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Tyrosinase Inhibitors Isolated from the Edible Brown Alga *Ecklonia stolonifera*

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Extracts from seventeen seaweeds were determined for tyrosinase inhibitory activity using mushroom tyrosinase with L-tyrosine as a substrate. Only one of them, *Ecklonia stolonifera* OKAMURA (Laminariaceae) belonging to brown algae, showed high tyrosinase inhibitory activity. Bioassay-guided fractionation of the active ethyl acetate (EtOAc) soluble fraction from the methanolic extract of *E. stolonifera*, led us to the isolation of phloroglucinol derivatives [phloroglucinol (**1**), eckstolonol (**2**), eckol (**3**), phlorofucofuroeckol A (**4**), and dieckol (**5**)]. Compounds **1**~**5** were found to inhibit the oxidation of L-tyrosine catalyzed by mushroom tyrosinase with IC_{50} values of 92.8, 126, 33.2, 177, and 2.16 μg/mL, respectively. It was compared with those of kojic acid and arbutin, well-known tyrosinase inhibitors, with IC_{50} values of 6.32 and 112 μg/mL, respectively. The inhibitory kinetics analyzed from Lineweaver-Burk plots, showed compounds **1** and **2** to be competitive inhibitors with K_i of 2.3×10^{-4} and 3.1×10^{-4} M, and compounds **3**~**5** to be noncompetitive inhibitors with K_i of 1.9×10^{-5}, 1.4×10^{-3} and 1.5×10^{-5} M, respectively. This work showed that phloroglucinol derivatives, natural compounds found in brown algae, could be involved in the control of pigmentation in plants and other organisms through inhibition of tyrosinase activity using L-tyrosine as a substrate.

Key words: *Ecklonia stolonifera*, Tyrosinase inhibitors, Phloroglucinol derivatives, Marine algae, Kinetics

INTRODUCTION

Tyrosinase (EC 1.14.18.1) is a multifunctional, copper-containing oxidase that catalyzes three distinct reactions of melanin synthesis, the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), the oxidation of DOPA to dopaquinone and the conversion of 5,6-dihydroxyindole to melanochrome (Mason, 1956; Wilcox *et al.*, 1985). Tyrosinase also known as polyphenol oxidase (PPO) is ubiquitously distributed from microorganisms to animals and plants (Mayer, 1987; Whitaker, 1995). The enzyme induces the browning of some fruits, vegetables, and crustaceans and causes a significant discount in their nutritional and market values. In addition, tyrosinase is a key enzyme in the insect molting process, so that its inhibitors might ultimately provide clues to control insect pests (Andersen, 1979). The enzymatic oxidation of L-tyrosine to melanin is of considerable importance because melanin has many functions, and alterations in melanin synthesis occur in many disease states. Recently, tyrosinase inhibitors have become increasingly important in cosmetic and medical products in relation to hyperpigmentation (Kim *et al.*, 2002; Pérez-Bernal *et al.*, 2000). Furthermore, it has been reported that tyrosinase could be central to dopamine neurotoxicity as well as contributing to the neurogeneration associated with Parkinson's disease (Xu *et al.*, 1997).

Ecklonia stolonifera OKAMURA (Laminariaceae), a perennial brown alga growing in a water depth of 2-10 m, is distributed in Korea and Japan, and is commonly used as a foodstuff along with *Laminaria japonica* and *Undaria pinnatifida*. Phloroglucinol and its derivatives (Taniguchi *et al.*, 1991), and ecklonialactones (Kurata *et al.*, 1989; 1993) have been isolated from *E. stolonifera*. This alga was found to have antioxidant (Choi *et al.*, 1993; Lee *et al.*, 1996), antimutagenic activity (Lee *et al.*, 1996; 1998; Han *et al.*, 2000), and feeding-deterrent effect (Taniguchi *et al.*, 1991). However, the inhibitory effect on the tyrosinase

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activity was not investigated. We report the phloroglucinol derivatives with tyrosinase inhibitory activity from *E. stolonifera*, and the inhibitory kinetics of the components on this enzyme activity.

MATERIALS AND METHODS

Plant material

Leafy thalli of the *E. stolonifera* were collected at Gijang-gun in Busan, South Korea in February 2000 and authenticated by Prof. H. G. Kim of the Faculty of Marine Bioscience and Technology, Kangnung National University. A voucher specimen (no. 20000228) has been deposited in the author's laboratory (JS Choi).

Chemicals and reagents

Column chromatography was done with silica gel 60 (70~230 mesh, Merck, Germany), RP-18 Lichroprep (Merck, Germany), and Sephadex LH-20 (Sigma, St. Louis, MO). TLC was carried out on a precoated Merck Kieselgel 60 F_{254} plate (0.25 mm), and 50% H_2SO_4 was used as spray reagent. L-Tyrosine was purchased from Janssen Chimica (Geel Belgium). Arbutin, kojic acid and mushroom tyrosinase (EC 1.14.18.1) were obtained from Sigma Chemical Co. K_2HPO_4 was obtained from Junsei Chemical Co. Ltd. (Tokyo, Japan), and KH_2PO_4 from Yakuri Pure Chemicals Co. Ltd. (Osaka, Japan). All the solvents for column chromatography were of reagent grade from commercial sources.

Isolation of active compounds

The lyophilized powder (3 kg) was refluxed with MeOH (3×9 L) for 3 h. The extract (700 g) was suspended in water and partitioned with *n*-hexane (27.9 g), CH_2Cl_2 (25.6 g), EtOAc (25.0 g), *n*-BuOH (99.6 g), in sequence. The EtOAc fraction (25.0 g) was applied to a silica gel (Merck, 70~230 mesh, 4×80 cm, 800 g) column. The column was eluted using mixtures of EtOAc/MeOH under stepwise gradient conditions (50:1~5:1) to yield the 10 subfractions (F1~F10), i.e., F1~F3; EtOAc/MeOH, 50:1 (5 L), F4~F6; EtOAc/MeOH, 10:1 (5 L), F7~F8; EtOAc/MeOH, 5:1 (5 L), and F9~F10; EtOAc/MeOH, 2:1 (2 L). The F1 (3.44 g, IC_{50} = 20 μg/mL) was subjected to a silica gel column chromatography (70~230 mesh, 3×70 cm, 250 g, *n*-hexane/EtOAc, 1:1) to get the 11 subfractions (F1-1~F1-11). Compound **1** (98 mg) was obtained from the RP-18 column chromatography (20% MeOH~100% MeOH, gradient) of F1-4 (257 mg). Compounds **2** (60 mg) and **3** (135 mg) were obtained from the RP-18 column chromatography (20% MeOH~100% MeOH, gradient) of F1-5 (1.01 g). Compounds **4** (57 mg) and **5** (87 mg) in F1-6 (945 mg) were obtained from the RP-18 column chromatography using a 20% MeOH~100% MeOH gradient, then finally purified by Sephadex LH-20 column chromatography with MeOH as a solvent.

The isolated compounds were identified as phloroglucinol (**1**), eckstolonol (**2**), eckol (**3**), phlorofucofuroeckol A (**4**), and dieckol (**5**) on the basis of chemical and physicochemical evidences, and compared the spectral data with those in the literatures reported previously (Fukuyama *et al.*, 1985; 1989; 1990; Nakamura *et al.*, 1996; Kang *et al.*, 2003).

Enzyme assay

Tyrosinase activity using L-tyrosine as a substrate was spectrophotometrically determined by a method (No *et al.*, 1999) described previously with a little modification. Ten μL of each sample solution with different concentrations (1~500 μg/mL) and 20 μL of mushroom tyrosinase (1000 units/mL) in a 50 mM phosphate buffer (pH 6.5) were added to 170 μL of an assay mixture containing with the ratio 10:10:9 of 1 mM L-tyrosine solution, 50 mM potassium phosphate buffer (pH 6.5) and distilled water in a 96-well microplate. One unit (U) of enzyme activity was defined as the amount of enzyme increasing 0.001 absorbance at 280 nm per min, in 3 mL reaction mix containing L-tyrosine at pH 6.5 at 25°C. The samples dissolved in DMSO were diluted to 30 times with distilled water before experiment. After incubation of the reaction mixture at 25 °C for 30 min, the absorbance of the mixture was determined at 490 nm ($\varepsilon = 3.3\times10^3\ M^{-1}cm^{-1}$) in a microplate reader (VERSA max, Molecular Device, CA). The extent of inhibition by addition of samples is expressed as a concentration necessary for 50% inhibition (IC_{50}). The percent inhibition of tyrosinase activity was calculated by the following equation: the % Inhibition = $[\{1 - (A_a - A_b)/A_c\} \times 100]$ where A_a is absorbance at 490 nm with test sample and enzyme, A_b is absorbance at 490 nm with test sample and without enzyme, and A_c is absorbance at 490 nm with enzyme and without test sample.

The preincubation mixture consisted of 60 μL of 50 mM phosphate buffer (pH 6.5), 50 μL of water, 10 μL of the samples solution, and 20 μL of the aqueous solution of the mushroom tyrosinase (1000 U/mL). The mixture was preincubated at 25°C for 5 min. Then, 60 μL of 1 mM L-tyrosine was added, and the reaction was monitored at 490 nm after 2 min incubation.

Kinetic analysis

The reaction mixture consisted of four different concentrations of L-tyrosine (0.5 to 2 mM) as a substrate and mushroom tyrosinase in 50 mM potassium phosphate buffer. Each sample of several concentrations was added to the reaction mixture, respectively. Michaelis constant (K_m) and maximal velocity (V_{max}) of the tyrosinase were determined by Lineweaver-Burk plots. The velocity equation

for the competitive inhibition in reciprocal form is: $1/V = K_m / V_{max} (1 + [I] / K_i) 1/[S] + 1/V_{max}$. Inhibition constants ($K_i$) of the competitive inhibitors were calculated by the following equation: $Km_{app} = K_m [1 + ([I] / K_i)]$ where Km_{app} is the apparent K_m in the presence of any inhibitor concentration. The reciprocal equation for the noncompetitive inhibition is: $1/V = K_m / V_{max} (1 + [I] / K_i) 1/[S] + 1/V_{max} (1 + [I] / K_i)$. K_i of the noncompetitive inhibitors were calculated by the following equation: $1/Vmax_{app} = (1 + [I] / K_i) / V_{max}$ where $Vmax_{app}$ is the apparent V_{max} in the presence of any inhibitor concentration.

RESULTS AND DISCUSSION

To discover the sources with tyrosinase inhibitory activity in marine algae, 17 seaweed extracts *i.e.* 2 of Chlorophyta, 5 of Phaeophyta, and 10 of Rhodophyta, were screened for their inhibitory effect on mushroom tyrosinase activity using L-tyrosine as a substrate (Table I). Only one of them, *Ecklonia stolonifera* OKAMURA (Laminariaceae), which belongs to brown algae, showed an inhibitory activity with an IC_{50} value of 345 μg/mL. The others showed an inhibitory activity of below fifty percent at the highest concentration of 500 μg/mL.

In our ongoing study to identify the active principles, we also evaluated the solvent soluble fractions including *n*-hexane, CH_2Cl_2, EtOAc, *n*-BuOH, as well as the H_2O layer derived from *E. stolonifera*. As summarized in Table II, some solvent partitioned fractions such as CH_2Cl_2 and EtOAc soluble fractions inhibited the oxidation of L-tyrosine catalyzed by tyrosinase with IC_{50} values of 183 and 67.0 μg/mL, respectively. However other fractions did not showed any activity at the highest concentration of 500 μg/mL. The EtOAc soluble fraction of the MeOH extract of *E. stolonifera* exhibited stronger inhibitory activity on the oxidation of L-tyrosine by the mushroom tyrosinase than that of arbutin, positive control with an IC_{50} value of 112 μg/mL. Column chromatography of the EtOAc fraction led us to the isolation of tyrosinase inhibitory phloroglucinol derivatives. These phloroglucinol derivatives were identified as phloroglucinol (**1**), eckstolonol (**2**), eckol (**3**), phlorofucofuroeckol A (**4**), and dieckol (**5**) on the basis of chemical and physicochemical evidences, and compared their spectral data with those in the literature as previously reported (Fukuyama *et al.*, 1985, 1989a, 1990; Nakamura *et al.*, 1996; Kang *et al.*, 2003). The chemical structures of the five phloroglucinol derivatives are presented in Fig. 1. IC_{50} values of compounds **1**~**5** on the oxidation of the L-tyrosine catalyzed by tyrosinase, were determined to be 92.8, 126, 33.2, 177, and 2.16 μg/mL, respectively, as shown at Table III. It was compared with kojic acid and arbutin, positive controls, with IC_{50} values of 6.32 and 112

Table I. Inhibitory activities of seaweeds on mushroom tyrosinase

	Seaweeds	IC_{50}[a] (μg/mL)
Chlorophyta	*Enteromorpha crinita*	>500
	Ulva pertusa	>500
Phaeophyta	*Ecklonia stolonifera*	345
	Hizikia fusiformis	>500
	Sargassum ringgoldianum	>500
	Sargassum miyabei	>500
	Sargassum thunbergii	>500
Rhodophyta	*Acrosorium flabellatum*	>500
	Carpopeltis cornea	>500
	Chondrus crispus	>500
	Gelidium amansii	>500
	Gigatina tenella	>500
	Gracilaria verrucosa	>500
	Grateloupia filicina	>500
	Pachymeniopsis elliptica	>500
	Rhodymenia intricate	>500
	Symphyocladia latiuscula	>500
Control	Kojic acid	4.57

[a]Inhibitory activity was expressed as the mean of 50% inhibitory concentrations of triplicate determinations, obtained by interpolation of concentration-inhibition curve.

Table II. Inhibitory activities of various fractions obtained from the MeOH extract of *E. stolonifera* on mushroom tyrosinase activity

Samples	IC_{50} (μg/mL)[a]
MeOH ex.	354.0
n-Hexane fr.	>500.0
CH_2Cl_2 fr.	183.0
EtOAc fr.	67.0
n-BuOH fr.	>500.0
H_2O fr.	>500.0
Arbutin	112.0
Kojic acid	6.32

[a]Inhibitory activity was expressed as the mean of 50% inhibitory concentrations of triplicate determinations, obtained by interpolation of concentration-inhibition curve.

Table III. Tyrosinase inhibitory activity of isolated compounds **1**~**5** from the EtOAc fraction of the MeOH extract of *E. stolonifera*

Compounds	IC_{50} (μg/mL)[a]
Phloroglucinol (**1**)	92.8
Eckstolonol (**2**)	126.0
Eckol (**3**)	33.2
Phlorofucofuroeckol A (**4**)	177.0
Dieckol (**5**)	2.16
Arbutin	112.0
Kojic acid	6.32

[a]Inhibitory activity was expressed as the mean of 50% inhibitory concentrations of triplicate determinations, obtained by interpolation of concentration-inhibition curve.

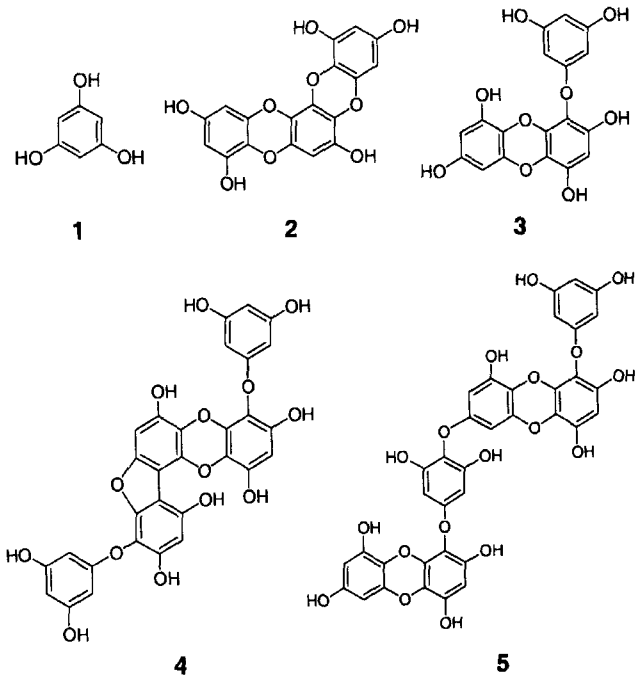

Fig. 1. The structures of the phlorotannins from *E. stolonifera*

μg/mL, respectively. All the compounds inhibited the monophenolase activity of the mushroom tyrosinase, and phloroglucinol (**1**) and eckol (**3**) showed stronger inhibitory activity than arbutin, and dieckol (**5**) displayed three times higher activity than kojic acid, a well-known tyrosinase inhibitor.

Lineweaver-Burk plots as shown in Fig. 2 and 3, depicted to determine the inhibitory modes of compounds **1**~**5**, respectively. The lines, obtained from the uninhibited enzyme and from three different concentrations of the test compounds, intersected on the vertical axis (Fig. 2). The results demonstrate that compounds **1** and **2** exhibited competitive inhibition against oxidation of L-tyrosine catalyzed by mushroom tyrosinase. K_i values of phloroglucinol (**1**) and eckstolonol (**2**) were estimated to be 2.3×10^{-4} and 3.1×10^{-4} M, respectively. The lines, obtained with the uninhibited enzyme and from different concentrations of eckol (**3**), phlorofucofuroeckol A (**4**) or dieckol (**5**), intersected on the horizontal axis (Fig. 3). The results demonstrate that compounds **3**, **4**, and **5** exhibited noncompetitive inhibitors for the oxidation of L-tyrosine catalyzed by mushroom tyrosinase. K_i values of **3**, **4**, and **5** which can be calculated from the slope or the 1/V-axis intercept, were 1.9×10^{-5}, 1.4×10^{-3}, and 1.5×10^{-5} M, respectively (Table IV).

A competitive inhibitor is a substance that combines with a free enzyme (E) in a manner that prevents substrate binding. That is, the inhibitor (I) and the substrate (S) are mutually exclusive, often because of true competition for the same site. A competitive inhibitor might be a nonmetabolizable analogue or derivative of the true substrate, or alternate substrate of the enzyme, or a product of the reaction. A classical noncompetitive inhibitor has no effect on substrate binding and *vice versa*. S and I bind

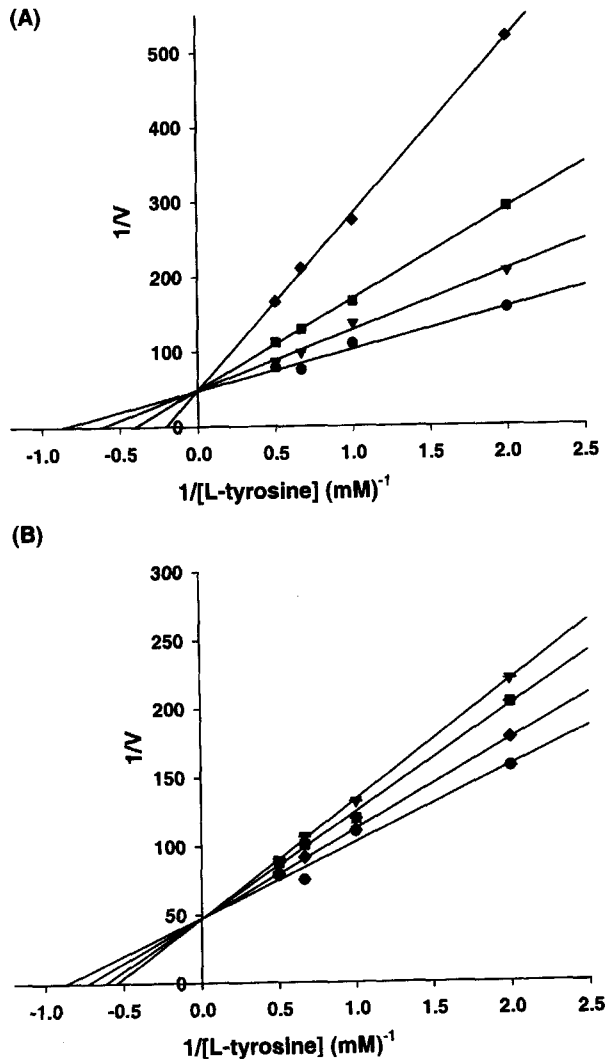

Fig. 2. Lineweaver-Burk plots for the inhibition of the mushroom tyrosinase by phloroglucinol (A) and eckstolonol (B). The reaction was done in the presence of phloroglucinol [final concentration of 0 (●), 125 (▼), 250 (■) and 500 μM (◆)] or eckstolonol [0 (●), 50 (◆), 100 (■) and 200 μM (▼)]. V_{max} = 0.02 $\triangle OD_{490}$/min, K_m = 1.2×10^{-3} M, 1/V : 1/($\triangle$ OD_{490}/min).

Table IV. Tyrosinase inhibitory modes and inhibitory constants (K_i) of phloroglucinol derivatives isolated from *E. stolonifera*

Compounds	Mode of inhibition[a]	K_i value (M)
Phloroglucinol (**1**)	Competitive	2.3×10^{-4}
Eckstolonol (**2**)	Competitive	3.1×10^{-4}
Eckol (**3**)	Noncompetitive	1.9×10^{-5}
Phlorofucofuroeckol A (**4**)	Noncompetitive	1.4×10^{-3}
Dieckol (**5**)	Noncompetitive	1.5×10^{-5}

[a]With respect to the oxidation of L-tyrosine by mushroom tyrosinase.

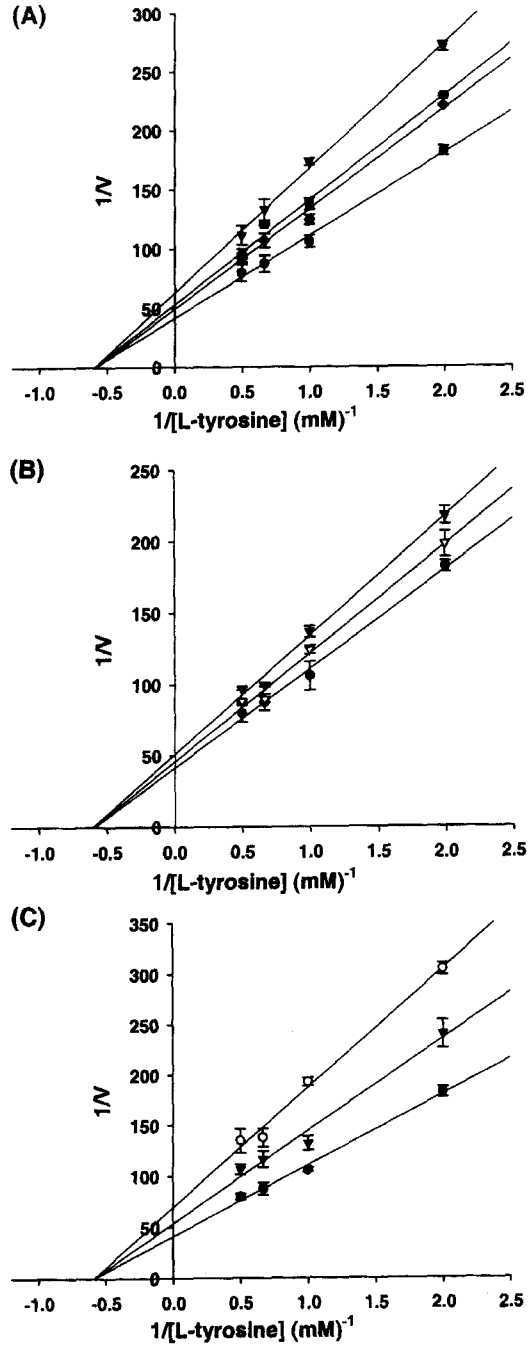

Fig. 3. Lineweaver-Burk plots for the inhibition of the mushroom tyrosinase by eckol (A), phlorofucofuroeckol A (B) and dieckol (C). The reaction was done in the presence of eckol [final concentration of 0 (●), 2.5 (◆), 5 (■) and 10 μM (▼)], phlorofucofuroeckol A [0 (●), 208 (▽), and 313 μM (▼)] or dieckol [0 (●), 5 (▼), and 10 μM (□)]. V_{max} = 0.02 $\triangle OD_{490}$/min, K_m = 1.7×10^{-3} M, 1/V : 1/($\triangle$ OD_{490}/min).

reversibly, randomly, and independently at different sites (Segel, 1976).

Phloroglucinol derivatives, common secondary metabolite constituents of brown algae, are polymers of acetate-malonate derived 1,3,5-trihydroxybenzene (phloroglucinol) (Higa, 1981). However, preincubation of the enzyme with these inhibitors in the absence of the substrate did not diminish enzyme activity significantly, suggesting that compounds **1-5** are inhibitors rather than inactivators of the enzyme (Khan and Andrawis, 1985).

Fukuyama *et al.* (1985; 1989a; 1989b; 1990) reported that phloroglucinol itself, and phlorethols did not show inhibitory activity on plasma plasmin at all, revealing the requirement of a dibenzo-1,4-dioxine ring for anti-plasmin inhibitory activity (Nakayama *et al.*, 1989; Hay, 1988). Phloroglucinol derivatives from *Eisenia bicyclis* showed a potent antioxidant effect which extended the induction time of autoxidation of methyl α-linolenate, seems to depend on the degree of polymeization of phloroglucinol. Generally, phloroglucinol derivatives of lower molecular weight were more effective than those of higher weight (Nakamura *et al.*, 1996). On the contrary, the feeding deterrent effect of marine herbivores (Taniguchi *et al.*, 1991; Hay, 1988; Altena and Steinberg, 1992; Boettcher and Targett, 1993; Targett *et al.*, 1995) and bactericidal activity (Nagayama *et al.*, 2002) of phloroglucinol derivatives tend to increase with polymerization of phloroglucinol. Although the mode of activity of phloroglucinol derivatives is still obscure; the interaction of phloroglucinol derivatives with bacterial proteins may play an important role in its bactericidal action (Stern *et al.*, 1996). Recently, Okada *et al.* reported the phloroglucinol derivatives from *E. bicyclis* exhibited inhibitory activity on glycation and α-amylase (Okada *et al.*, 2004). Although tyrosinase inhibitory activities of phloroglucinol derivatives such as phloroglucinol and eckol have been reported (Mitani, and Sakai, 1992; Onodera *et al.*, 1992), the tyrosinase inhibitors from *E. stolonifera* and their inhibitory kinetics have not been reported so far.

Nerya *et al.* (2003) suggested that one of the structural requirements for noncompetitive inhibitors on the tyrosinase is the presence of a resorcinol moiety, and that most of the competitive inhibitors have the ability to chelate copper in this enzyme, implying a possible inhibitory mechanism. All the phloroglucinol derivatives isolated from *E. stolonifera* contain resorcinol moieties, their tyrosinase inhibitory mode, however, exhibited two different types as described above. It will need the further study for the structure-activity relationships of tyrosinase inhibitors.

Dioxygen or reactive oxygen species (ROS) are required by the enzyme for the oxidation of L-tyrosine to 3-hydroxy-L-tyrosine (L-dopa) and then to L-dopaquinone (Schallreuter, 1999). Yokochi *et al.* (2003) reported that the vitamin B_6 inhibited the diphenolase activity by quenching ROS (probable singlet oxygen) generated during some reaction step of the diphenolase reaction. Further, they suggested that the inhibition is dependent on the quenching of ROS produced in some intermediary step of the tyrosinase

reaction. Previously, we investigated that the phloroglucinol derivatives from *E. stolonifera* inhibited total ROS generation (Kang *et al.*, 2004). Thus, it may be one of the probable inhibitory mechanisms that phloroglucinol derivatives inhibit tyrosinase by scavenging ROS produced during the catalytic cycle of the enzyme.

Safety is a primary consideration for tyrosinase inhibitors, especially for those used in food and cosmetic products, as these may be regularly utilized in unregulated quantities.In higher plants and fungi, tyrosinase inhibitors occur in various compounds, such as oxyresveratrol (Shin *et al.*, 1998), quercetin (Kubo and Kinst-Hori, 1999), (-) epigallocatechin 3-*O*-gallate (EGCG) (No *et al.*, 1999), and metallothionein (Goetghebeur and Kermasha, 1996), however, its inhibitors from edible alga *E. stolonifera* have not been found. Safety is a primary consideration for tyrosinase inhibitors, especially for those used in food and cosmetic products, as these may be regularly utilized in unregulated quantities. This work showed that phlorotannins, natural compounds found in an edible brown alga, could be involved in the control of pigmentation in plants and other organisms through inhibition of tyrosinase activity using L-tyrosine as a substrate.

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