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Tyrosinase Inhibitor from the Flowers of *Impatiens balsamina*

LIM, YOUNG-HEE[1]*, IN-HWAN KIM[2], JUNG-JU SEO[3], AND JEONG-KEUN KIM[4]

[1]*Department of Clinical Laboratory Science and* [2]*Department of Food and Nutrition, College of Health Sciences, Korea University, Seoul 136-703, Korea*
[3]*Hazardous Substance Research Team, Korea Basic Science Institute, Seoul 136-701, Korea*
[4]*Department of Chemical Engineering and Biotechnology, Korea Polytechnic University, Kyunggi-do 429-793, Korea*

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Abstract Kaempferol was isolated and identified from the methanol extract of the flowers of *Impatiens balsamina*. Kaempferol showed inhibitory activity against mushroom tyrosinase with an ID_{50} of 0.042 mM. Inhibition kinetics, as determined using a Lineweaver-Burk plot, showed kaempferol to be a competitive inhibitor of mushroom tyrosinase with a K_i value of 0.011 mM. The lag phase of tyrosine hydroxylation catalyzed by mushroom tyrosinase clearly increased on increasing the concentration of kaempferol. In addition to its tyrosinase inhibiting activity, kaempferol strongly inhibited melanin production by *Streptomyces bikiniensis*, in a dose-dependent manner, without inhibiting cell growth. For comparative purposes, the tyrosinase inhibitory activity of kaempferol was also assayed versus quercetin, a positive standard.

Key words: Tyrosinase inhibitor, kaempferol, *Impatiens balsamina*, melanin

Tyrosinase (polyphenol oxidase, EC 1.14.18.1) is a copper-containing oxidase with a ubiquitous distribution, which participates in the biosynthesis of the skin pigment melanin in vertebrates, in the sclerotization of insect cuticles, and in the biosynthesis of phenolic compounds in plants and microbes [13, 23]. Tyrosinase has been purified from a variety of sources, which include mushrooms, the silkworm, and human melanocytes [1, 18, 19, 28]. It is also responsible for the browning reactions of some fruits and vegetables [26, 33], and this undesired browning due to the enzymatic oxidation of phenols by tyrosinase is of great practical concern [7, 25]. Therefore, tyrosinase inhibitors are widely utilized by the food industry, and are becoming more important in medicinal and cosmetic products intended to reduce hyperpigmentation. Various tyrosinase inhibitors isolated from natural and synthetic sources have become important industrially [5, 14, 17, 22, 29]. Moreover, the relationships between inhibitory activities and structures of tyrosinase inhibitors have also been investigated [11, 12, 27]. However, primarily because of safety concerns, relatively few are used in cosmetic products.

The colored flowers of *Impatiens balsamina* contain flavonoid pigments, flavonols, phenolic compounds, and lawsone, which are known to possess antifungal, anticancer, and antioxidant activities [19, 30, 32]. In Korea, *Impatiens balsamina* L. has been used to treat scrofulosis, carbuncles, and dysentery, and juices obtained from its flowers and leaves have been used to dye nails [10]. In addition, it continues to be used in traditional oriental medicine as an antimicrobial [8]. Moreover, 2-methoxy-1,4-naphthoquinone isolated from the stems and flowers of *Impatiens balsamina* has been found to possess strong antifungal activity against *Candida albicans*, *Aspergillus fumigatus*, and *Trichophyton mentagrophytes*, and antibacterial activity against *Bacillus subtilis* and *Salmonella typhimurium* [10, 34].

In this paper, we report on the tyrosinase inhibitor kaempferol, which was isolated from the flowers of *Impatiens balsamina*, and in particular, our study focused on a kinetic study of the inhibition of monophenolase and diphenolase activities of mushroom tyrosinase by kaempferol (a monophenol inhibitor) and quercetin (a diphenol inhibitor) with L-tyrosine (a monohydroxyphenol) and L-DOPA (a dihydroxyphenol) as substrates of tyrosinase. In addition, kaempferol was studied for its inhibitory activity on melanin production in *Streptomyces bikiniensis*, which we viewed as a melanin-producing indicator organism.

*Corresponding author
Phone: 82-2-940-2815; Fax: 82-2-917-2388;
E-mail: yhlim@korea.ac.kr

MATERIALS AND METHODS

Bacterial Strain

Streptomyces bikiniensis NRRL B-1049 was purchased from Korean Collection for Type Culture (Daejon, Korea).

Plant Material

The flowers of *Impatiens balsamina* were collected in Koyang-si, Kyunggi-do, Korea, during July and August 2003. A voucher specimen has been deposited at the Department of Clinical Laboratory Science, College of Health Sciences, Korea University, Korea.

Extraction and Isolation

The flowers of *Impatiens balsamina* (1.2 kg) were extracted using methanol (MeOH) at 60°C. The extract was filtered and concentrated in a vacuum, and the resulting concentrate (35 g) was successively partitioned between n-hexane, $CHCl_3$, ethyl acetate (EtOAc), and H_2O. The active fraction (2.4 g) partitioned into the EtOAc extract was subjected to silica gel column chromatography using different solvents ($CHCl_3$-MeOH) to produce 6 fractions. The active fraction ($CHCl_3$-MeOH, 9:1) (0.7 g) was fractionated by silica gel column chromatography using different solvents ($CHCl_3$-Acetone) to give 6 fractions. The active fraction ($CHCl_3$-Acetone, 9:1) (37 mg) was subjected to TLC using a benzene-MeOH-acetic acid (90:16:8) mobile phase, and the active fraction (26 mg) was chromatographed on TLC (RP-18) using acetonitrile-H_2O (75:25). The final active compound (6.2 mg) obtained was purified by HPLC (C18 column) by eluting with a gradient of 5% to 95% acetonitrile containing 0.1% formic acid.

Analysis of the Isolated Compound

Direct Electrospray Ionization (ESI). The sample was analyzed using an LCQ Deca ion trap mass spectrometer (Thermofinigan, San Jose, CA, U.S.A.) in the negative-ion mode, at an Ispray voltage of 3.5 kV.

Electron Impact-Mass Spectrometry (EI-MS). The EI experiment was performed on a SX-102 double focusing magnetic sector mass spectrometer (JEOL, Japan), operated in positive-ion mode, at an acceleration voltage of 10 kV, an electron energy of 70 eV, and a source temperature of 230°C. TLC was carried out on precoated silica gel 60 F254 and RP-18 plates (Merck), and spots were visualized by spraying with 50% H_2SO_4. HPLC was performed using a Jasco unit equipped with a PU-1580 pump, a CO-1560 column thermostat, a UV-2075 plus detector, and a LG-1580-04 Quaternary gradient unit. A prepacked column (Luna 3 C18, 150×4.6 mm, Phenomenex) was used and eluted with a gradient mix (solvent A: 5% acetonitrile containing 0.1% formic acid; solvent B: 95% acetonitrile containing 0.1% formic acid) at a flow rate of 0.3 ml/min. Eluate was monitored at 254 nm.

3,4',5,7-Tetrahydroxy flavone (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one). Yellow powder; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.14 (br d, 2H, J=9 Hz), 7.05 (br d, 2H, J=8.5 Hz), 6.53 (d, 1H J=1.5 Hz), 6.26 (d, 1H, J=2 Hz); ^{13}C NMR (500 MHz, DMSO-d_6) δ 175.89, 163.89, 160.69, 159.18, 156.16, 146.81, 135.64, 129.49, 122.21, 121.65, 115.42 (2 C), 103.03, 98.18, 93.46.

MS (ESI, negative) m/z 285[M-H], MS (EI) m/z 286 (M+, 100), 258 (26), 121 (48); high-resolution electron ionization mass spectral (HREIMS) calculated for $C_{15}H_{10}O_6$: 286.0477. Found: 286.0475

Tyrosinase Inhibitory Activity Assay

Mushroom tyrosinase and L-tyrosine were purchased from Sigma (St. Louis, MO, U.S.A.). Assays were performed as previously described with slight modifications [16]. Briefly, reaction mixtures, consisting of 150 μl of 0.1 M phosphate buffer (pH 6.8), 10 μl of mushroom tyrosinase (2,000 U/ml), 10 μl of a sample in DMSO, and 100 μl of distilled water, were mixed in a 96-well microplate and pre-incubated at 25°C for 10 min, and then 30 μl of 2 mM L-tyrosine was added. Optical densities (OD) were measured at 475 nm after incubation at 25°C for 30 min using a Spectra max 340pc microplate reader (Molecular Devices). Tyrosinase inhibition percent was calculated as follows: % inhibition=$\{[(A-B)-(C-D)]/(A-B)\}\times100$ (A=OD without the test substance but with tyrosinase; B=OD without the test substance and without tyrosinase; C=OD with the test substance and with tyrosinase; D=OD with the test substance but without tyrosinase; all ODs were determined at 475 nm).

Determination of the Inhibition of Melanin Production by *Streptomyces bikiniensis*

A preserved culture of *S. bikiniensis* NRRL B-1049 was inoculated onto a Papavizas' VDYA agar slant containing V-8 juice 200 ml (Campbell Soup Co.), glucose 2 g, yeast extract 2 g (Difco), $CaCO_3$ 1 g, agar 20 g, and distilled water 800 ml (pH 7.2). After incubating at 28°C for 2 weeks, 2 ml of sterile water was added onto the slant culture and the spore mass formed on the aerial mycelium was removed with an inoculating loop. Spore suspensions of *S. bikiniensis* in sterilized saline were

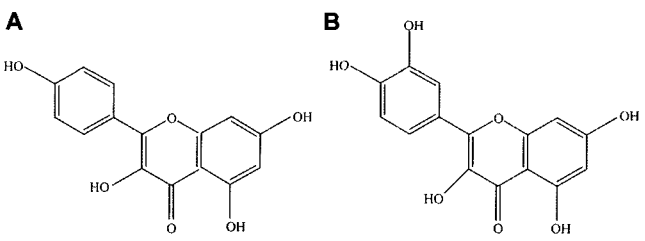

Fig. 1. Chemical structures of kaempferol (**A**) and quercetin (**B**).

Table 1. ID_{50} and K_i values for the monophenolase and diphenolase activities of mushroom tyrosinase in the presence of kaempferol and quercetin.

Substrate	Inhibitory agent	ID_{50} (mM)	K_i (mM)	Mode of inhibition
L-Tyrosine	Kaempferol	0.042±0.002	0.011±0.002	Competitive
	Quercetin	0.031±0.006	0.008±0.001	Competitive
L-DOPA	Kaempferol	0.250±0.02	0.182±0.05	Competitive
	Quercetin	0.139±0.04	0.101±0.02	Competitive

then inoculated onto agar medium ISP No. 7 supplemented with Bacto-yeast extract (0.2%) and uniformly spread over the agar surface. After drying the agar surfaces, paper discs (8 mm diameter), soaked with a sample solution, were placed on the agar plates, which were then incubated at 28°C for 48 h. The resulting melanin formation inhibitory zones were measured from the reverse sides of the plates [31].

RESULTS AND DISCUSSION

Kaempferol inhibits mushroom tyrosinase and its catalytic characteristics have been documented [16, 17]. In the present study, we found that the methanol extract of the flowers of *Impatiens balsamina* suppressed melanin biosynthesis in *Streptomyces bikiniensis*. In addition, we isolated kaempferol as a yellow powder from the methanol

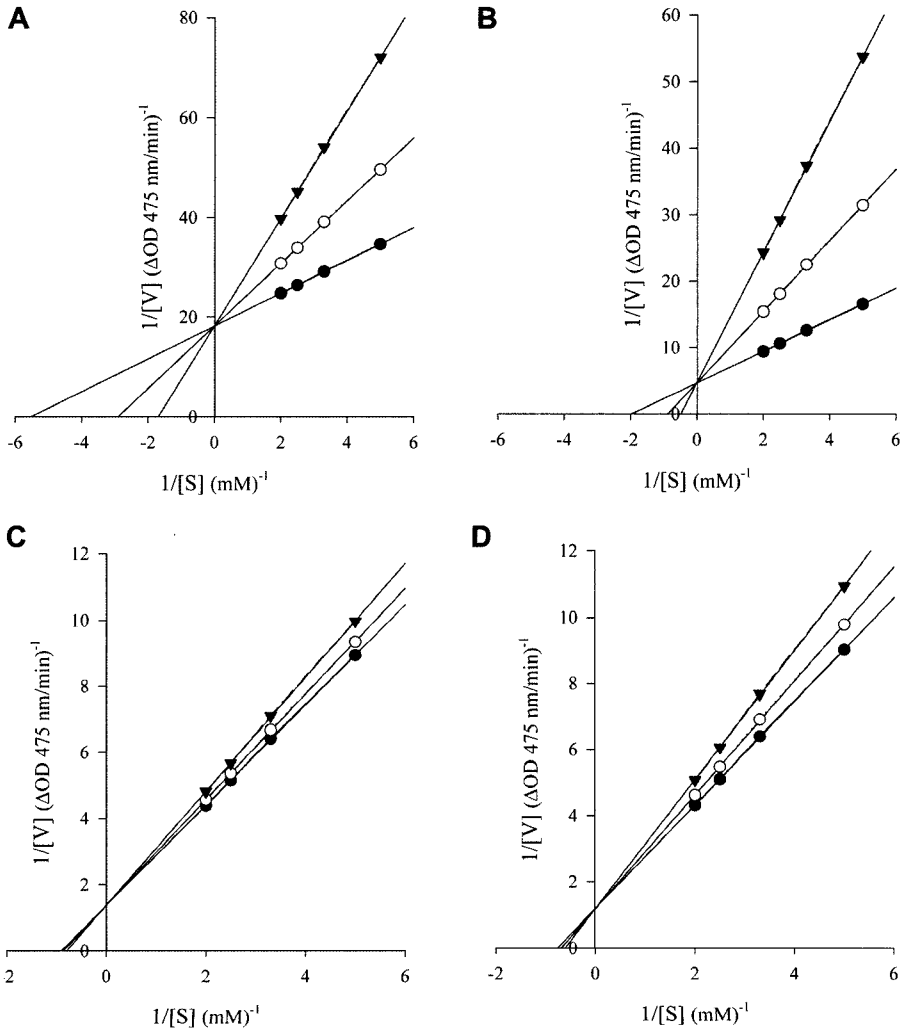

Fig. 2. Lineweaver-Burk plots of mushroom tyrosinase and L-tyrosine with kaempferol (**A**) and quercetin (**B**), and L-DOPA with kaempferol (**C**) and quercetin (**D**).
The symbols represent without (●) and with [(○) 0.01 mM and (▼) 0.025 mM] kaempferol or quercetin.

extract of the flowers of *Impatiens balsamina*, and established its molecular formula, $C_{15}H_{10}O_6$, by HREIMS. Kaempferol is relatively insoluble in water, and therefore, it was dissolved in dimethylsulfoxide (DMSO) and its antityrosinase activity evaluated and compared with that of the known tyrosinase inhibitor, quercetin (Fig. 1). The inhibitory dose (ID_{50}) required to reduce tyrosinase activity to 50% was determined by adding kaempferol or quercetin to an assay system containing L-tyrosine or L-DOPA, and their respective ID_{50} values were determined to be 0.042 and 0.031 mM, respectively, in the presence of L-tyrosine, and 0.250 and 0.139 mM when L-DOPA was used as tyrosinase substrate (Table 1). A previous study obtained a similar result when kaempferol and L-DOPA were used as an inhibitor and a substrate for tyrosinase, although the ID_{50} value (0.139 mM) that we obtained using an assay system containing quercetin and L-DOPA was twice as high as the previous result [16], which may have been due to the different assay systems used. In the present study, we used inhibitors dissolved in DMSO to determine ID_{50} values, whereas inhibitors were dissolved in water in the previous study. We adjusted the final concentration of DMSO to below 5%, which is unlikely to affect enzyme activity. In addition, we conducted a kinetic study of L-tyrosine hydroxylation catalyzed by mushroom tyrosinase in the presence of kaempferol and quercetin, and found that they were competitive inhibitors of mushroom tyrosinase with K_i values of 0.011 and 0.008 mM against L-tyrosine and 0.182 and 0.101 mM against L-DOPA, respectively (Fig. 2).

The effects of different concentrations of kaempferol on the oxidation of L-tyrosine by tyrosinase were studied at various L-tyrosine concentrations (Fig. 3). Our results showed that kaempferol inhibited enzyme activity in a dose-dependent manner, and that the inhibition rate was reduced on increasing the concentration of L-tyrosine. Tyrosinase catalyzes two different reactions, the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) [9, 15, 26]. Monophenolase activity shows a characteristic lag period before the hydroxylation step

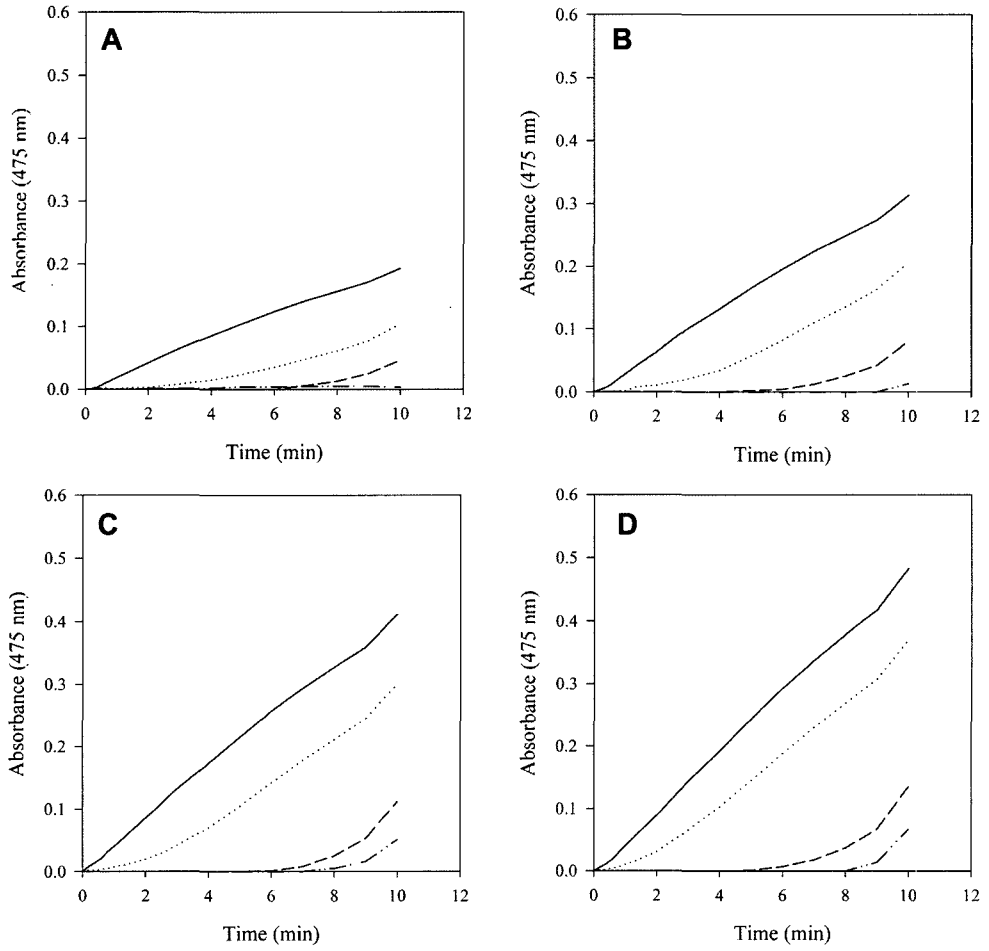

Fig. 3. Effect of the presence of kaempferol on the lag period of L-tyrosine hydroxylation by mushroom tyrosinase.
The concentrations of L-tyrosine corresponding to (**A**), (**B**), (**C**), and (**D**) were 0.1, 0.2, 0.3, and 0.4 mM, respectively. The concentrations of kaempferol were 0 (——), 0.01 (·····), 0.025 (– – –), and 0.05 (–·–·–) mM.

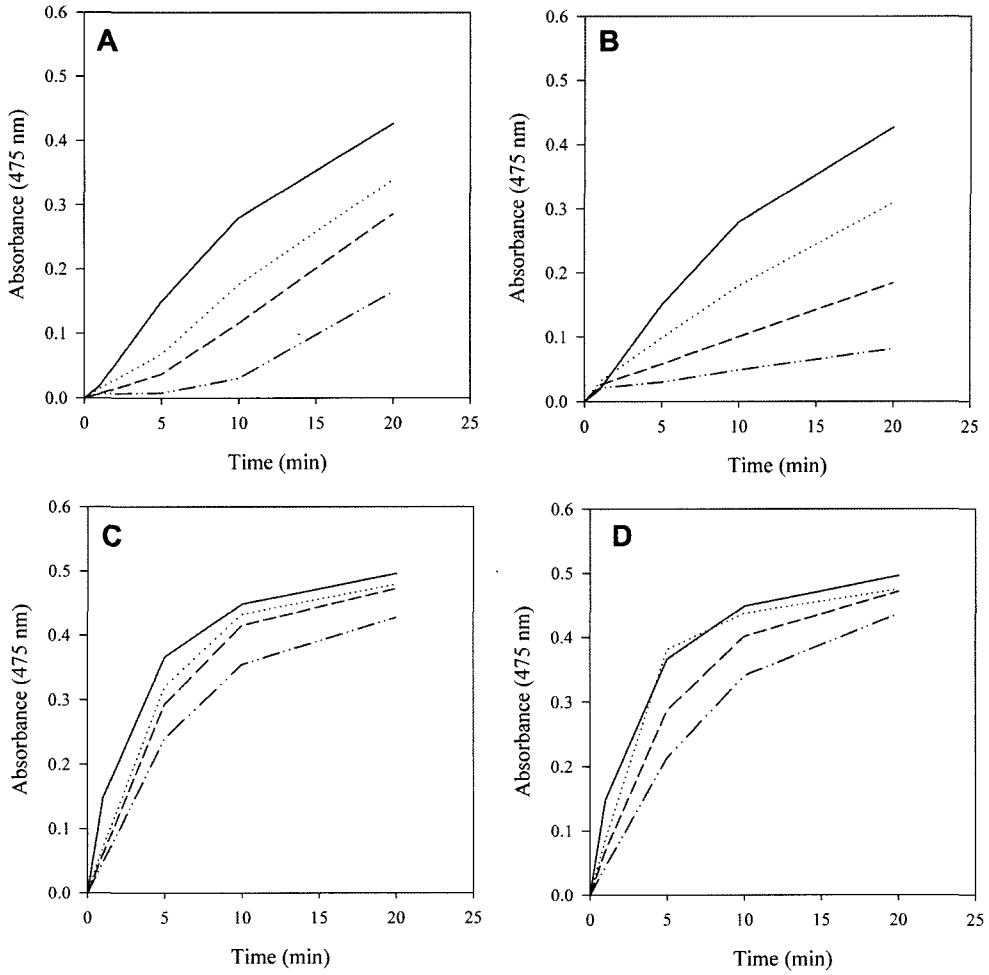

Fig. 4. Inhibitory effects of kaempferol and quercetin on tyrosinase activity in the presence of 0.2 mM L-tyrosine (**A** and **B**) or 0.2 mM L-DOPA (**C** and **D**).
The concentrations of kaempferol (**A** and **C**) and quercetin (**B** and **D**) were 0 (——), 0.01 (·····), 0.025 (— —), and 0.05 (—·—) mM, respectively.

commences [2]. On the other hand, the absence of a lag time demonstrates diphenolase activity. Moreover, the lag period on monophenolase activity is absent in the presence of diphenols [20]. In our study, the lag phase was found to be reduced in the presence of a quercetin, a diphenol, and an inhibitor of tyrosinase (Fig. 4B). The lag phase clearly increased 20, 13, 14, and 12 times on increasing the concentration of kaempferol [0 to 0.05 mM] at a constant L-tyrosine concentration [0.1, 0.2, 0.3, and 0.4 mM, respectively], but decreased approximately 0.4 and 0.6 times on increasing the L-tyrosine concentration [0.1 to 0.4 mM] in the presence of kaempferol [0.01 and 0.05 mM, respectively] (Table 2). These results demonstrate that the inhibitory activity of kaempferol is suppressed in systems containing high concentrations of L-tyrosine, which concurs with the fact that kaempferol is a competitive inhibitor of mushroom tyrosinase. In a previous study, quercetin was found to completely suppress the lag time of L-tyrosine [17]. Our result also showed a similar suppressive effect by quercetin (Fig. 4B). In the present study, kaempferol and quercetin were found to have similar inhibitory effects on the diphenolase activity of mushroom tyrosinase when L-DOPA was used as a substrate (Figs. 4C and 4D). Moreover, kaempferol and quercetin were shown to exhibit a concentration-dependent inhibitory effect on the oxidation of L-DOPA. In addition, the inhibitory effects of

Table 2. Lag times of mushroom tyrosinase at different concentrations of L-tyrosine and kaempferol.

L-Tyrosine (mM)	Lag time (min)			
	Kaempferol (mM)			
	0	0.01	0.025	0.05
0.1	0.7±0.2	3.1±0.3	6.9±1.1	14.0±0.1
0.2	0.7±0.2	2.6±0.2	6.7±1.2	9.3±0.8
0.3	0.6±0.2	1.4±0.2	6.9±1.1	8.3±1.2
0.4	0.7±0.2	1.1±0.1	6.6±1.0	8.3±1.1

the diphenol, quercetin, on the activity of tyrosinase versus the diphenol substrate, L-DOPA, were greater than those of the monophenol inhibitor, kaempferol (Figs. 4C and 4D). However, the inhibitory effects of kaempferol and quercetin on the oxidation of L-DOPA were less than their inhibitory effects on the hydroxylation of L-tyrosine, which supports the observations shown in Table 1. Our results show that the monophenolase activity of mushroom tyrosinase is profoundly more influenced than the diphenolase activity of the enzyme by both inhibitors. Tyrosinase purified from frog epidermis was found to have a tyrosinase activity lag period that decreased on increasing the concentration of caffeic acid in the reaction mixture [4]. In the melanin biosynthesis pathway from tyrosine, the hydroxylation of tyrosine to L-DOPA is considered to be the rate-determining step [2]. In a previous report, the lag period reduced on increasing the mushroom tyrosinase concentration [24]. Moreover, the lag period was found to depend on the generation of DOPA when tyrosinase acted on a tyrosine substrate [6]. In another study, a low level of DOPA quinone-H^+ was found to be responsible for the lag period, which lasted until the steady-state DOPA quinone-H^+ concentration had been reached [3, 21]. The time required to reach this concentration is determined by the total tyrosinase concentration and by the substrate concentration. Thus, the lag period would increase on reducing the concentration of the enzyme capable of binding with the substrate. Kaempferol acted as a competitive inhibitor of mushroom tyrosinase, thus the amount of enzyme available for hydroxylation diminished as the kaempferol concentration was increased, which explains the lag period increase on increasing the concentration of kaempferol.

A previous report found that kaempferol does not extend the lag period when L-tyrosine is used as a substrate, whereas a flavonol, galangin, significantly lengthened the lag time [31]. However, we found that the lag time was extended when kaempferol was present. Kaempferol acts as a competitive inhibitor, as exhibited in Table 1, thus the amount of enzyme bound to L-tyrosine decreased in the presence of kaempferol, which resulted in a longer lag period. Kubo *et al.* concluded that kaempferol does not activate monophenolase activity but rather inhibits *o*-diphenolase activity, and that quercetin activates monophenolase activity but inhibits *o*-diphenolase activity [16]. The results obtained in the present study support this conclusion. Furthermore, the inhibition of tyrosinase activity increased more rapidly as the concentration of inhibitors increased at a constant substrate concentration. On the other hand, the inhibitors showed less influence on enzyme inhibition as the concentration of substrate was increased. Kubo *et al.* also reported that kaempferol does not extend the lag time when L-tyrosine is used as a substrate [16]. However, we found that kaempferol significantly increased the lag time under this condition, which suggests that kaempferol inhibits the monophenolase activity of mushroom tyrosinase.

Table 3. Inhibitory activity of kaempferol on melanin formation in *Streptomyces bikiniensis* NRRL B-1049.

Amount used (μg)[a]	Inhibition zone (mm)
0[b]	0
1.14	20±1.0
2.28	35±1.2

[a]Dissolved in DMSO.
[b]DMSO only.

In systems as diverse as fungi and vertebrates, tyrosinase catalyzes the initial step in the formation of the pigment melanin from tyrosine. We examined the inhibitory effect of kaempferol on melanin production in *S. bikiniensis*, and found that it clearly showed inhibited melanin production in a concentration-dependent manner, without inhibiting cell growth (Table 3), which does not imply that kaempferol inhibits the production of tyrosinase in *S. bikiniensis*. Our kinetic study results lead us to believe that kaempferol is possibly partly responsible for melanin production inhibition in *S. bikiniensis*, and that it achieves this by inhibiting the reaction between tyrosinase and L-tyrosine.

Recently, we found that both 2-methoxy-1,4-naphthoquinone and kaempferol isolated from *Impatiens balsamina* inhibit the growth of *Propionibacterium acnes*, which is the major causative agent of acne (personal communication, data not shown). The ethanol extract of *Impatiens balsamina* has been approved as a food additive (used as an antioxidant) in Korea and Japan, thus underwriting its safety. In addition to the anti-acne and melanin biosynthesis inhibitory activities of extracts of *Impatiens balsamina*, its extracts are also relatively safe. Thus, kaempferol has a huge advantage regarding its use in the cosmetic and pharmaceutical industries, and for the treatment of some dermatological disorders associated with melanin hyperpigmentation and acne.

REFERENCES

1. Bouchilloux, S., P. McMahill, and H. S. Mason. 1963. The multiple form of mushroom tyrosinase. Purification and molecular properties of the enzymes. *J. Biol. Chem.* **238:** 1699–1707.
2. Cabanes, J., F. G. Cánovas, J. A. Lozano, and F. G. Carmona. 1987. A kinetic study of the melanization pathway between L-tyrosine and dopachrome. *Biochim. Biophys. Acta* **923:** 187–195.
3. Cánovas, F. G., F. G. Carmona, J. V. Sánchez, J. L. I. Pastor, and J. A. L. Teruel. 1982. The role of pH in the melanin biosynthesis pathway. *J. Biol. Chem.* **257:** 8738–8744.
4. Carmona, F. G., E. Pedreño, J. D. Galindo, and F. G. Cánovas. 1979. A new spectrophotometric method for the

determination of cresolase activity of epidermis tyrosinase. *Anal. Biochem.* **95:** 433–435.
5. Chen, J. S., C. I. Wei, and M. R. Marshall. 1991. Inhibition mechanism of kojic acid on polyphenol oxidase. *J. Agric. Food Chem.* **39:** 1897–1901.
6. Cooksey, C. J., P. J. Garratt, E. J. Land, S. Pavel, C. A. Ramsden, P. A. Riley, and N. P. M. Smit. 1997. Evidence of the indirect formation of the catecholic intermediate substrate responsible for the autoactivation kinetics of tyrosinase. *J. Biol. Chem.* **272:** 26226–26235.
7. Friedman, M. 1996. Food browning and its prevention: An overview. *J. Agric. Food Chem.* **44:** 631–653.
8. Fukumoto, H., K. Ishiguro, T. Murashima, and M. Isoi. 1994. Structure determination of a kaempferol 3-rhamnosyldiglucoside from *Impatiens balsamina*. *Phytochemistry* **37:** 1486–1488.
9. Hearing, V. J. and M. Jiménez. 1987. Mammalian tyrosinase - The critical regulatory control point in melanocyte pigmentation. *Int. J. Biochem.* **19:** 1141–1147.
10. Kang, S. C. and Y. Moon. 1992. Isolation and antimicrobial activity of a naphthoquinone from *Impatiens balsamina*. *Saengyak Hakhoechi* **23:** 240–247.
11. Khan, M. T. H., M. I. Choudhary, K. M. Khan, M. Rani, and A. Rahman. 2005. Structure-activity relationships of tyrosinase inhibitory combinatorial library of 2,5-disubstituted-1,3,4-oxadiazole analogues. *Bioorg. Med. Chem.* **13:** 3385–3395.
12. Khan, M. T. H., S. B. Khan, and A. Ather. 2006. Tyrosinase inhibitory cycloartane type triterpenoids from the methanol extract of the whole plant of *Amberboa ramosa* Jafri and their structure-activity relationship. *Bioorg. Med. Chem.* **14:** 938–943.
13. Kim, J.-H., J. J. Song, B.-G. Kim, M.-H. Sung, and S. C. Lee. 2004. Enhanced stability of tyrosine phenol-lyase from *Symbiobacterium toebii* by DNA shuffling. *J. Microbiol. Biotechnol.* **14:** 153–157.
14. 14. Kim, W.-G., I.-J. Ryoo, S.-H. Park, D.-S. Kim, S. Lee, K.-C. Park, and I.-D. Yoo. 2005. Terrein, a melanin biosynthesis inhibitor, from *Penicillium* sp. 20135. *J. Microbiol. Biotechnol.* **15:** 891–894.
15. Körner, A. M. and J. Pawelek. 1982. Mammalian tyrosinase catalyzes three reactions in the biosynthesis of melanin. *Science* **217:** 1163–1165.
16. Kubo, I. and I. K. Hori. 1999. Flavonols from Saffron flower: Tyrosinase inhibitory activity and inhibition mechanism. *J. Agric. Food Chem.* **47:** 4121–4125.
17. Kubo, I., I. K. Hori, S. K. Chaudhuri, Y. Kubo, Y. Sánchez, and T. Ogura. 2000. Flavonols from *Heterotheca inuloides*: Tyrosinase inhibitory activity and structural criteria. *Bioorg. Med. Chem.* **8:** 1749–1755.
18. Kupper, U., D. M. Niedermann, G. Travaglini, and K. Lerch. 1989. Isolation and characterization of the tyrosinase gene from *Neurospora crassa*. *J. Biol. Chem.* **264:** 17250–17258.
19. Kwon, B. S., A. K. Haq, S. H. Pomerantz, and R. Halaban. 1987. Isolation and sequence of a cDNA clone for human tyrosinase that maps at the mouse c-albino locus. *Proc. Natl. Acad. Sci. USA* **84:** 7473–7477.
20. Lerch, K. and L. Ettlinger. 1972. Purification and characterization of a tyrosinase from *Streptomyces glaucescens*. *Eur. J. Biochem.* **31:** 427–437.
21. López, J. N. R., M. B. Arnao, F. M. Ortiz, J. Tudela, M. Acosta, R. Varón, and F. G. Cánovas. 1992. Catalytic oxidation of 2,4,5-trihydroxyphenylalanine by tyrosinase: Identification and evolution of intermediates. *Biochim. Biophys. Acta* **1160:** 221–228.
22. Maeda, K. and M. Fukuda. 1991. *In vitro* effectiveness of several whitening cosmetic components in human melanocytes. *J. Soc. Cosmet. Chem.* **42:** 361–368.
23. Malmstrom, B., L. Andreasson, and B. Reinhammar. 1975. Copper-containing oxidases and superoxide dismutase, pp 507–579. *In* Boyer, P. (ed.), *The Enzymes*, 3rd Ed. Vol. 12. Academic Press, New York.
24. Martínez, J. R. R., J. N. R. López, R. V. Castellanos, and F. G. Cánovas. 1993. Discrimination between two kinetic mechanisms for the monophenolase activity of tyrosinase. *Biochem. J.* **294:** 621–623.
25. Martinez, M. V. and J. R. Whitaker. 1995. The biochemistry and control of enzymatic browning. *Trends Food Sci. Technol.* **6:** 195–200.
26. Mayer, A. M. 1987. Polyphenol oxidases in plants: Recent progress. *Phytochemistry* **26:** 11–20.
27. Nihei, K., Y. Yamagiwa, T. Kamikawa, and I. Kubo. 2004. 2-Hydroxy-4-isopropylbenzaldehyde, a potent partial tyrosinase inhibitor. *Bioorg. Med. Chem. Lett.* **14:** 681–683.
28. Nishioka, K. 1978. Particulate tyrosinase of human malignant melanoma. Solubilization, purification following trypsin treatment and characterization. *Eur. J. Biochem.* **85:** 137–146.
29. No, J. K., D. Y. Soung, Y. J. Kim, K. H. Shim, Y. S. Jun, and S. H. Rhee. 1999. Inhibition of tyrosinase by green tea components. *Life Sci.* **65:** 241–246.
30. Park, K. J. and H. H. Lee. 2005. *In Vitro* antiviral activity of aqueous extracts from korean medicinal plants against influenza virus type A. *J. Microbiol. Biotechnol.* **15:** 924–929.
31. Tomita, K., N. Oda, M. Ohbayashi, and H. Kamei. 1990. A new screening method for melanin biosynthesis inhibitors using *Streptomyces bikiniensis*. *J. Antibiotics* **43:** 1601–1605.
32. Wang, X.-L., K.-T. Kim, J.-H. Lee, H.-G. Hur, and S.-I. Kim. 2004. C-Ring cleavage of isoflavones daidzein and genistein by a newly-isolated human intestinal bacterium *Eubacterium ramulus* Julong 601. *J. Microbiol. Biotechnol.* **14:** 766–771.
33. Whittaker, J. R. 1995. Phenol oxidase, pp. 271–307. *In* Wong, D. (ed.), *Food Enzymes, Structure and Mechanism*. Chapman and Hall, New York.
34. Yang, X., D. K. Summerhurst, S. F. Koval, C. Ficker, M. L. Smith, and M. A. Bernards. 2001. Isolation of an antimicrobial compound from *Impatiens balsamina* L. using bioassay-guided fractionation. *Phytother. Res.* **15:** 676–680.