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Isolation of 1',3'-Dilinolenoyl-2'-Linoleoylglycerol with Tyrosinase Inhibitory Activity from *Flammulina velutipes*

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This study was carried out to evaluate the inhibitory effect of *Flammulina velutipes* extracts on tyrosinase activity and to identify its biologically active component. The ethyl acetate and *n*-butanol extracts showed potent tyrosinase inhibitory activities. Subsequently, fractions of the *n*-butanol extract showed only a partial tyrosinase inhibitory activity. The most active compound of tyrosinase inhibitory activity was identified from the ethyl acetate extract as 1',3'-dilinolenoyl-2'-linoleoylglycerol (LnLLn) by comparing its mass, ^{1}H-, and ^{13}C-NMR spectral data with those previously reported in the literature. LnLLn showed tyrosinase inhibitory activity with an IC_{50} value of 16.1 μg/ml. These results suggest that the ethyl acetate extract of *F. velutipes* could be applicable for the development of a new whitening agent.

Keywords: 1',3'-Dilinolenoyl-2'-linoleoylglycerol (LnLLn), *Flammulina velutipes*, tyrosinase inhibitory activity, whitening effect

Flammulina velutipes is a basidomycete fungus and one of the most popular edible mushrooms in Asia. The fruiting body of *F. velutipes* contains a variety of nutrients such as essential amino acids, dietary fibers, vitamins, and minerals as general components of the mushroom [2]. There have also been several reports of physiologically active materials originating from the mushroom *F. velutipes*. These active components include proteins, polysaccharides, and glycoproteins, related to antitumor effects and immunoregulation [11–14] as well as sesquiterpenes of curarine-type with antibacterial activity [7]. Monoterpentriols with growth-promoting activity [6] were also separated from *F. velutipes* fruit bodies and mycelium. Furthermore, many studies of *F. velutipes* have demonstrated biological activities like cholesterol-lowering, antioxidant, and tyrosinase inhibitory activities for skin-whitening effect [4, 8, 16].

Tyrosinase is responsible for not only melanization in animals but also browning in fruits and vegetables. Furthermore, it has been reported that tyrosinase is associated with neurodegenerative diseases [15]. Therefore, the development of tyrosinase inhibitors has been the subject of continuing interest in the food, pharmaceutical, and cosmetic industries. In contrast to many studies on the proteins and polysaccharides associated with its biological activities, there are no reports on a tyrosinase inhibitor associated with the skin-whitening effects of *F. velutipes*.

The present study was therefore undertaken to search for a tyrosinase inhibitor from *F. velutipes*. From extracts showing a maximal tyrosinase inhibitory activity, the most active component was separated through refinement of fragments by using solvent polarity and column chromatography. We report herein the finding that *F. velutipes* fruiting bodies exert tyrosinase inhibitory activity by producing a triacylglycerol characterized structurally as 1',3'-dilinolenoyl-2'-linoleoylglycerol (LnLLn).

Materials and Methods

Materials

Fresh fruiting bodies of *F. velutipes*, obtained from the Rural Development Administration (RDA, Suwon, Korea), were shade-dried and fine-cut. Mushroom tyrosinase and all other reagents were purchased from Sigma.

Instruments and Reagents

Silica gel 60 (Merck, 70–230 μm) was used for the purification of a tyrosinase inhibitor from the fruiting bodies of *F. velutipes*. Fourier transform-nuclear magnetic resonance (FT-NMR) spectra were obtained with Varian Mercury 300 MHz for ^{13}C- and ^{1}H- NMR, with tetramethylsilane (TMS) as the internal reference; the chemical

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shifts (δ) are reported in parts per million. Infrared (IR) spectra were obtained on a Nicolet Impact 420 FT-IR spectrophotometer, and gas chromatography-mass spectrometry (GC-MS) spectra were obtained on a GC-MS system combining the GC model of Varian CP-3800 and Saturn 2000 Mass Selective Detector. Ultraviolet (UV) spectra were recorded using a Milton-Roy spectronic Genesys-5 UV. For thin-layer chromatography (TLC), Kiesel gel $60F_{254}$ (Art. 5715, Merck) was used.

Extraction and Isolation of a Tyrosinase Inhibitor from the Fruiting Bodies of *F. velutipes*.

The shade-dried *F. velutipes* fruit bodies (397 g) were thrice refluxed with methanol (3,000 ml) for 5 h to give a methanol extract (161 g). The extract was suspended in distilled water, and re-extracted with *n*-hexane, ethyl acetate, and *n*-butanol. Each solute fraction was concentrated under vacuum to give *n*-hexane (1.4 g), ethyl acetate (6.8 g), *n*-butanol (16.7 g), and water (128.0 g) fractions. The ethyl acetate fraction (5 g) showing the highest tyrosinase inhibitory activity was fractionated by silica gel 60 column chromatography with *n*-hexane/acetone (10:1) to give four fractions; E-1 (1.69 g), E-2 (1.05 g), E-3 (0.13 g), and E-4 (1.57 g). Fraction E-1 showed the highest tyrosinase inhibitory activity. The tyrosinase inhibitory compounds contained in the E-1 fraction were subject to silica gel 60 column chromatography and gradually developed with an *n*-hexane/ethyl acetate solvent system at the given ratio (hexane/ethyl acetate=20:1→10:1) to gain the compound I (95.1 mg).

Compound I - Light yellow liquid; UV λmax: 233, 270 (*n*-hexane); IR: liquid film 2,927, 1,746 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, t, *J*=7.2 Hz, L-CH_3), 0.97 (6H, t, *J*=7.5 Hz, Ln-CH_3), 1.24–1.29 (30H, m, chain CH_2), 1.58–1.59 (6H, m, -CH_2CH_2CO-), 2.02–2.09 (12H, m, allylic CH_2), 2.27–2.32 (6H, m, -CH_2CO-), 2.73–2.81 (10H, m, diallylic CH_2), 4.13 (2H, dd, *J*=11.7, 6 Hz, glycerol H-3'), 4.28 (2H, dd, *J*=12, 4.2 Hz, glycerol H-1'), 5.25–5.40 (17H, m, glycerol H-1'+olefinic H); ^{13}C-NMR($CDCl_3$) δ: 173.4 (Ln-C-1), 173.0 (L-C-1), 132.1 (Ln-C-16), 130.4 (Ln-C-9, L-C-13), 130.2 (L-C-9), 128.4 (Ln-C-13), 128.4 (Ln-C-12), 128.3 (L-C-10), 128.1 (L-C-12), 128.0 (Ln-C-10), 127.3 (Ln-C-15), 69.1 (C-2'), 62.3 (C-1', 3'), 34.4 (L-C-2), 34.2 (Ln-C-2), 31.7 (L-C-16), 29.8, 29.6, 29.4, 29.3 (Ln-C-7, 6, 5, 4, L-C-7, 15, 6, 5, 4), 27.4 (Ln-C-8, 17, L-C-8, 14), 25.8 (Ln-C-11, 14), 25.7 (L-C-11), 25.0 (L-C-3), 22.8 (L-C-17), 20.7 (Ln-C-3), 14.5 (Ln-C-1), 14.3 (L-C-1).

Preparation and Analysis of Fatty Acid Methyl Esters

To identify compositions of fatty acids bonded with compound I by using GC-MS, the methyl ester derivative of fatty acid was prepared according to the method described by Murrieta *et al.* [10]. Fatty acid methyl esters were separated using a GC-MS system. The GC conditions were as follows; column: VF-5 ms (Varian, 30 m×0.32 mm, 0.25 μm); oven temperature program: the column was held initially at 100°C for 5 min after injection, then increased to 280°C with 10°C/min heating ramp for 5 min; injector temperature: 250°C; carrier gas: Helium; column flow: 1.0 ml/min; injection volume: 2.0 μl. MS conditions were regulated as follows; ionization energy: 70 eV; ion source temperature: 280°C; interface temperature: 250°C; mass range: 35–450 atomic mass units.

Identification of fatty acids on compound I was assigned by comparison of their retention times and mass spectra with corresponding data from reference compounds and by comparison of their mass spectra with Wiley and Nist libraries.

Table 1. Inhibitory effects of the *F. velutipes* fruit bodies extracted with the given solvent on mushroom tyrosinase activity.

Test sample	Inhibitory activity (%)[a]
Ascorbic acid	99.7±0.6
Methanol extract	97.5±1.1
n-Hexane extract	-17.1±6.7
Ethyl acetate extract	90.5±0.8
n-Butanol extract	97.1±2.0
H_2O extract	85.3±1.2

[a]Each value represents the averages±standard deviations based on the results obtained from at least three independent experiments.

Enzymatic Assay of Tyrosinase

Inhibitory activities of mushroom tyrosinase were determined according to the methods described by Chang *et al.* [3] with some modifications. A stock solution of the tested sample was diluted to varying concentrations (0, 12.5, 25, 50, 100, 250, and 500 μg/ml) using dimethyl sulfoxide (DMSO) before assay of tyrosinase inhibitory activity. Each dilution (0.2 ml) was thoroughly mixed with 2.7 ml of a solution of 0.3 mM L-tyrosine dissolved in 200 mM potassium phosphate buffer (pH 6.5). Then, 0.1 ml of mushroom tyrosinase (110 U/ml in phosphate buffer) was added to each mixture to initiate the reaction. The assay mixture was incubated at 37°C for 30 min. The increase in absorbance at 475 nm due to the formation of dopachrome was monitored using a spectrophotometer. The percentage of inhibition of tyrosinase activity was calculated as follows: % inhibition=[1−{(B−C)/(A−D)}]×100, where A is the absorbance at 475 nm with the enzyme, B is the absorbance at 475 nm with the assay mixture, C is the absorbance at 475 nm with the tested sample, and D is the absorbance at 475 nm with sterile distilled water. The concentration of a compound at which 50% of the enzyme activity was inhibited (the IC_{50} value) was obtained by linear curve fitting.

Results and Discussion

Inhibitory Effects of *F. velutipes* Extracts on Tyrosinase Activity

We were interested to identify a compound with tyrosinase inhibitory activity from *F. velutipes*, since it was previously reported that the extracts of *F. velutipes* significantly inhibited mushroom tyrosinase activity [8]. The methanol

Table 2. Percentage of unsaturated fatty acid methyl esters analyzed by GC-MS and their retention times (Rt) from compound I.

Unsaturated fatty acid methyl esters	Rt (min)	Percentage (%)[a]
Linoleic acid	18.55	32.64
Linolenic acid	18.64	66.99
Total unsaturated fatty acid	NA	99.63

[a]The values were obtained from the peak area of the GC-MS chromatogram. Values represent the averages of three replicates and the standard deviations were, in all cases, below 10%. NA, not applicable.

Table 3. Partial ^{13}C-NMR spectra of olefinic carbon of the compound I and reference compound LnLLn [1].

Carbon number	Chemical shift of the compound I (ppm)	Chemical shift of the reference compound (ppm)
1',3'- C-16(Ln)	132.1	130.9
1',3'- C- 9(Ln)	130.4	130.2
2'- C-13(L)	130.4	130.2
2'- C- 9(L)	130.2	129.9
1',3'- C- 13(Ln)	128.4	128.2
1',3'- C- 12(Ln)	128.4	128.2
2'- C-10(L)	128.3	128.1
2'- C-12(L)	128.1	127.9
1',3'- C- 10(Ln)	128.0	127.7
1',3'- C- 15(Ln)	127.3	127.1

extract of *F. velutipes* fruit bodies was prepared and assayed for tyrosinase inhibitory activity, together with ascorbic acid as a positive control. As shown in Table 1, the tyrosinase inhibitory activity of the methanol extract of *F. velutipes* was comparable to that of the positive control. Thus, the methanol extract was fractioned with *n*-hexane, ethyl acetate, *n*-butanol, and water in succession. The active fractions, the ethyl acetate and *n*-butanol layers, were further separated by silica gel column chromatography with the enzymatic assay of tyrosinase as a guide. Finally, an active compound (compound I) was isolated from the ethyl acetate extract. However, fractions of *n*-butanol extract showed only 10% to 30% of tyrosinase inhibitory activity compared with that of the positive control (data not shown), suggesting that these fractions did not contain any compounds exhibiting significant tyrosinase inhibitory activity.

Identification of Tyrosinase Inhibitor from *F. velutipes*

The ^{1}H and ^{13}C chemical shift assignments of the compound I were compared with spectral data reported previously [5, 17]. The absorption band at 1,746 cm^{-1} indicated the carbonyl group. The ^{1}H-NMR spectra showed a methylene group of glycerol (4.13 and 4.28 ppm), methyl group of unsaturated fatty acids (0.87 and 0.97 ppm), and olefinic protons of unsaturated fatty acids (5.25–5.40 ppm). The potent tyrosinase inhibitor, compound I, was found to be a triglyceride composed of unsaturated fatty acids. These unsaturated fatty acids were composed of linoleic and linolenic acid in a molar ratio of 1:2 as shown by the GC-MS analysis

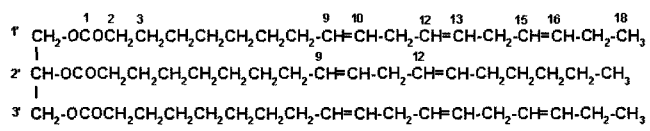

Fig. 1. Structure of the compound I isolated from *F. velutipes* fruit bodies.

Table 4. Inhibitory effect of 1',3'-dilinolenoyl-2'-linoleoylglycerol on mushroom tyrosinase activity.

Test sample	IC_{50} (μg/ml)[a]
Ascorbic acid	34.2±3.7
1',3'-Dilinolenoyl-2'-linoleoylglycerol	16.1±0.5

[a]Each value represents the averages ±standard deviation based on the results obtained from at least three independent experiments.

(Table 2). As shown in Table 3, the ^{13}C-NMR spectra showed the presence of olefinic carbon (132.1–127.3 ppm), which was consistent with the findings of Awl *et al.* [1]. The carbons of glyceryl group (69.1 and 62.3 ppm) were also shown in the ^{13}C-NMR spectra as mentioned in Materials and Methods. From these results, it was concluded that compound I was 1',3'-dilinolenoyl-2'-linoleoylglycerol (LnLLn) (Fig. 1).

As shown in Table 4, we examined the inhibitory effect of LnLLn on mushroom tyrosinase. The IC_{50} value of LnLLn for tyrosinase inhibitory activity was 16.1 μg/ml, which was about 2-fold higher than that of the positive control, suggesting that the compound was sufficient to act as a tyrosinase inhibitor. It was recently shown that triacylglycerols such as trilinolein (1,2,3-trilinoleoylglycerol: LLL) and triolein (1,2,3-trioleoylglycerol: OOO), contained in sake lees, function as a noncompetitive tyrosinase inhibitor, and a higher inhibitory effect of trilinolein compared with triolein correlates with the higher number of fatty acid double bonds [9]. Therefore, it may be concluded that the tyrosinase inhibitory effect of LnLLn isolated from the *F. velutipes* fruit bodies is associated with noncompetitive inhibition.

In conclusion, the results of this study demonstrated for the first time that the *F. velutipes* fruit bodies contain triacylglycerols like LnLLn, which has the potential to be further developed as a natural source of anti-browning agents for foods or skin-whitening agents in cosmetics. It is expected that further studies on the production process for this compound will highlight its future ranking as effective anti-browning agents or skin-whitening agents.

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References

1. Awl, R. A., E. N. Frankel, and D. Weisleder. 1989. Synthesis and characterization of triacylglycerols containing linoleate and linolenate. *Lipids* **24:** 866–872.

2. Breene, W. M. 1990. Nutritional and medicinal value of specialty mushrooms. *J. Food Prot.* **53:** 883–893.
3. Chang, T. S., H. Y. Ding, and H. C. Lin. 2005. Identifying 6,7,4'-trihydroxyisoflavone as a potent tyrosinase inhibitor. *Biosci. Biotechnol. Biochem.* **69:** 1999–2001.
4. Fukushima, M., T. Ohashi, Y. Fujiwara, K. Sonoyama, and M. Nakano. 2001. Cholesterol-lowering effects of maitake (*Grifola frondosa*) fiber, shiitake (*Lentinus edodes*) fiber, and enokitake (*Flammulina velutipes*) fiber in rats. *Exp. Biol. Med.* **226:** 758–765.
5. Guillen, M. D. and A. Ruiz. 2001. High resolution ^{1}H nuclear magnetic resonance in the study of edible oils and fats. *Trends Food Sci. Technol.* **12:** 328–338.
6. Hirai, Y., M. Ikeda, T. Murayama, and T. Ohata 1998. New monoterpentriols from the fruiting body of *Flammulina velutipes*. *Biosci. Biotechnol. Biochem.* **62:** 1364–1368.
7. Ishikawa, N. K., Y. Fukushi, K. Yamaji, S. Tahara, and K. Takahashi. 2001. Antimicrobial cuparene-type sesquiterpenes, enokipodins C and D, from a mycelial culture of *Flammulina velutipes*. *J. Nat. Prod.* **64:** 932–934.
8. Jang, M. S., A. Sanada, H. Ushio, M. Tanaka, and T. Ohshima. 2003. Inhibitory effect of enokitake extracts on melanosis of shrimp. *Fish. Sci.* **69:** 379–384.
9. Jeon, H. J., M. Noda, M. Maruyama, Y. Matoba, T. Kumagai, and M. Sugiyama. 2006. Identification and kinetic study of tyrosinase inhibitors found in sake lees. *J. Agric. Food Chem.* **54:** 9827–9833.
10. Murrieta, C. M., B. W. Hess, and H. D. C. Rule. 2003. Comparison of acidic and alkaline catalysts for preparation of fatty acid methyl esters from ovine muscle with emphasis on conjugated linoleic acid. *Meat Sci.* **65:** 523–529.
11. Pang, X. B., W. B. Yao, X. B. Yang, C. Xie, D. Liu, J. Zhang, and X. D. Gao. 2007. Purification, characterization and biological activity on hepatocytes of a polysaccharide from *Flammulina velutipes* mycelium. *Carbohydr. Polym.* **70:** 291–297.
12. Smiderle, F. R., E. R. Carbonero, G. L. Sassaki, P. A. J. Gorin, and M. Iacomini. 2008. Characterization of a heterogalactan: Some nutritional values of the edible mushroom *Flammulina velutipes*. *Food Chem.* **108:** 329–333.
13. Wang, P. H., C. I. Hsu, S. C. Tang, Y. L. Huang, J. Y. Lin, and J. L. Ko. 2004. Fungal immunomodulatory protein from *Flammulina velutipes* induces interferon-γ production through p38 mitogen-activated protein kinase signaling pathway. *J. Agric. Food Chem.* **52:** 2721–2725.
14. Wasser, S. P. and A. L. Weis. 1999. Therapeutic effects of substances occurring in higher basidiomycetes mushrooms: A modern perspective. *Crit. Rev. Immunol.* **19:** 65–96.
15. Xu, Y., A. H. Stokes, W. M. Freeman, S. C. Kumer, B. A. Vogt, and K. E. Vrana. 1997. Tyrosinase mRNA is expressed in human substantia nigra. *Mol. Brain Res.* **45:** 159–162.
16. Yang, J. H., H. C. Lin, and J. L. Mau. 2002. Antioxidant properties of several commercial mushrooms. *Food Chem.* **77:** 229–235.
17. Yeung, D. K. W., S. L. Lamb, J. F. Griffith, A. B. W. Chan, Z. Chen, P. H. Tsang, and P. C. Leung. 2008. Analysis of bone marrow fatty acid composition using high-resolution proton NMR spectroscopy. *Chem. Phys. Lipids* **151:** 103–109.