry.

Experimental

Materials

Mushroom tyrosinase, L-tyrosine, L-3,4-dihydroxyphenylalanine (L-DOPA) was purchased from Sigma Chemical Co. USA. 2'-Hydroxy-4'-methoxyacetophenone was purchased from National Institute for the Control of Pharmaceutical and Biological Product (NICPBP). Dimethyl sulfoxide (DMSO) and other reagents were of analytical grade and obtained from commercial suppliers. Double distillated and de-ionized water was used unless stated otherwise.

Methods

Enzyme activity was monitored by dopachrome formation at 475 nm [ε=3700 (mol•L - 1·cm) - 1] accompanying the oxidation of the substrate (L-DOPA). 1 U/min of enzymatic activity was defined as the amount of enzyme increasing 0.001 absorbance in a minute at 475 nm in this condition. The assay was performed as Tsou's method [11] with slight modificatios. In this method, 1 mL of a 5.0 mmol·L - 1 L-DOPA aqueous solution was mixed first with 3.6 mL of Na2HPO4-NaH2PO4 buffer (pH6.8), and incubated at 30 ºC for 10 min. Then, 0.1 mL of 2 mmol•L - 1 2'-hydroxy-4'-methoxyacetophenone solution (dissolved in DMSO) and 0.4 mL of 50 μg /mL tyrosinase solution [dissolved in Na2HPO4-NaH2PO4 buffer (pH6.8)] were added to the mixture. This solution was immediately monitored for the formation of dopachrome by measuring the absorbance (A475) at 475 nm with a spectrophotometer (HP 6010) until 12 min. The reaction was carried out under a constant temperature of 30 ºC.

Results and Discussion

Concentration effects of 2'-hydroxy-4'-methoxyacetophenone on the diphenolase activity of tyrosinase

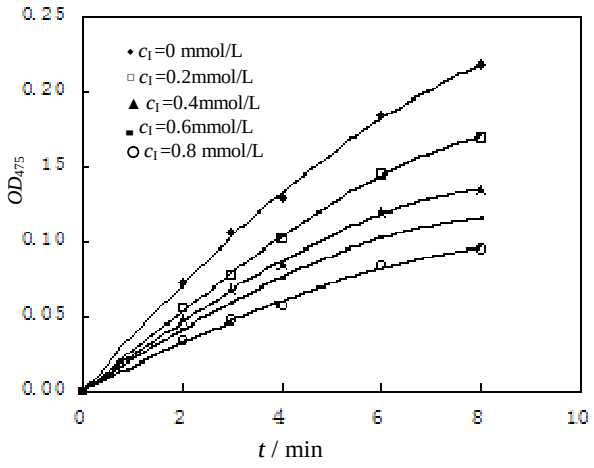


Fig.1 Progress curves for the inhibition against diphenolase by 2'-hydroxy-4'-methoxyacetophenone

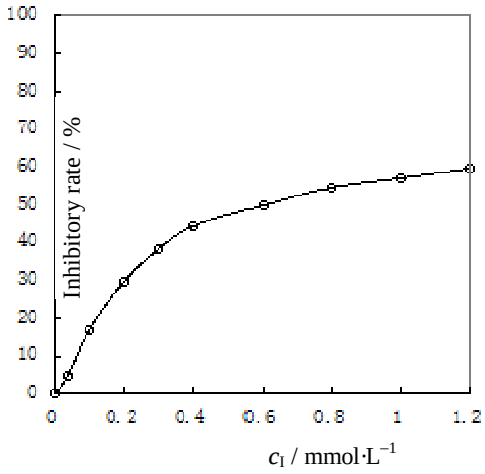


Fig.2 Effect of concentration of 2'-hydroxy-4'-methoxyacetophenone on inhibitory rate to diphenolase

The inhibitory effects of different concentrations of 2'-hydroxy-4'-methoxyacetophenone on the oxidation of L-DOPA by tyrosinase were studied. The kinetics course of the oxidation of L-DOPA in

the presence of different concentrations of 2'-hydroxy-4'-methoxyacetophenone is shown in Fig.1. There is no lag period as shown in Fig.1. Increasing the concentration of 3-(4-Hydroxyphenyl)-2-propenoic acid (cI) resulted in a increase of the inhibitory rate as shown in Fig.2. The inhibitor concentration leading to 50% inhibitory rate (IC50) was estimated to be 0.60 mmol/L.

The reversible inhibition of 2'-hydroxy-4'-methoxyacetophenone on the diphenolase activity of tyrosinase

Fig.3 shows that in the presence of different concentrations of 2'-hydroxy-4'-methoxyacetophenone the enzyme activity was dependent on the enzyme concentration. The plots of the residual enzyme activity versus the concentrations of 2'-hydroxy-4'-methoxyacetophenone gave a family of straight lines which passed through the origin, indicating that the decrease of catalytic efficiency is result by the inhibitory effects of 2'-hydroxy-4'-methoxyacetophenone on the activity of mushroom tyrosinase, but not the decrease of enzyme concentration, therefore the inhibition of the enzyme by 2'-hydroxy-4'-methoxyacetophenone was reversible. Increasing the 2'-hydroxy-4'-methoxyacetophenone concentration resulted in the descending of the slopes of the line.

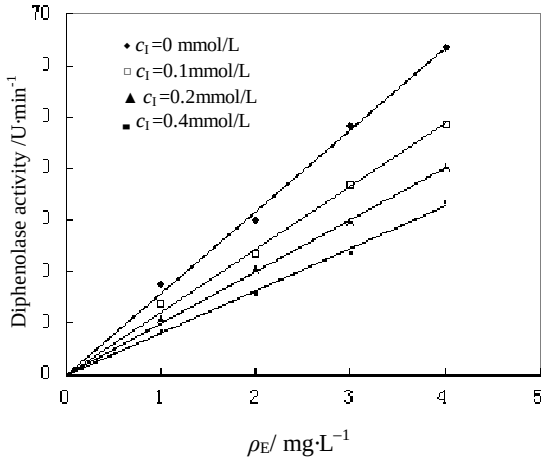


Fig.3 Effect of concentration of tyrosinase on diphenolase activity at different concentration of 2'-hydroxy-4'-methoxyacetophenone

Inhibitory kinetic parameters of 2'-hydroxy-4'-methoxyacetophenone on the diphenolase

A steady-state analysis was performed to estimate the inhibition type and the kinetic parameters of tyrosinase during the oxidation of L-DOPA. Lineweaver-Burk plots for inhibitory effect of 2'-hydroxy-4'-methoxyacetophenone on diphenolase were obtained at different concentrations of 2'-hydroxy-4'-methoxyacetophenone (cI) and L-DOPA (cS) as shown in Fig.4.

Under the conditions employed in the current investigation, the oxidation reaction of L-DOPA by tyrosinase follows Michaelis-Menten kinetics. The Michaelis constant (Km) and the maximum reaction rate (vm) determined under these conditions were only apparent because the assay was carried out in air-saturated aqueous solutions. The effect of oxygen concentration on these parameters is unknown in the present study.

As shown in Figure 5, a Lineweaver-Burk plot results in a family of lines intersecting in the second quadrant on the coordinate plain, with the intercepts on the Y axis being corelated to the concentrations of 2'-hydroxy-4'-methoxyacetophenone. As shown in Table 1, with the increasing in the concentrations of 2'-hydroxy-4'-methoxyacetophenone, the apparent Michaelis constant (Km) increases, while the maximum reaction rate (vm) decreases, indicating a hybrid inhibition mechanism. Derived from a linear plot of the intercepts on Y axis versus the corresponding 2'-hydroxy-4'-methoxyacetophenone concentrations, the inhibition constant to the free enzyme (KI) and to the enzyme-substrate complex (KIS) are calculated to be 0.084 mmol/L and 0.12 mmol/L, respectively.

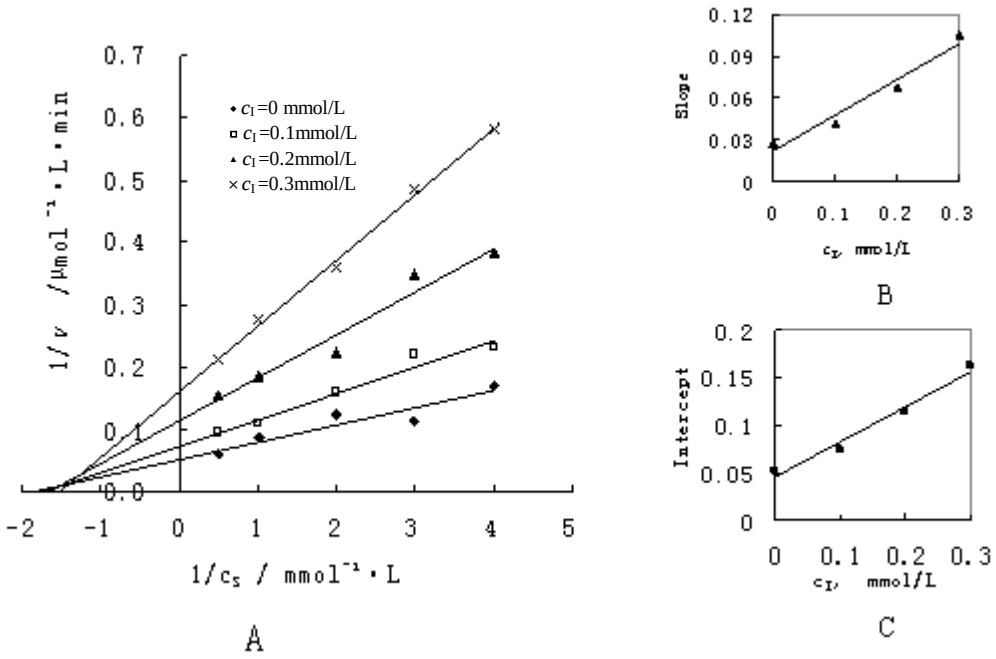


Fig. 3 （A）Lineweaver-Burk plots for inhibition of 2'-hydroxy-4'-methoxyacetophenone on tyrosinase for the catalysis of *L*-DOPA. （B）Relationship between slope of line 1-4 and concentration of 2'-hydroxy-4'-methoxyacetophenone. （C）Relationship between intercept on vertical axis of line 1-4 and concentration of 2'-hydroxy-4'-methoxyacetophenone.

Table 1 Kinetic Parameters of Tyrosinase by different 2'-hydroxy-4'-methoxyacetophenone concentration

c_I mmol•L^{-1}	Michaelis-Menten equation	K_m, mmol•L^{-1}	v_m, μmol•L^{-1}	inhibition type	IC_{50}, mmol•L^{-1}	K_I, mmol•L^{-1}	K_{IS}, mmol•L^{-1}
0	$1/v = 0.0274\times1/c_S + 0.0529$	0.52	18.9	mixed	0.60	0.084	0.12
0.1	$1/v = 0.0421\times1/c_S + 0.0742$	0.57	13.5				
0.2	$1/v = 0.0687\times1/c_S + 0.1143$	0.60	8.75				
0.3	$1/v = 0.1051\times1/c_S + 0.1625$	0.65	6.15				

Model analysis of 2'-hydroxy-4'-methoxyacetophenone interacts with the enzyme

The precise explanation how 2'-hydroxy-4'-methoxyacetophenone interacts with the enzyme on a molecular basis is still unknown, but it can be explained as follows. First, ketone is known to react with amino groups to form Schiff bases and can be stabilized by aromatic nucleus. Thus, 2'-hydroxy-4'-methoxyacetophenone may form a Schiff base with a primary amino group in the enzyme and result to the decrease of enzyme activity. This inhibition is a noncompetitive type and 2'-hydroxy-4'-methoxyacetophenone (I) can not only bind with free enzyme (E) to produce enzyme-2'-hydroxy-4'-methoxyacetophenone complex (E-I), and also bind with substrate-enzyme to produce DOPA-enzyme-2'-hydroxy-4'-methoxyacetophenone complex (S-E-I). Moreover, the introduction of an electron-donating methoxy group at the para position should stabilize the Schiff base and increase the inhibitory activity.In addition,the ortho hydroxyl group in 2'-hydroxy-4'-methoxyacetophenone forms a quasi six-membered ring through intramolecular hydrogen bonding and produces a more stable chelate structure. Second, the hydroxyl group and ketone are known as chelate agents. Thus, 2'-hydroxy-4'-methoxyacetophenone may chelate copper in the binuclear active center of the enzyme, and competes with substrate (L-DOPA) resulting to inhibit enzyme's activity.In general, the mixed inhibition exerted by 2'-hydroxy-4'-methoxyacetophenone comes from its ability to form a Schiff base with a primary amino group and to chelate copper in the active site of tyrosinase.

Conclusion

In this paper, we investigate the inhibitory mechanism of 2'-hydroxy-4'-methoxyacetophenone on the diphenolase. The results showed that 2'-hydroxy-4'-methoxyacetophenone was found to inhibit diphenolase activity of tyrosinase well. The inhibition type of 2'-hydroxy-4'-methoxyacetophenone on the diphenolase was determined to be a mixed inhibitor. The 2'-hydroxy-4'-methoxyacetophenone concentrations leading to 50 % inhibitory rate (IC50) were0.60 mmol·L - 1 for diphenolase, much less than that of arbutin (IC50 =5.3 mmol·L - 1 for diphenolase activity).

Acknowledgements

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