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Inhibitory kinetics of enterolactone on mushroom tyrosinase

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ABSTRACT

Tyrosinase (EC 1.14.18.1) is a key enzyme in post-harvest browning of fruit and melanin formation in human skin. Tyrosinase inhibitors are important in agricultural and cosmetic chemistry. In the present study, the inhibitory kinetics of enterolactone on themonophenolase and diphenolase activities of mushroom tyrosinasewere investigated using the kinetic method of substrate reaction. The results showed that enterolactone inhibited themonophenolase and diphenolase activities of mushroom tyrosinase, with IC_{50} values of 0.42 and 0.124 mM, respectively. The inhibition kinetics showed that enterolactone displayed non-competitive mechanism. The inhibition constants were determined to be 0.8315 and 0.6956mM, respectively. Furthermore, enterolactone exerted a potent inhibitory effect on intracellular melanin formation in B16/F10 murine melanoma cells and did not cause cytotoxicity. These results provide a comprehensive understanding of the inhibitory mechanisms underlying enterolactoneactivity and its inhibition of melanin formation, suggesting the potential benefits of using this compound.

Keywords: Mushroom tyrosinase, enterolactone, monophenolase activity, diphenolase activity, inhibition kinetics

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INTRODUCTION

Tyrosinase (EC 1.14.18.1) is a copper containing multifunctional oxidase with diverse physiological roles related to melanin production[1-3]. Thise enzyme displays two different enzymatic reactions : the hydroxylation of monophenol to o-diphenol (cresolase activity) and the oxidation of diphenol to corresponding o-quinones (catecholase activity).

Tyrosinase is also responsible not only for enzymatic browning, an unfavorable darkening of fruit and vegetables that occurs when they undergo processing, but also for the molting process of insects and adhesion of marine organisms[4]. Therefore, tyrosinase inhibitors with low Ki values have attracted substantial attention recently for applications, such as whitening agents, anti-browning agents, and bio-insecticides to prevent hyperpigmentation by inhibiting enzymatic oxidation. A number of tyrosinase inhibitors have already been reported, the majority of which comprise compounds structurally analogous to phenolic substrates. Generally, their inhibitory mechanism is competitive displacement of these substrates, L-tyrosine and L-DOPA with other chemically related dihydroxybenzene derivatives[5]. However, interestingly, many phenolic inhibitors such as cinamic acid,kurarinone, and ρ-methoxybenzoic acid show a mechanism of non-competitive inhibition[6-8].

Enterolactone, also known as trans-α,β-bis(3-hydroxybenzyl)butyrolactone, is an enterobacterial breakdown product of plant lignans. Recent epidemiological studies have shown that there are lower concentrations of enterolactone in breast cancer patients than in healthy controls[9,10]. For this reason, it have been suggested to possess several beneficial health effects in humans, which may be protective against cardiovascular disease[11,12].

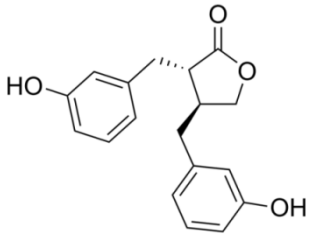
Although many tyrosinase inhibitors are structurally analogous to phenolic substrates, there have been few studies on the effects of enterolactone on tyrosinase.

Therefore, the aims of this work are to determine the kinetics of the inhibition of the mono- and diphenolase activities of tyrosinase and to evaluate the kinetic parameters and inhibitory mechanisms involved. Mushroom tyrosinase was used throughout our studies. We also evaluated the anti-melanogenic effect of enterolactone in B16/F10 murine melanoma cells.

MATERIALS AND METHODS

Chemicals

Enterolactone (the structures are shown below), mushroom tyrosinase (EC 1.14.18.1),L-3,4-dihydroxyphenylalanine (L-DOPA) and L-tyrosine (L-Tyr) were purchased from SigmaAldrich (St. Louis, MO). Allother reagents were of analytical grade. The water used wasre-distillated and deidnized.



Tyrosinase inhibition assay

The mushroom tyrosinase inhibition activities were determined according to the method described in the literature with some modifications[13]. Briefly, mushroom tyrosinase (1250 units/mL) and 0.07 mL of 2 mM L-tyrosine were added to 0.09 mL of 0.1 M phosphate buffer containing the test sample. A total of0.2 mL of test mixture was incubated for 10 min at 37°C and the absorption due to the formation of L-DOPA or dopaquinone was monitored at 475 nm. The same mixture except for the plant extract was used as a control. Hydroquinone-O-β-glucopyranoside (arbutin) was used as a positive control. Each treatment was performed in triplicate.

Kinetic analysis of tyrosinase inhibition

To a 96-well plate in a total assay mixture of 200 μL were added various concentrations of L-tyrosine and L-DOPA(0.2 to 0.6 mM) as substrates, 5 μL of mushroom tyrosinasesolution (1250 units) and 100 mM potassium phosphate buffer (pH 6.8) with (0.25, 0.5, and 1 mMenterolactone) or without test sample[14]. Using a microplate reader, the initial rate of dopachrome formation in the reaction mixture was determined by the linear increase in absorbance at 475 nm. The Michaelis constant (Km) and maximal velocity (Vmax) of the tyrosinase were determined by Lineweaver–Burk plots at various concentrations of L-tyrosine and L-Dopa as substrates. The reaction kinetics required a modification of the Michaelis–Menten equation due to the uncompetitive inhibition by enterolactone together with substrate inhibition by L-tyrosine and L-Dopa.

Measurement of melanin contents

Melanin contents were measured as reported by Friedmann and Gilchrest with slight modification. The cells were treated with enterolactone and α-MSH(50 nM) for 3 days. After treatment, the cells were detached by incubation in trypsin/ethylenediaminetetraacetic acid. After precipitation, the color of the cell pellets was evaluated visually and cell pellets containing a known number of cells were solubilized in boiling 0.1M NaOH for 1h. Spectrophotometric analysis of melanin contents was performed at an absorbance wavelength of 475 nm[15].

MTT assay

Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. B16F10 cells were cultured in 24-well plates for 18 h, followed by treatment with various concentrations (0.075, 0.15, and 0.3 mM) of enterolactone for 48 h. Briefly, MTT was added to the cells and the formazan crystals were dissolved in dimethyl sulfoxide. The absorbance was measured at 540 nm. The percentage of cells showing cytotoxicity was determined relative to that in the control group.

RESULTS AND DISCUSSION

Effects of Enterolactone on the Monophenolase and DiphenolaseActivity of Mushroom Tyrosinase

To clarify the effects of enterolactone on mushroom tyrosinase activity, we first performed a tyrosinase assay to investigate the mono- and diphenolase activities using L-tyrosine and L-DOPA as substrates. The results showed that enterolactone had inhibitory effects on tyrosinase activity, as shown in Fig. 1.With increasing concentrations of enterolactone, the mono- and diphenolase activities of mushroom tyrosinasemarkedly decreased. The inhibitor concentrations leading to a 50% decrease in the activity (IC_{50}) of enterolactone were estimated to be 0.42 mM(L-

tyrosine)and 0.124 mM(L-DOPA).Enterolactone inhibited diphenolase activity to a higher extent thanmonophenolase activity. The IC_{50} value of monophenolase activity was about three times greater than that of diphenolase activity.

The Mechanism of Inhibition on Monophenolase Activity of Mushroom Tyrosinase by Enterolactone

We also investigated the type of inhibition of enterolactoneon the monophenolase activity of mushroom tyrosinase. The effects on the enzymatic oxidation of L-tyrosine of enterolactone at different concentrations were investigated. Enterolactone was found to inhibit the monophenolase activity of tyrosinase. The double reciprocalLineweaver–Burk plot for the monophenolase activity of mushroom tyrosinase assayed as the hydroxylation of L-tyrosine in the presence of different concentrations of enterolactoneis shown in Fig. 2. This plot shows a set of straight lines which intersect exactly on the vertical axis: the value of Km was unchanged by the inhibitor, but the maximum velocity(Vmax) was decreased, indicating the non-competitive inhibition of L-tyrosine. The equilibrium constants(K_i)for inhibitor binding with free enzyme or enzyme-substrate complex was determined(K_i=0.8315 mM) from a plot of the intercept on the *Y*-axis *versus* the inhibitor concentration, which was linear as shown in the inset. The inhibitor constants are summarized in Table 1.

The Mechanism of Inhibition on Diphenolase Activity of Mushroom Tyrosinase by Enterolactone

We also investigated the effect of enterolactoneon diphenolase activity using L-DOPA as a substrate. In the presence of enterolactone, the diphenolase activity of mushroom tyrosinase was clearly inhibited. We studied the inhibitory mechanism of enterolactoneon diphenolase activity of mushroom tyrosinase. Lineweaver–Burk plots(Fig. 3) showed that enterolactone was also a non-competitive inhibitor. The results revealed that the value of Km remained the same and the value of Vmax decreased with increasing enterolactone concentration.The equilibrium constant(K_i)for inhibitor binding with free enzyme or enzyme-substrate complex was determined(K_i=0.6956 mM) from a plot of the intercept on the *Y*-axis *versus* the inhibitor concentration, which was linear as shown in the inset. The inhibitor constants are summarized in Table 1.

Cell viability and melanin content after exposure to enterolactone

In the present study, B16/F10murine melanoma cells were used as the cell model for examining the inhibitory effect of enterolactone on melanogenesis. To show that the enterolactonedid not have cytotoxic effects on murine B16/F10 melanoma cells, an MTT assay was first performed. Our results showed that the cell viability of murine B16 melanoma treated in 0.3mMenterolactone was almost 100%; this suggests that, after treatment for 72 h, enterolactone did not affect cell viability. Given the lack of a cytotoxic effect of enterolactone on murine B16/F10 melanoma cells, the inhibitory effects of enterolactoneon melanin contents were assessed. The melanin content of enterolactone-treated melanocytes is shown in Fig. 4. Unlike in the control group, treatment with enterolactone (125, 250, and 500 μM) for 72 h reduced melanin contents in a dose-dependent manner.

Table 1.The kinetic parameters of mushroom tyrosinase for the oxidation of mono- and diphenol substrates

	Substrate	
	L-tyrosine	L-DOPA
IC_{50}	0.42 mM	0.124 mM
K_m	0.96 ± 0.024 mM	0.647 ± 0.01 mM
V_{max}	0.209 ±0.018 mM	0.05 ± 0.0067 mM
Inhibition type	Non-competitive	Non-competitive
K_i	0.8315 mM	0.6956 mM

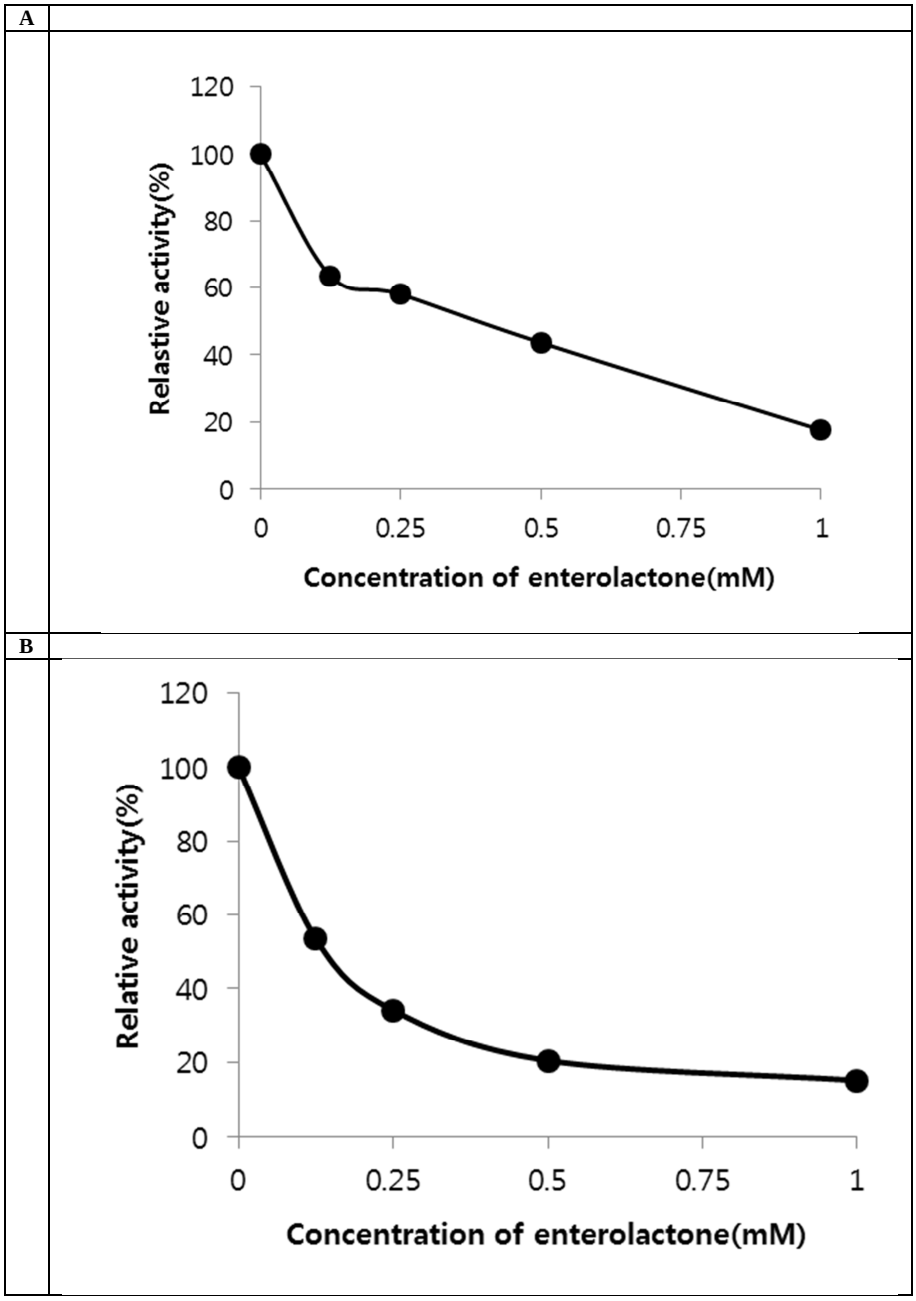


Figure 1. Effects of the substrate analogue enterolactone on the oxidation of L-DOPA (A)and L-tyrosine (B) by mushroom tyrosinase

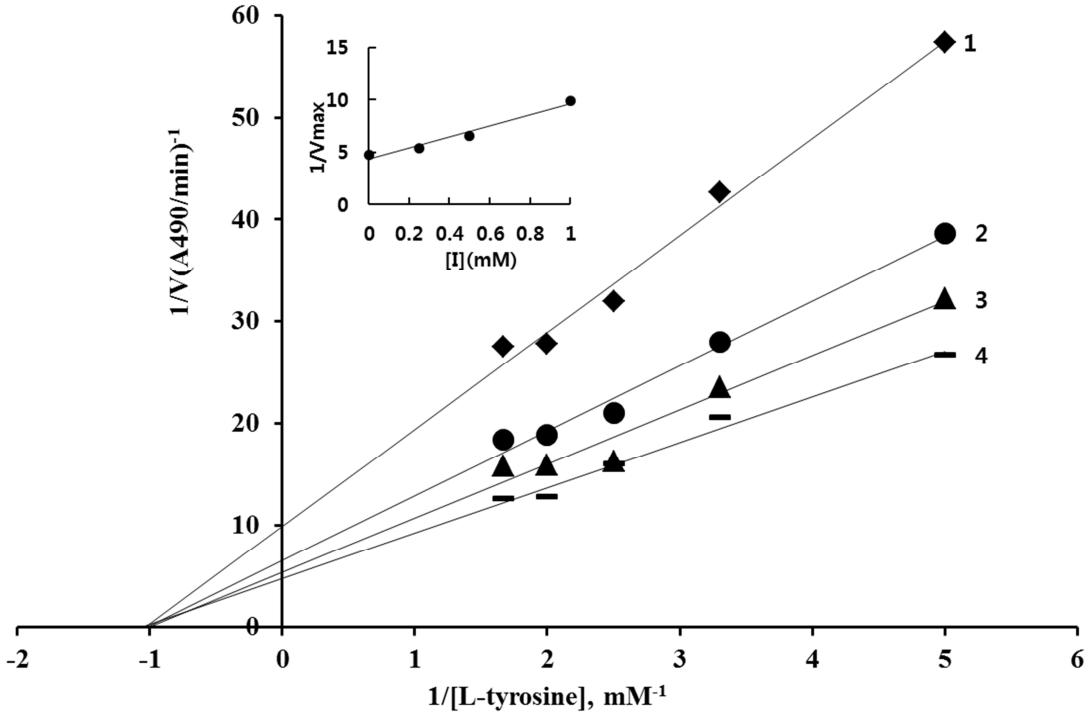


Figure 2.Lineweaver–Burk's double reciprocal plots for the inhibition by enterolactone of the oxidation of L-tyrosine by mushroom tyrosinase.

The data include mean values of 1/V, and the inverse of the absorption increased at 475 nm per min, in three independent tests with various concentrations of L-tyrosine.The concentrations of enterolactone for curves 1–4 were 0, 0.25, 0.5, and 1 mM, respectively. The inset shows the plot of $1/V_{max}$ *versus* the concentration of enterolactone, to determine the inhibition constant.

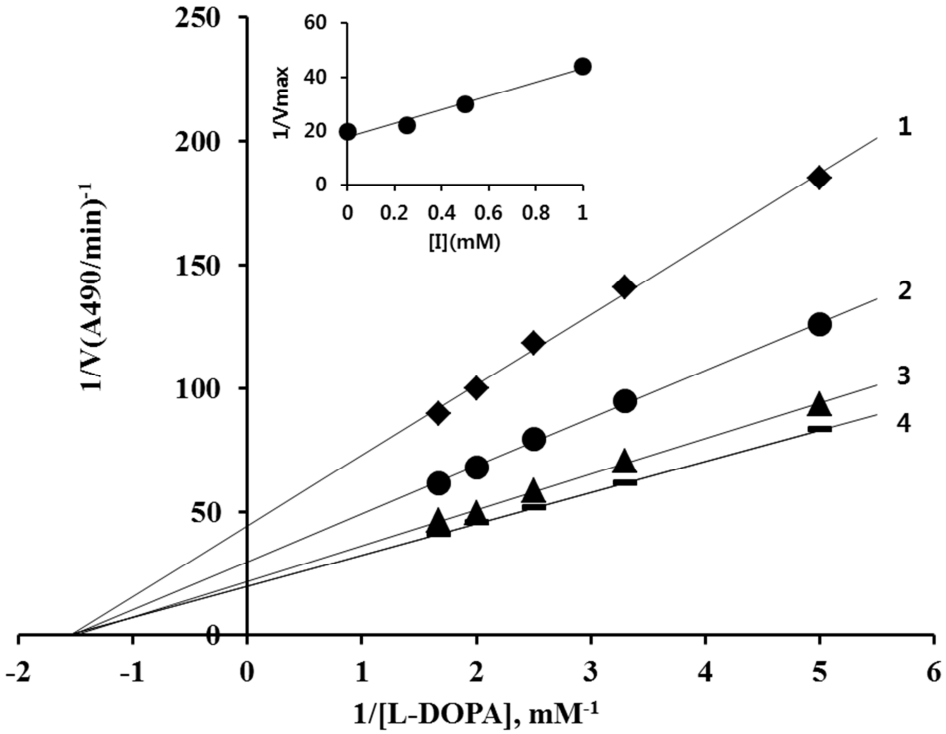


Figure 3.Lineweaver–Burk's double reciprocal plots for the inhibition by enterolactone of the oxidation of L-DOPA by mushroom tyrosinase.The data include mean values of 1/V, and the inverse of the absorption increase at 475 nm per min in three independent tests with various concentrations of L-tyrosine.The concentrations of enterolactone for curves 1–4 were 0, 0.25, 0.5, and 1 mM, respectively. The inset shows the plot of $1/V_{max}$ *versus* the concentration of enterolactone, to determine the inhibition constant

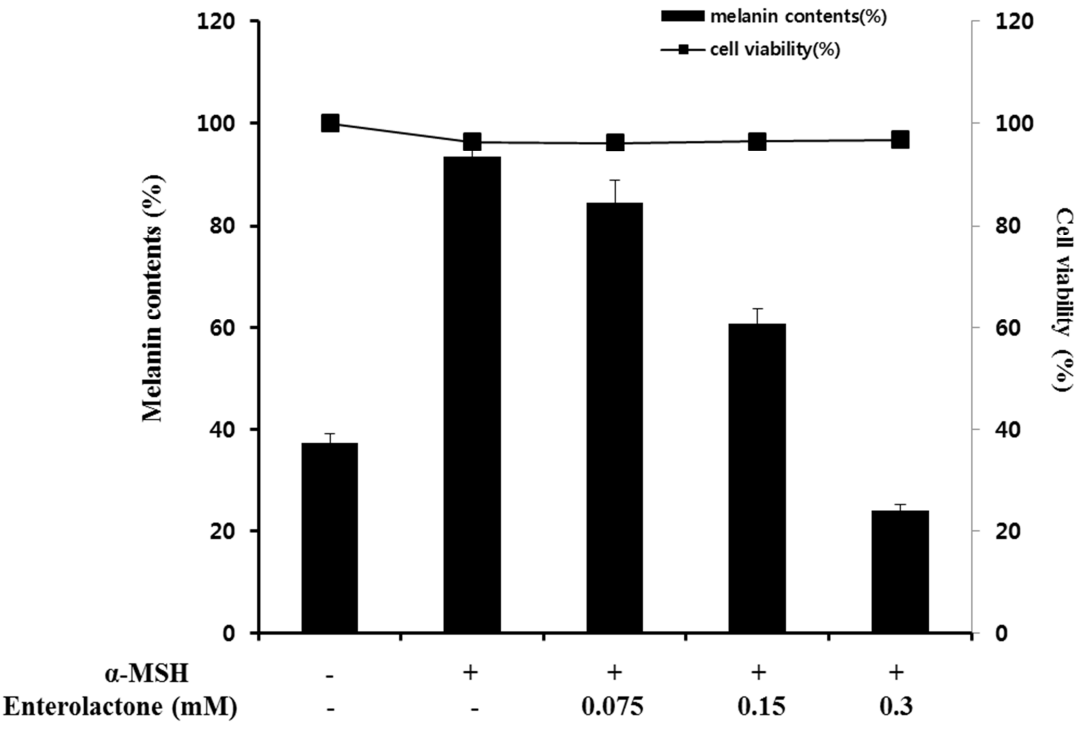


Figure 4.Effect of enterolactone on melanin contents and cytotoxicity.After incubation of B16F10 murine melanoma cells with various concentrations of enterolactone for 72 h, melanin contents and cytotoxicitieswere determined. Data are expressed as a percentage of the control and are mean±SEA of triplicate experiments

CONCLUSION

In this study, we investigated the inhibitory effect of enterolactone on mushroom tyrosinase. The results showed that enterolactone significantly inhibited its monophenolase and diphenolase activities. The kinetic studies showed that both substrates, L-tyrosine and L-Dopa, were non-competitive inhibitors. In addition, a cytotoxicity study showed the enterolactone is not cytotoxic and is safe at concentration as high as 0.3 mM. Furthermore, it exerted a potent inhibitory effect on intracellular melanin formation in B16/F10 murine melanoma cells. Therefore, it is suggested that the application of enterolactone may be promising for inhibiting the melanin formation and browning.

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