J. Cosmet. Sci., **63,** 225–232 (July/August 2012)

Crude ethanol extracts from grape seeds and peels exhibit anti-tyrosinase activity

CHENG-KUANG HSU, SU-TZE CHOU, PEI-JANE HUANG, MEI-CHIN MONG, CHIEN-KUO WANG, YU-PIN HSUEH, and JYUN-KAI JHAN, *Department of Food Science, National Chiayi University, No.300, Syuefu Rd., Chiayi City 60004, (C.-K.H.)*; *Department of Health Nutrition and Biotechnology, Asia University, No.500, Lioufeng Road, Wufeng Shiang, Taichung 413 (C.-K.H., P.-J.H., M.-C.M.); Department of Biotechnology, Asia University, No. 500, Lioufeng Road, Wufeng Shiang, Taichung 413 (C.-K.W., Y.-P.H.); and Department of Food and Nutrition, Providence University, No. 200, Chungchi Rd., Shalu, Taichung 433 (J.-K.J.), Taiwan, ROC*.

Accepted for publication November 30, 2011.

Synopsis

This study aimed to evaluate the anti-tyrosinase activites of ethanol extracts from the peels and the seeds of Kyoho grapes and Red Globe grapes (KG-PEE, KG-SEE, RGG-PEE, and RGG-SEE). The total phenolic content in KG-SEE and RGG-SEE was 400±11 and 339±7 mg gallic acid equivalent/g, respectively, about 22 times and 13 times that in KG-PEE and RGG-PEE, respectively. Both seed extracts showed significantly higher anti-tyrosinase activity than the peel extracts due to their high total phenolic content. The gallic acid content in RGG-SEE was twice that in KG-SEE, and gallic acid showed high anti-tyrosinase activity; thus, RGG-SEE had higher anti-tyrosinase activity than KG-SEE. Lineweaver-Burk plots revealed that the inhibitory mechanism of the ethanol extracts from the grapes was a mix-type inhibition. Grape seed has a greater total phenolic content and has potential as a skin-lighting agent.

INTRODUCTION

Tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase EC 1.14.18.1) is a multifunctional copper-containing enzyme and commonly present in microorganisms, plants, and animals. This enzyme mainly is involved in the first two steps of the melanin biosynthesis pathway; first, L-tyrosine (monophenolase activity) is hydroxylated and the hydroxylation product, L-dopa (diphenolase activity), is further oxidized into the corresponding *o*-quinone (1).

Address all correspondence to Cheng-Kuang Hsu at ckhsu@mail.nycu.edu.tw.

Tyrosinase is responsible for enzymatic browning in plants and therefore is considered to produce undesirable changes in color, flavor, and nutritive values of plant-derived foods (2,3). Therefore, tyroinase inhibitors may prevent the browning reaction caused by tyroinase and maintain the appearance of plant foods.

Furthermore, tyrosinase catalyzes the reaction of melanin biosynthesis in human skin and results in dark skin (4). Recently, safe and effective tyrosinase inhibitors have become important for their potential applications in preventing pigmentation in human beings (1,4,5). Therefore, tyrosinase inhibitors are also important in cosmetic applications for skin-whitening effects, since certain people prefer a lighter skin color (6).

Plants are rich sources of bioactive chemicals and mostly free from harmful side effects, and there is an increasing interest in finding natural tyrosinase inhibitors from them. Some potent tyrosinase inhibitors, such as cuminaldehyde (7), oxyresveratrol (8), kaempferol (9), quercetin (10), and gallic acid derivatives (11) have been isolated from various plants. Phenolic compounds are rich in many plants and they have been shown to possess anti-tyrosinase activity (10–13).

In this study, the effects of ethanol extracts from the peels and the seeds of two grape cultivars, Kyoho grapes and Red Globe grapes, on mushroom tyrosinase activity were investigated.

MATERIALS AND METHODS

CHEMICALS

Mushroom tyrosinase (4,000 units/mg), and 3,4-dihydroxyphenylalanine (L-dopa) were purchased from Sigma Chemicals Co. (St. Louis, MO). HPLC-grade ellagic acid, gallic acid, cinnamic acid, resveratrol, and catechin hydrate were also purchased from Sigma. Chlorogenic acid and kuromanin chloride were purchased from Extrasynthese (Genay, France). Kyoho grapes (*Vitis vinifera* × *Vitis labrusca*) and Red Globe grapes (*Vitis vinifera*) were purchased from a local farmer in Taichung city, Taiwan, ROC.

PREPARATION OF ETHANOL EXTRACT FROM THE PEEL AND THE SEED OF GRAPES

Grape peel and seed were homogenized with 95% ethanol (1: 10, weight (g)/volume (ml)) for 1 min and then set in a refrigerator at 4°C for 12 h. After centrifuging the mixture at 7000*g* at 4°C for 20 min, the ethanolic solution was filtrated with Whatman No.l paper, and then ethanol was removed in an evaporator at a temperature lower than 40°C.

MEASUREMENT OF TOTAL PHENOLIC CONTENT

The total phenol content was measured using Folin-Ciocalteu's reagent method (14). The sample (0.5 ml, 200 mg sample/ml) was mixed with 0.5 ml of Folin-Ciocalteu's reagent for 3 min and then mixed with 0.05 ml of 10 % Na_2Co_3. The absorbance of the mixture was measured at 735 nm after 1-hr incubation at room temperature. Gallic acid was used as the

standard for the calibration curve, and the total phenolic content was expressed as gallic acid equivalents (mg/g dry material).

CHARACTERIZATION OF PHENOLIC COMPOUNDS IN EXTRACTS

Phenolic compounds in the tested extract were analyzed based on the method described by Li *et al*. (15), with a slight modification. Dried extract (10 mg) was dissolved in 1 ml of 0.1% phosphoric acid, filtered through a 0.45-μm filter, and analyzed by HPLC. HPLC analysis was performed using a Hewlett- Packard HPLC system (HP 1100 series, Waldron, Germany), consisting of a quaternary pump and a variable wavelength detector (VWD) at 270 nm and equipped with a Li-Chrospher RP-18 cartridge column (Merck, 250 mm × 4.6 mm, 5 μm). The mobile phase was a stepwise gradient of water (0.1% v/v phosphoric acid)–acetonitrile (0.01 min, 100:0; 50 min, 20:80), and the injection volume was 30 μl. The identification of each compound was based on a combination of retention time and spectral matching by comparison with those of known standards.

ENZYMATIC ASSAY OF TYROSINASE

The tyrosinase activity using L-dopa as substrate was measured according to the method of Kubo and Kinst-Hori (7), with slight modifications. First, 0.29 ml of 4.5 mM L-dopa solution (the substrate for tyrosinase) was mixed with 0.3 ml of 25 mM phosphate buffer (pH 6.8) and incubated at 25°C for 10 min. Then, 0.3 ml of tested samples of different concentrations (1, 5, 10, and 15 mg/ml) was added to the mixture followed by the addition of 0.01 ml of 4000 units/ml mushroom tyrosinase. The formation of dopachrome was immediately monitored by measuring the linear increase in optical density at 475 nm. Triplicate measurements were recorded. The increased absorbance at 475 nm was recorded during 10 min at room temperature. Deionized water was used instead of the extract for the blank. One unit (U) of enzymatic activity was defined as the amount of enzyme needed for increasing 0.001 absorbance per min at 475 nm under the experimental conditions.

DETERMINATION OF KINETIC PARAMETERS

Mushroom tyrosinase (0.03 ml, 4000 units/ml) was incubated with various concentrations of 0.27-ml enzyme substrates (L-dopa, 0.6–0.66 mg/ml) in 0.3 ml of 25-mM phosphate buffer (pH 6.8) at room temperature, and tested samples (0.3 ml) were added to the reaction mixture simultaneously. The kinetic parameters, K_m and V_{max}, of the tyrosinase activity were calculated by linear regression from Lineweaver-Burk plots.

STATISTICAL ANALYSIS

For each measurement, three replicates were conducted. The data were presented as the mean ± standard deviation. One-way analysis of variance (ANOVA) was conducted using a package (SAS Institute Inc., Cary, NC). Duncan's multiple ranges test was used to determine the significant difference between different treatments.

RESULTS AND DISCUSSION

ANTI-TYROSINASE ACTIVITY OF GRAPE EXTRACTS

L-dopa is an endogenous phenolic substrate for polyphenol oxidase in apple and potato sources (16). It is an intermediate product during oxidation of L-tyrosine and is used commonly as an enzyme substrate of tyrosinase, a polyphenol oxidase (1,9–11,17). In this study, the inhibitory effects of ethanol extracts from peels and seeds of Kyoho grapes and Red Globe grapes (KG-PEE, KG-SEE, RGG-PEE, and RGG-SEE, respectively) toward mushroom tyrosinase activity were evaluated. Therefore, L-dopa was used as the substrate of mushroom tyrosinase to screen the inhibitory activities of ethanol extracts from the grapes.

The inhibitory effect of KG-PEE on mushroom tyrosinase activity depended on the concentrations (Figure 1). As the concentrations of KG-PEE increased from 0 mg/ml to 5 mg/ml, the absorbance of OD_{475nm}, an index of dopachrome formation, decreased accordingly, indicating a decrease in tyrosinase activity. All the ethanol extracts showed anti-tyrosinase activity, with the following order: RGG-SEE > KG-SEE > KG-PEE > RGG-PEE. It was noted that the seed extracts from both the Kyoho grapes and Red Globe grapes had higher anti-tyrosinase activities than the peel extracts.

To evaluate the inhibitory mechanism of the ethanol extracts, mushroom tyrosinase activity was determined under various L-dopa concentrations. It was found that the reaction rate of tyrosinase decreased as the concentrations of KG-SEE increased from 0.33 mg/ml to 5 mg/ml (Figure 2). KG-PEE, RGG-PEE, and RGG-SEE also reduced the tyrosinase reaction rate in different magnitudes (data not shown). The Lineweaver-Burk plots revealed that these extracts served as mix-type inhibitors, and a represented plot for KG-SEE is shown in Figure 3.

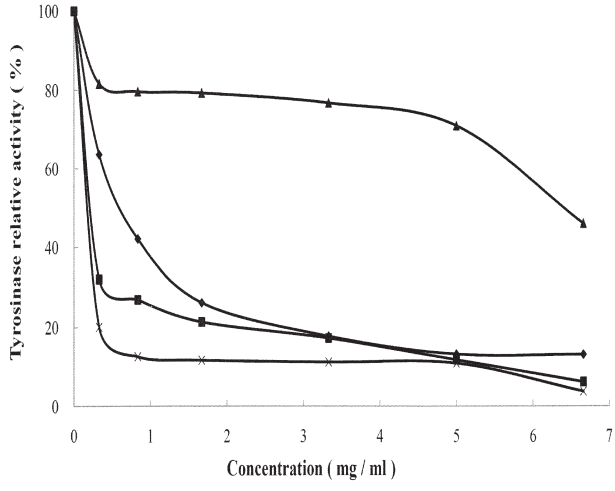

Figure 1. Relative inhibitory effect of grape extracts on mushroom tyrosinase activity using L-dopa (0.33 mg/ml) as the substrate. Mushroom tyrosinase (44 units/ml) reacted at room temperature for 10 min. Symbols: -◆- (KG-PEE); -■- (KG-SEE); -▲- (RGG-PEE); -×- (RGG-SEE).

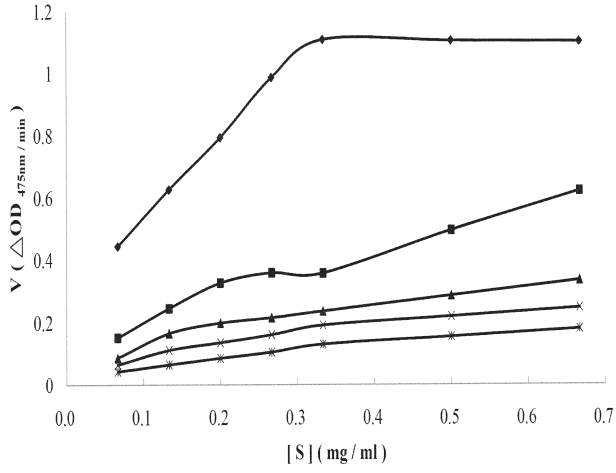

Figure 2. Michaelis-Menton equation of V versus [S] with KG-SEE and L-dopa. The enzymatic assay was performed with mushroom tyrosinase (44 units/ml) at room temperature for 10 min. Symbols: -◆- (0 mg/ml KG-SEE); -■- (0.33 mg/ml KG-SEE); -▲- (1.67 mg/ml KG-SEE); -×- (3.33 mg/ml KG-SEE); -*- (5 mg/ml KG-SEE).

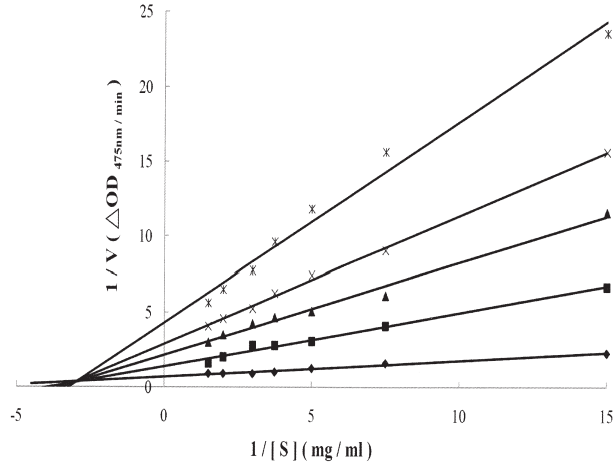

Figure 3. Lineweaver-Burk plot of 1/V versus 1/[S] with KG-SEE and L-dopa. The enzymatic assay was reformed with mushroom tyrosinase (44 mg/ml) at room temperature for 10 min. Symbols: -◆- (0 mg/ml KG-SEE); -■- (0.33 mg/ml KG-SEE); -▲- (1.67 mg/ml KG-SEE); -×- (3.33 mg/ml KG-SEE); -*- (5 mg/ml KG-SEE).

THE EFFECT OF PHENOLIC COMPOUNDS

We suspected that phenolic compounds played important roles in anti-tyrosinase activity. Thus, the total phenolic content in the ethanol extracts from Kyoho grapes and Red Globe grapes was determined and presented as the amount of gallic acid equivalent. The total phenolic content in KG-SEE and RGG-SEE was 400±11 and 339±7 mg gallic acid equivalent/g, respectively, while KG-PEE and RGG-PEE showed 18±1 and 27±0 mg

gallic acid equivalent/g (Table I). Our data indicated that the total phenolic content in ethanol extracts from grape seeds was much higher than in grape peels. The amount of phenolic compounds in the various grape derivatives parallels the anti-tyrosinase activity. Therefore, it was quite possible that phenolic compounds contributed to the anti-tyrosinase activity in the grape extracts.

As the anti-tyrosinase activity in the seed extracts are the main interest, it was noted that even though KG-SEE had a higher total phenolic content than RGG-SEE, the anti-tyrosinase activity of KG-SEE was lower than that of RGG-SEE. This indicated that total phenolic content was not the only factor affecting the anti-tyrosinase activity, and that the profile of phenolic compounds in the extracts might also be an important variable. The phenolic compounds in KG-SEE and RGG-SEE were determined by using HPLC, and the results are shown in Table II. Among the detected phenolic compounds, there were four compounds that showed a significant difference between KG-SEE and RGG-SEE. RGG-SEE had higher gallic acid and kuromanin chloride content than KG-SEE, but lower catechin hydrate and ellagic acid content. After testing the anti-tyrosinase activity of these four pure phenolic compounds, we found that the anti-tyrosinase activity followed the order: gallic acid > catechin hydrate > kuromanin chloride > ellagic acid (Figure 4).

Table I
Total Phenolic Content in the Ethanol Extracts from the Peels and Seeds of Kyoho Grapes and Red Globe Grapes*

KG-SEE	KG-PEE	RGG-SEE	RGG-PEE
400 ± 11^{a}	18 ± 1^{d}	339 ± 7^{b}	27 ± 0^{c}

*Total phenolic content is presented as the amount of gallic acid equivalent (mg gallic acid equivalent/g).
[a,b,c,d] The values in the same row followed by different superscripts were significantly different ($p < 0.05$).

Table II
Phenolic Compounds in the Ethanol Extracts from the Seeds of Kyoho Grapes and Red Globe Grapes Determined by HPLC

	Content (g kg^{-1})	
Compounds	KG-SEE	RGG-SEE
Gallic acid	2.22^{b}	4.60^{a}
Catechin hydrate	5.49^{a}	2.27^{b}
Kuromanin chloride	—	2.75
Ellagic acid	1.61^{a}	0.05^{b}
Flavon	0.60*	0.65
Rutin	—	—
Chlorogenic acid	—	—
Resveratrol	—	—
Quercetin	—	—
Cinnamic acid	—	—

—Not detected.
*The values in the same row are not significantly different.
[a,b]The values in the same row followed by different superscripts are significantly different ($p<0.05$).

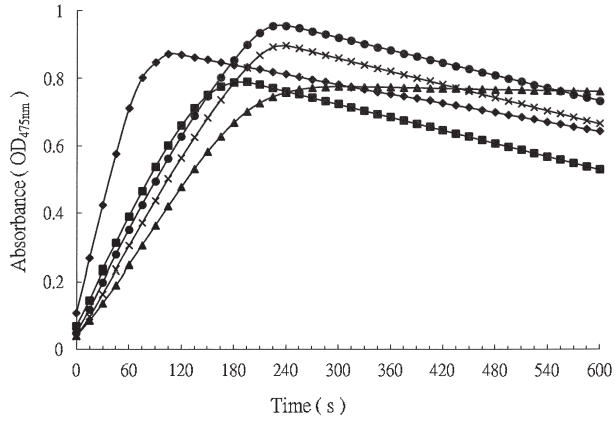

Figure 4. Effect of pure phenolic compounds on mushroom tyrosinase activity using L-dopa (0.33 mg/ml) as the substrate. Mushroom tyrosinase (44 units/ml) reacted at room temperature for 10 min. Symbols: -◆- (no inhibitor); -■- (ellagic acid 0.03 mg/ml); -▲- (gallic acid 0.03 mg/ml); -●- (kuromanin chloride 0.03 mg/ml); -×- (catechin hydrate 0.03 mg/ml).

Gallic acid has been identified as a tyrosinase inhibitor from many plants, and it inhibits the diphenolase activity of mushroom tyrosinase (18,19). Since the gallic acid content in RGG-SEE was about twice that in KG-SEE and gallic acid showed high anti-tyrosinase activity, we suggested that gallic acid was the main compound affecting the anti-tyrosinase activity in the ethanol extracts from grape seeds.

CONCLUSION

Grape seeds had higher anti-tyrosinase activity than grape peels because grape seeds had higher total phenolic compounds. The profile of phenolic compounds was also an important factor affecting anti-tyrosinase activity. It was suggested that gallic acid was the main compound affecting the anti-tyrosinase activity in the ethanol extracts from grape seeds. The inhibitory mechanism of the ethanol extracts from the grape seeds was a mix-type inhibition. Since grape seeds were the potential source of the tyrosinase inhibitor, they might serve as a skin-lighting agent.

ACKNOWLEDGMENT

This study was supported by the National Science Council (NSC 97-2313-B-468-002-MY3), Taiwan, ROC. Their financial support is greatly appreciated.

REFERENCES

(1) S. Y. Seo, V. K. Sharma, and N. Sharma, Mushroom tyrosinase: Recent prospects, *J. Agr. Food Chem.,* **51,** 2837–2853 (2003).

(2) A. Sánchez-Ferrer, J. N. Rodríguez-López, F. García-Cánovas, and F. García-Carmona, Tyrosinase: A comprehensive review of its mechanism, *Biochim. Biophys. Acta,* **1247,** 1–11 (1995).

(3) M. Friedman, Food browning and its prevention: An overview, *J. Agr. Food Chem.,* **44,** 631–653 (1996).
(4) D. B. Mosher, M. A. Pathak, and T. B. Fitzpatrick, "Vitiligo: Ethology, Pathogenesis, Diagnosis and Treatment," in *Updates: Dermatology in General Medicine,* T. B. Fitzpatrick, A. Z. Eisen, K. Wolff, I. M. Freedberg, and K. F. Austen. Eds. (McGraw-Hill, New York, 1983), pp. 373–398.
(5) K. Maeda and M. Fukuda, *In vitro* effectiveness of several whitening cosmetic components in human melanocytes, *J. Soc. Cosmet. Chem.,* **42,** 361–368 (1991).
(6) T. P. Dooley, Topical skin depigmentation agents: Current products and discovery of novel inhibitors of melanogenesis, *J. Dermatol. Treat.,* **8,** 275–279 (1997).
(7) I. Kubo and I. Kinst-Hori, Tyrosinase inhibitors from anise oil, *J. Agr. Food Chem.,* **46,** 1268–1271 (1998).
(8) N. H. Shin, S. Y. Ryu, E. J. Choi, S. H. Kang, I. M. Chang, K. R. Min, and Y. Kim, Oxyresveratrol as the potent inhibitor on dopa oxidase activity of mushroom tyrosinase, *Biochem. Bioph. Res. Co.,* **243,** 801–803 (1998).
(9) I. Kubo and I. Kinst-Hori, Flavonols from saffron flower: Tyrosinase inhibitory activity and inhibition mechanism, *J. Agr. Food Chem.,* **47,** 4121–4125 (1999).
(10) Q. X. Chen and I. Kubo, Kinetics of mushroom tyrosinase inhibition by quercetin, *J. Agr. Food Chem.,* **50,** 4108–4112 (2002).
(11) J. K. No, D. Y. Soung, Y. J. Kim, K. H. Shim, Y. S. Jun, S. H. Rhee, T. Yokozawa, and H. Y. Chung, Inhibition of tyrosinase by green tea components, *Life Sci.,* **65,** 241–246 (1999).
(12) A. Karioti, A. Protopapa, N. Megoulas, and H. Skaltsa, Identification of tyrosinase inhibitors from *Marrubium velutinum* and *Marrubium cylleneum, Bioorg. Med. Chem.,* **15,** 2708–2714 (2007).
(13) H. Y. Ding, H. C. Lin, and T. S. Chang, Tyrosinase inhibitors isolated from the roots of *Paeonia suffruticosa, J. Cosmet. Sci.,* **60,** 347–352 (2009).
(14) M. Sato, N. Ramarathnam, Y. Suzuki, T. Ohkubo, M. Takeuchi, and H. Ochi, Varietal differences in the phenolic content and superoxide radical scavenging potential of wines from different sources, *J. Agr. Food Chem.,* **44,** 37–41 (1996).
(15) Q. Li, Y. Jia, L. Xu, X. Wang, Z. Shen, Y. Liu, and K. Bi, Simultaneous determination of protocatechuic acid, syringin, chlorogenic acid, caffeic acid, liriodendrin and isofraxidin in *Acanthopanax senticosus* Harms by HPLC-DAD, *Biol. Pharm. Bull.,* **29,** 532–534 (2006).
(16) T. O. C. Ndubizu, Relations of phenolic inhibitors to resistance of immature apple fruits to rot, *J. Hortic. Sci.,* **51,** 311–319 (1976).
(17) W. Madani, S. Kermasha, and B. Bisakowaki, Inhibition of tyrosinase activity by a polyphenol esterase using selected phenolic substrates, *Phytochemistry,* **52,** 1001–1008 (1999).
(18) I. Kubo, I. Kinst-Hori, Y. Kubo, Y. Yamagiwa, T. Kamikawa, and H. Haraguchi, Molecular design of antibrowning agents, *J. Agr. Food Chem.,* **48,** 1393–1399 (2000).
19) I. Kubo, Q. X. Chen, and K. Nihei, Molecular design of antibrowning agents: Antioxidative tyrosinase inhibitors, *Food Chem.,* **81,** 241–247 (2003).