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Characteristics of Tyrosinase Inhibitory Extract from *Ecklonia stolonifera*

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Tyrosinase inhibitory activities of 14 kinds of seaweed, *Ecklonia stolonifera, Ecklonia cava, Undaria pinnatifida, Laminaria japonica, Sargassum fulvellum, Sargassum miyabei, Sargassum thunbergii, Porphyra yezoensis, Gracilaria verrucosa, Carpopeltis affinis, Pachymeniopsis elliptica, Gelidium amansii, Codium fragile* and *Ulva pertusa* were determined using commercially available mushroom tyrosinase in an *in vitro* assay system. The 1% (w/v) methanol extract from *E. stolonifera* showed the highest tyrosinase inhibitory activity of 79.0%, electron donating activity of 79.0% and total phenol content of 3.75 mg/100 g. Ethyl acetate-methanol-water (7 : 2 : 0.2, v/v) fraction (0.5%, w/v) isolated from the methanol extract showed tyrosinase inhibitory activity of 75.9%, electron donating activity of 88.1% and total phenol content of 4.38 mg/100 g. Tyrosinase inhibitory activity was closely associated with total phenol content (R=0.99) and electron donating activity (R=0.99). Maximum absorption wavelength of the fraction was 218 nm and that of phenolic compounds showed about a range from 210 to 220 nm. The inhibition mode of the fraction was noncompetitive inhibition.

Key words: Tyrosinase, enzyme inhibition, enzymatic browning, *E. stolonifera*

Introduction

Tyrosinase (EC 1. 14. 18. 1), copper-containing monooxygenase, is one of the most important causes of enzymatic browning. It catalyzes the *o*-hydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones as affected by the specificity of its substrates then *o*-quinone produces melanin through concurrent or sequent nonenzymatic oxidation, reaction with amino compounds, condensation and polymerization (Lee and Whitaker, 1994).

This enzyme causes significant commercial losses of marine *crustacea,* such as lobster, krill, crab and shrimp by the browning (George et al., 1992) and also catalyzes biological damage, such as cell toxicity (Gantchev et al., 1994), aging by the active oxygen produced during the enzymatic reaction (Jiang and Miles, 1993; Nagano and Koga, 1994) and DNA damage by phenoxyl radicals produced from the oxidation of etoposide (VP-16), an antitumor drug (Kurella et al., 1995).

For these reasons, it has been widely studied for the inhibition of tyrosinase. Sulfites, cysteine (Richard-Forget et al., 1992) and other sulfhydryl compounds (Friedman and Bautista, 1995), Maillard reaction products (Nicoli et al., 1991), ascorbic acid derivatives (Sapers and Miller, 1992), benzoic acid (Conrad et al., 1994) and azide (Sugumaran, 1995) were investigated for the inhibition of tyrosinase. In addition, phenolic compounds, such as 4-hexyl resorcinol (McEvily et al., 1991), *p*-nitrophenol (Conrad et al., 1994), dihydroxyindole, dihydroxy phenylalanine (Sugumaran, 1995), salicylhydroxamic acid and tropolone (Ferrar

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and Walker, 1996) have been found for the inhibitory activity. Jung et al. (1995) and Choi et al. (1995) screened plants, such as vegetables, fruits, mushrooms, teas and medicinal plants for tyrosinase inhibition, while seaweed was little studied except the tyrosinase inhibitive fluoroglucine polymer separated from 20% methanol extract of *Ishige okamurai* (Takabayashi and Noda, 1996).

Therefore, this study was conducted to investigate tyrosinase inhibitory effect of seaweed and to prepare the inhibitive material from seaweed. Fourteen kinds of seaweed were screened for the tyrosinase inhibitory activity and the mode of inhibition of fraction from *E. stolonifera* was investigated.

Materials and Methods

Materials

Eight kinds of fresh seaweed, *Ecklonia stolonifera, Sargassum fulvellum, Sargassum miyabei, Sargassum thunbergii, Gracilaria verrucosa, Carpopeltis affinis, Pachymeniopsis elliptica* and *Codium fragile* were collected at Pusan (Yeong-Do and Kijang) and Taebyun, Korea and six kinds of dried seaweed, *Ecklonia cava, Undaria pinnatifida, Laminaria japonica, Porphyra yezoensis, Gelidium amansii* and *Ulva pertusa* were purchased at Kijang and Chagalchi market, Pusan, Korea.

Preparation and fractionation of seaweed extracts

The seaweed was washed with running tap water, dried at 50℃ for 24 hr and then ground to fine powder (200 mesh). Each powder was extracted with 10 volumes (v/w) of methanol at an ambient temperature for 48 hrs and filtrated (Toyo, 5A). The filtrates were evaporated under a low pressure at 40 ℃ to obtain methanol extract.

The methanol extract was stepwise fractionated with hexane-chloroform-methanol (6 : 4 : 0.8), ethyl acetate-methanol-water (7 : 2 : 0.2) and methanol-water (10 : 1) by silica gel column chromatography (silica gel 60; Merck, Germany).

Tyrosinase activity

Tyrosinase activity was determined by the modified method of Opoku-Gyamfua et al. (1992) using L-dihydroxy phenylalanine (L-DOPA) as a substrate. The reaction medium consists of 1.7 mL of 50 mM phosphate buffer (pH 7.5), 0.2 mL of tyrosinase (500 unit/mℓ), 0.1 mL of sample solution (DMSO as a blank) and 1 mL of 5 mM L-DOPA solution. A unit of enzyme activity was defined as the change of absorbance of 0.001/min at 475 nm and 25℃. The tyrosinase inhibitory activity was calculated as follows; Inhibition (%) $=[1-(A/B)]\times 100$, where A and B represent the absorbance of sample and blank solution which were incubated for 15 min prior to the addition of L-DOPA.

Total phenol content

Total phenol content was determined by the method of Folin-Denis (AOAC, 1996). Folin-Denis reagent comprised 11.2 g sodium tungstate, 2.0 g phosphomolibdic acid and 12.85 g metaphosphoric acid in 75 mL distilled water. Prior to preparation, all chemicals were dried at 75℃ for 12 hrs. The solution was refluxed for 2 hrs, cooled to an ambient temperature and then filled up to 100 mL. Aliquot (0.5 mL) of 1% (w/v) sample solution was placed in a test tube with 2.5 mL of distilled water, 2 mL of Folin-Denis reagent and 5 mL saturated sodium carbonate solution, and allowed it to stand for 1 hr. Total phenol content (mg/100 g) was determined by the absorbance at 660 nm. The contents were calculated from the calibration curve developed using tannic acid.

Electron donating activity

Electron donating activity was determined from the reducing ability by the electron donation to 1,1-diphenyl-2-picryl-hydrazyl (DPPH). Three milliliters of 1% (w/v DMSO) mathanol extract and 3 mL of 100 μM DPPH (dissolved in 99.9% ethanol) were mixed for 10 seconds and then incubated at 37℃ for 30 min. The absorbance was measured at 516 nm. The electron donating activity was calculated as follows; activity (%) $=[1-(A/B)]\times 100$, where A and B represent the absorbance of sample and blank solution, respectively.

Determination of maximum absorption wavelength

Each maximum absorbance wavelength of fraction from *E. stolonifera* and 13 kinds of phenolic com-

pounds was scanned in a range from 200 to 500 nm with spectrophotometer (Shimadzu, UV-160A).

Results and Discussion

Table 1 shows tyrosinase inhibitory activity of each methanol extract from the 14 kinds of seaweed. The extract of *E. stolonifera* showed the highest inhibitory activity of 79.0% followed by extract of *E. cava* (72.1 %), *S. miyabei* (48.5%) and *S. thunbergii* (46.7%). Other extracts, however, were less than 25%. Therefore, *E. stolonifera* was a good source for the inhibition of tyrosinase.

Table 1. Tyrosinase inhibitory activity of methanol extracts[a] from seaweed

Seaweed		Inhibitory activity (%)
Division	Species	
Phaeophyta	*Ecklonia stolonifera*	79.0
	Ecklonia cava	72.1
	Undaria pinnatifida	6.3
	Laminaria japonica	2.7
	Sargassum fulvellum	7.3
	Sargassum miyabei	48.5
	Sargassum thunbergii	46.7
Rhodophyta	*Porphyra yezoensis*	-[b]
	Gracilaria verrucosa	13.3
	Carpopeltis affinis	21.5
	Pachymeniopsis elliptica	23.6
	Gelidium amansii	24.7
Chlorophyta	*Codium fragile*	12.3
	Ulva pertusa	7.3

[a]Each 1% (w/v) extract was added into the reaction medium.
[b]Not detected

Tyrosinase inhibitory activity of the methanol extract from the *E. stolonifera* was compared with some compounds, such as 4-hexylresorcinol, ascorbic acid, cysteine, benzoic acid, EDTA (ethylenediaminetetraacetic acid) and sodium sulfite (Table 2). Tyrosinase inhibitory activity (79.0%) of 1% methanol extract of the *E. stolonifera* was a little lower than 91.8% of 1 mM 4-hexylresorsinol, publicly approved as a tyrosinase inhibitor but it was higher than that of other compounds (<35%).

A lot of polar and anti-oxidative phenolic compounds occurred in plant kingdom (Jaime et al., 1989). Thereby, to investigate characteristics of the inhibitory fractions from *E. stolonifera* against tyrosinase, total phenol contents, electron donating activity and tyrosinase inhibitory activity of every water extract, methanol extract and the ethyl acetate-methanol-water fraction were compared (Fig. 1). The 1% (w/v) methanol extract contained 3.75 mg/100 g of phenolic compounds similar to 3.88 mg/100 g of the methanol soluble fraction from steamed green tea (Yeo et al., 1994) and it showed electron donating activity of 79.0%, which was evidently higher than 22.7% of water extract showing low total phenol content (2.27 mg/100 g) and tyrosinase inhibitory activity (24.8%). The 0.5% ethyl acetate-methanol-

Table 2. Comparision of tyrosinase inhibitory activity of methanol extract from *E. stolonifera* and some compounds

Compounds (Concentration)	Inhibitory activity (%)
E. stolonifera extract (1%)	79.0
4-Hexylresorcinol (1 mM)	91.8
Ascorbic acid (1 mM)	-[a]
Cysteine (1 mM)	8.2
Benzoic acid (1 mM)	19.0
EDTA (1 mM)	33.3
Sodium sulfite (1 mM)	33.6

[a]Not detected.

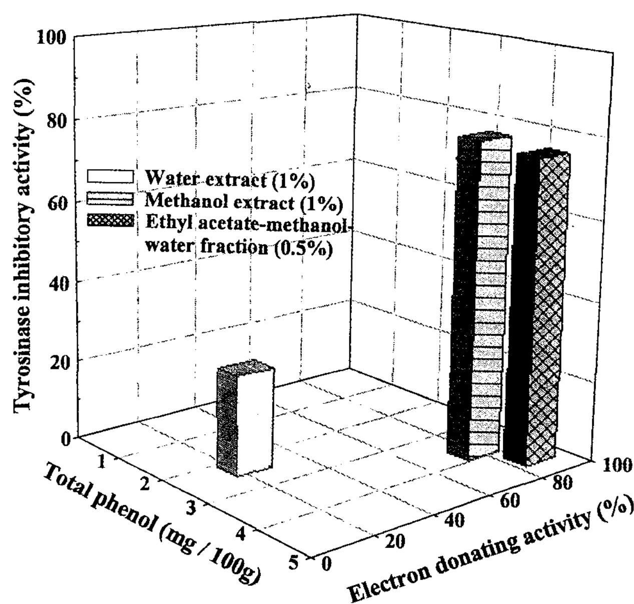

Fig. 1. Comparision of tyrosinase inhibitory activity, electron donating activity and total phenol content of every water extract, methanol extract and ethyl acetate-methanol-water fraction from *E. stolonifera*.

water fraction showed the highest degrees of electron donating activity (88.1%) and total phenol contents (4.38 mg/100 g) and also exhibited high tyrosinase inhibitory activity (76%), which was almost same to 79% of the 1% methanol extract. Furthermore, tyrosinase inhibitory activity of the every extract was closely related to the both total phenol content (R =0.989) and electron donating activity (R=0.999). Kang et al. (1996) reported high electron donating activity of phenolic compounds showing strong reducing ability. Therefore, it could be deduced that the inhibitive components of *E. stolonifera* were phenolic compounds containing reducing ability.

Thereby, the ethyl acetate-methanol-water fraction was compared with 13 kinds of phenolic compounds in both maximum absorption wavelength and tyrosinase inhibitory activity (Table 3). The fraction showed the maximum absorption wavelength at 218 nm and those of phenolic compounds showed about a range from 210 to 220 nm. Because most glycosides commonly have the maximal absorption around at 217~219 nm (Yamaguchi, 1970) and that of phenolic glucosides is at 222 nm (Nakatani and Kikuzaki, 1987) the fraction was supposed as a phenolic glycosides. But, tyrosinase inhibitory activities of the phenolic compounds were different. Phenol (96.8%),

Table 3. Maximum absorption wavelength and tyrosinase inhibitory activity of the ethylacetate-methanol-water fraction and some phenolic compounds

Compoundsa	Maximum absorption wavelength (nm)	Inhibitory activity (%)
Ethyl acetate-methanol-water fraction	218.0	75.9
Phenol	219.0	96.8
Benzene	211.5	49.3
4-Hexylresorcinol	211.0	88.3
Resorcinol	210.0	-0.9
Phloroglucinol	211.5	-8.8
Pyrogallol	212.0	23
Vanillic acid	218.0	17.5
Bergenin	219.5	24.3
Gentisic acid	214.5	20.1
Protocatechuic acid	225.5	25.6
Syringic acid	217.0	22.7
p-Hydroxybenzoic acid	207.5	29.2
Tetrahydroxy-1,4-quinone hydrate	206.0	17.9

[a]Ethyl acetate-methanol-water fraction is 0.5% (w/v) and each phenolic compounds is 1 mM

4-hexylresorcinol (88.3%) and the fraction (75.9%) showed high inhibitory activities while others exhibited no significantly high activity (<50%). Especially, the (−) levels of resorcinol and phloroglucinol describe the catalytic activity on tyrosinase. Related this result, shannon et al. (1967) reported that resorcinol and phloroglucinol increased the velocity of oxidation reaction of tyrosinase in apple and Duckworth and Coleman (1970) also reported that phenolic compounds were substrates of tyrosinase, however, benzoic acid and cyanide showed noncompetitive inhibition against oxygen and catechol varied, respectively. From these resuts, it was identified that phenolic compound could be an inhibitor or a substrate of tyrosinase as affected by its structural difference.

Kinetic tests for mode of inhibition of the ethyl acetate-methanol-water fraction were conducted with double reciprocal Lineweaver-Burk plots. Figure 2 shows inhibition mode of the fraction against tyrosinase activity in a correlation of 1/V (tyrosinase activity) vs 1/S (L-DOPA concentration). The fraction exhibited noncompetitive inhibition against tyrosinase. From these results, a reducing ability of phenolic compounds which reduce *o*-quinones formed back to the colorless *o*-dihydroxyphenolic substrates as well as direct inhibitive effect of themselves by binding to a site other than active site (Lee and Whitaker, 1995) were deduced as basic reason of the non-

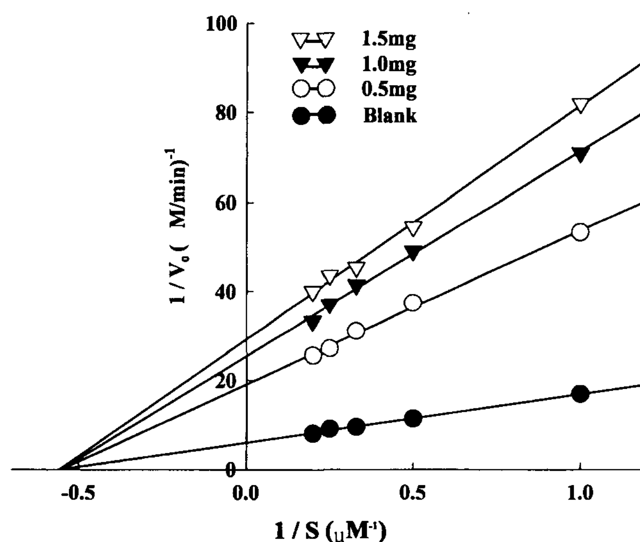

Fig. 2. Lineweaver-Burk plots of ethyl acetate-methanol-water fraction from the methanol extract of *E. stolonifera*.

competitive inhibition. (+) Catechin glucoside was reported as a noncompetitive inhibitor for tyrosinase activity by Funayama et al. (1993).

Conclusively, it was suggested that tyrosinase inhibitor of the fraction from *E. stolonifera* were a phenolic glycosides and the inhibitory activity was due to a nonenzymatic reduction of the quinones formed and direct inhibitive effect by binding of inhibitors to a site other than active site of tyrosinase. Further research to purify the inhibitive compound and to prepare an antibrowning agent are to continue.

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