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Identification and Kinetic Study of Tyrosinase Inhibitors Found in Sake Lees

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The present study found that the *n*-hexane extract of freeze-dried sake lees inhibits tyrosinase activity and showed that the constituents isolated from the *n*-hexane extract are the mixture of triacylglycerols. The inhibitory effects of triolein and trilinolein found as the triacylglycerols were examined using tyrosinases from mushroom and *Streptomyces castaneoglobisporus*. The IC_{50} values of the triacylglycerol mixture for the oxidase activity on mushroom and *Streptomyces* tyrosinases were 20 and 0.14 μg/mL, respectively. The IC_{50} values of trilinolein for the oxidase activity on mushroom and *Streptomyces* tyrosinases were 8.4 and 0.1 μM, respectively. However, the inhibitory effect of triolein ($IC_{50} = 30$ μM) was lower than that of trilinolein, even when the *Streptomyces* tyrosinase was used for the assay. Kinetic analyses indicate that both trilinolein and triolein inhibit the tyrosinase activity noncompetitively. When transformed with a plasmid carrying the *Streptomyces* tyrosinase gene, the melanin-synthesizing ability of the transformed *Escherichia coli* host was dose-dependently interfered with by trilinolein.

KEYWORDS: Sake lees; triacylglycerol; trilinolein; triolein; tyrosinase inhibitor

INTRODUCTION

The melanin-synthesizing ability is widely distributed in animals, plants, and microorganisms. The role of melanin in mammalians is to protect the skin from UV-induced injury. However, the overexpression of melanin production in the skin may cause melasma, freckles, ephelides, and age spots (*1*, *2*). Hyperpigmentation can be dependent on the activity of melanogenic enzymes and the function of tyrosinase, which is a key enzyme for melanin synthesis (*3*).

Tyrosinase (EC 1.14.18.1), which is a copper-containing enzyme, catalyzes two distinct reactions of melanin synthesis, the hydroxylation of a monophenol and the conversion of an *o*-diphenol to the corresponding *o*-quinone (*4*, *5*). The melanin pigment is generated as a result of spontaneous polymerization of the quinones. Tyrosinase is also known as a polyphenol oxidase (PPO) (*6*), which is responsible for not only melanization in animals but also browning in fruits and vegetables. This undesirable enzymatic browning of fruits and vegetables causes a significant decrease in their nutritional and market value (*7*, *8*). Furthermore, tyrosinase has been reported to be associated with neurodegenerative diseases as well as Parkinson's disease (*9*). Therefore, the development of tyrosinase inhibitors has been anticipated in the food, pharmaceutical, and cosmetic industries (*10*, *11*).

The notable feature observed in tyrosinases from different sources is that the central copper-binding domain is conserved. However, except for the central copper-binding domain, the similarity of the primary structures of tyrosinase is very low among different sources. For instance, the number of cysteine residues that form disulfide linkages and stabilize the protein structure differs from one organism to another (*12*). Furthermore, the N-terminal 18 amino acids are putative signal peptides of human and mouse tyrosinases but not of fungal ones (*13*, *14*). Thus, the difference of the sequences may reflect the structural difference of tyrosinases from different sources. In spite of the differences, almost all studies on tyrosinase inhibitors so far conducted have used mushroom tyrosinase because the enzyme is commercially available. A competitive inhibitor, which inhibits the catalytic activity of tyrosinase by binding to the active site, may exhibit almost the same inhibitory effects among tyrosinases from different sources. However, there are tyrosinase inhibitors that do not bind to the catalytic site. In this case, the use of tyrosinases from different sources will be valuable for understanding the inhibitory effect of binding to another site.

Hydroquinone, kojic acid, ascorbic acid, and sulfites have been used as antibrowning agents contained in cosmetic products. However, because kojic acid exhibits cytotoxicity, some safe tyrosinase inhibitors, which are derived from food

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Table 1. ^{1}H NMR Spectra of the Mixture of Triacylglycerols Originated from Sake Lees

chemical shift (ppm)	functional group
0.86–0.91	–C**H**$_3$ (on the saturated, palmitoleic, oleic and linoleic acyl groups)
0.95–1.00	–C**H**$_3$ (on linolenic acyl groups)
1.22–1.42	–(C**H**$_2$)$_n$– (in position β or further from double bonds, in position γ or further from carbonyl groups)
1.55–1.67	–OCO–CH$_2$–C**H**$_2$– (in position β in relation to the carbonyl groups)
1.97–2.13	–C**H**$_2$–CH=CH– (in position α in relation to a single double bond)
2.26–2.36	–OCO–C**H**$_2$– (in position α in relation to the carbonyl groups)
2.73–2.84	=HC–C**H**$_2$–CH= (in position α in relation to two double bonds)
4.10–4.35	–C**H**$_2$OCOR (on the 1 and 3 carbons of the glyceryl groups)
5.23–5.28	>C**H**OCOR (on the 2 carbon of the glyceryl groups)
5.28–5.44	–C**H**=C**H**– (on the olefinic groups)

materials, are necessary (*15*−*18*). Sake lees, which are generated as a byproduct in the production of sake, are rich in nutrition; however, most of them are treated as industrial waste. Therefore, an effective use of sake lees would be valuable from an environmental point of view. Sake lees have been reported to be effective in the removal of organochlorine compounds or benzene from wastewater and to contain some peptides that inhibit the catalytic activity of the angiotensin I-converting enzyme (*19*, *20*). Furthermore, the qualities of moisture retention and whitening have been attributed to sake lees because sake brewers (*Tohji* in Japanese) frequently have light and smooth skin.

The objective of this study is to identify the compounds in sake lees that inhibit the catalytic activity of tyrosinases. The candidates were subjected to a kinetic study to evaluate their ability as tyrosinase inhibitors. In this experiment, two kinds of tyrosinases, which were derived from mushroom and *Streptomyces castaneoglobisporus*, were used to examine whether the inhibitory effect of candidates contained in sake lees is distinguishable by tyrosinase from different sources.

MATERIALS AND METHODS

Materials. Freeze-dried sake lees were kindly provided by Chugoku-Jozo Co., Ltd. (Hiroshima, Japan). Mushroom tyrosinase, triolein, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO). L-DOPA (3,4-dihydroxy-L-phenylalanine) and trilinolein were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Kojic acid [5-hydroxy-2-(hydroxymethyl)-4*H*-pyron-4-one] and all other reagents were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan).

General Methods. Silica gel 60 (Merck, 63−200 μm) was used for the purification of tyrosinase inhibitors from sake lees. NMR spectra were obtained in $CDCl_3$ with a JEOL Lambda 400 spectrometer operating at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR, with TMS as the internal reference; the chemical shifts (δ) are reported in parts per million. IR spectra were obtained on a Shimadzu FTIR-8100A spectrometer, and GC-MS spectra were obtained on a Shimadzu GCMS-QP5050A spectrometer (70 eV) fitted with a column (UA-65, 30 m length × 0.25 mm i.d.). UV spectra were recorded using a Molecular Devices VERSA$_{max}$ microplate reader. For thin-layer chromatography (TLC), Merck silica gel F_{254} plates (20 × 20 cm, 0.2 mm thick) were used.

Preparation of Tyrosinase from *S. castaneoglobisporus*. The tyrosinase from *S. castaneoglobisporus* is overproduced using *Streptomyces lividans* 66 harboring pAKM1 (*21*); in other words, the spore suspension of *S. lividans* 66 harboring pAKM1 was inoculated to 400 mL of a TSB medium supplemented with thiostrepton (45 μg/mL) and $CuSO_4$ (8 μM) at a final concentration and grown at 28 °C for 46 h. The purification of tyrosinase secreted into the culture medium was carried out at 4 °C. After the mycelium was eliminated by centrifugation at 27000*g* for 20 min, the resulting supernatant fluid was applied on a DEAE-cellulose column (400 mL at wet volume; Whatman) equilibrated with a 10 mM sodium phosphate buffer (pH 6.8). After the majority of the melanin pigment was eliminated from the supernatant by the DEAE-cellulose column, the solution was supplemented with solid NaCl (4 M) at a final concentration and then applied on a Phenyl-Sepharose column (50 mL at wet volume; Amersham) equilibrated with 10 mM sodium phosphate buffer (pH 6.8) containing 4 M NaCl. The fractions containing tyrosinase were eluted with a 10 mM sodium phosphate buffer (pH 6.8) without 4 M NaCl, concentrated using Macrosep 10K (PALL), and added to DEAE-cellulose resin to completely eliminate the melanin pigment. After the DEAE-cellulose resin was removed by low-speed centrifugation, the resulting supernatant was filtered with Acrodisk syringe filters (0.45 μL HT Tuffryn Membrane, PALL). The filtrate was subjected to a Phenyl-Sepharose (Amersham) column (10 mL wet volume) equilibrated with a 10 mM sodium phosphate buffer (pH 6.8) containing 4 M NaCl and washed with 10 bed-column volumes of the same solution. The *Streptomyces* tyrosinase was eluted with a linear gradient of NaCl (from 4 to 0 M) in a 10 mM sodium phosphate buffer (pH 6.8). The purity of the final tyrosinase preparation was checked with SDS-PAGE. The enzyme purified to homogeneity was concentrated by ultrafiltration (Macrosep 10K, PALL) and stored at 4 °C until use.

Table 2. ^{13}C NMR Spectra of the Mixture of Triacylglycerols Originated from Sake Lees

chemical shift (ppm)	functional group
172.8–173.2	–CH$_2$O**C**OR (carbonyl groups)
127.8–130.2	–**C**H=**C**H– (olefinic groups)
68.8	>**C**HOCOR (2 carbon of the glyceryl groups)
62.1	–**C**H$_2$OCOR (1 and 3 carbons of the glyceryl groups)
14.0–34.2	–(**C**H$_2$)$_n$–, –**C**H$_3$ (acyl groups)

Extraction, Isolation, and Identification of a Tyrosinase Inhibitor from Sake Lees. A preliminary extraction from freeze-dried sake lees using several organic solvents determined the presence of some compounds that significantly inhibit tyrosinase activity in the *n*-hexane extract; therefore, the freeze-dried sake lees (700 g) were extracted with *n*-hexane (21 L) at room temperature for 3 h. The extract was concentrated to dryness at 40 °C under vacuum to produce the *n*-hexane extract (22.64 g).

The tyrosinase inhibitory compounds contained in the *n*-hexane extract were subjected to silica gel 60 column chromatography on a small scale and gradually developed with an *n*-hexane/EtOAc solvent system at the given ratio (*n*-hexane/EtOAc = 1:0 → 32:1 → 16:1 → 8:1 → 0:1) to give five fractions. The fraction developed with hexane/EtOAc (32:1) was subjected to column chromatography (silica gel 60, 5 cm diameter × 20 cm; *n*-hexane/EtOAc = 8:1) and divided into fractions 1−4. Fraction 2, which was purified using column chromatography, was developed with the same silica gel. In this case, after the fraction was developed with *n*-hexane until contaminant spots on TLC disappeared, it was developed with *n*-hexane/$CHCl_3$ = 9:1. The yield of the compound, which was obtained as one spot on TLC (R_f = 0.4, $CHCl_3$), was 1.817 g. By comparison with the spectral data reported previously (*22*−*25*), the compound was identified as a mixture of triacylglycerols. ^{1}H and ^{13}C NMR and GC-MS (EI, CI) data are shown in **Tables 1**, **2**, and **3**, respectively. The mixture exhibits IR absorption bands at 3011, 2955, 2855, 1747, 1464, 1215, 1163, 1099, and 760 cm^{-1}.

Table 3. Triacylglycerols Contained in the Mixture Originated from Sake Lees

	EI		CI							
no.	$[M]^+$	retention time (min)	$[M + NH_4]^+$	retention time (min)	acyl chains and fragments					
1	694	31.59	712	31.59	10	14	16	523 $[M - COOC_9H_{19}]^+$	467 $[M - COOC_{13}H_{27}]^+$	439 $[M - COOC_{15}H_{31}]^+$
2	722	33.34	740	33.34	10	16	16	551 $[M - COOC_9H_{19}]^+$	467 $[M - COOC_{15}H_{31}]^+$	
3	718	33.80	736	33.80	10	14	18:2	547 $[M - COOC_9H_{19}]^+$	491 $[M - COOC_{13}H_{27}]^+$	439 $[M - COOC_{17}H_{31}]^+$
4	746	35.54	764	35.55	10	16	18:2	575 $[M - COOC_9H_{19}]^+$	491 $[M - COOC_{15}H_{31}]^+$	467 $[M - COOC_{17}H_{31}]^+$
5	774	36.96	792	36.96	12	16	18:2	575 $[M - COOC_{11}H_{23}]^+$	519 $[M - COOC_{15}H_{31}]^+$	495 $[M - COOC_{17}H_{31}]^+$
6	770	37.40	788	37.40	10	18:2	18:2	599 $[M - COOC_9H_{19}]^+$	491 $[M - COOC_{17}H_{31}]^+$	
7	802	38.36	820	38.36	14	16	18:2	575 $[M - COOC_{13}H_{27}]^+$	547 $[M - COOC_{15}H_{31}]^+$	523 $[M - COOC_{17}H_{31}]^+$
8	798	38.78	816	38.79	12	18:2	18:2	599 $[M - COOC_{11}H_{23}]^+$	519 $[M - COOC_{17}H_{31}]^+$	
9	830	40.10	848	40.21	16	16	18:2	575 $[M - COOC_{15}H_{31}]^+$	551 $[M - COOC_{17}H_{31}]^+$	
10	828	40.23	846	40.37	14	18:1	18:2	601 $[M - COOC_{13}H_{27}]^+$	547 $[M - COOC_{17}H_{33}]^+$	549 $[M - COOC_{17}H_{31}]^+$
11	858	41.21	876	41.57	16	18:1	18:1	603 $[M - COOC_{15}H_{31}]^+$	577 $[M - COOC_{17}H_{33}]^+$	
12	856	41.74	874	42.13	16	18:1	18:2	601 $[M - COOC_{15}H_{31}]^+$	575 $[M - COOC_{17}H_{33}]^+$	577 $[M - COOC_{17}H_{31}]^+$
13	854	42.23	872	42.59	16	18:2	18:2	599 $[M - COOC_{15}H_{31}]^+$	575 $[M - COOC_{17}H_{31}]^+$	
14	852	42.37	870	42.56	16:1	18:1	18:3	599 $[M - COOC_{15}H_{29}]^+$	571 $[M - COOC_{17}H_{33}]^+$	575 $[M - COOC_{17}H_{29}]^+$
15	882	43.20	900	43.36	18:1	18:1	18:2	601 $[M - COOC_{17}H_{33}]^+$	603 $[M - COOC_{17}H_{31}]^+$	
16	880	43.53	898	43.73	18	18:2	18:3	597 $[M - COOC_{17}H_{35}]^+$	601 $[M - COOC_{17}H_{31}]^+$	603 $[M - COOC_{17}H_{29}]^+$
17	878	43.83	896	44.00	18:2	18:2	18:2	599 $[M - COOC_{17}H_{31}]^+$		

Inhibition of Tyrosinase in Vitro. To identify the tyrosinase inhibitor, we used a method described previously (*26*) with some modifications: 100 μL of 10 mM L-DOPA solution and 74 μL of a 0.1 M phosphate buffer (pH 6.8) were mixed and placed into 96-well plates. The mixture was preincubated at 25 °C for 5 min; after that, 6 μL of DMSO with or without a sample and 20 μL of 200 units of tyrosinase/mL were added, and the linear increase in the optical density of the solution was measured at 475 nm for 5 min. The reactions were carried out under a constant temperature of 25 °C. Inhibitory activity was expressed as the concentration that inhibited 50% of tyrosinase activity, which is designated IC_{50}. A control reaction was conducted with DMSO. Kojic acid was used as a positive control for the evaluation of the tyrosinase inhibitor. The inhibition mode was estimated by Lineweaver−Burk plots and Dixon plots at various L-DOPA and tyrosinase inhibitor concentrations with 20 μL of 400 units/mL of tyrosinase in an aqueous solution. To ensure the reproducibility of the results, all studies were performed at least in triplicate, and the mean values are reported here.

Assay of Chelating between Triacylglycerol and Copper(II) Ion. To test the chelating of copper(II) ion to triacylglycerols, the UV−vis (240−540 nm) spectra of trilinolein and their copper-complexed counterparts were measured. The reaction mixture, consisting of 1.8 mL of a 0.1 M phosphate buffer (pH 6.8), 1.0 mL of water, 0.1 mL of the sample solution (0.05 mM), and 0.1 mL of the aqueous solution of tyrosinase (100 units) or $CuSO_4$ (125 μM), was incubated at 25 °C for 30 min; then, the spectra of the mixture were recorded.

Free Radical Scavenging Activity Assay. The effect of triacylglycerol on the DPPH radical was estimated according to the method of Yamaguchi et al. (*27*) with minor modifications. The trilinolein or triolein solution (0.1 mL in DMSO) was mixed with 0.1 mL of 0.1 mM DPPH in ethanol. The mixture was vigorously shaken and allowed to stand for 30 min at room temperature in the dark. The decrease in the optical density of the resulting solution at 517 nm was monitored.

Inhibition of the Melanin Pigment Synthesizing Activity by Trilinolein. The inhibitory effect on tyrosinase activity was assayed in vivo using *Escherichia coli* BL21(DE3)-pLysS harboring pET-mel3 (*28*). The plasmid, pET-mel3, carries a tyrosinase gene from *S. castaneoglobisporus*. The assay of tyrosinase inhibition in vivo was conducted by observing the brownish color formed on an assay plate by the recombinant *E. coli*. The assay plate consists of a 5-mL upper layer and a 15-mL lower layer. The former is an LB soft agar medium containing ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL) at final concentrations. The latter medium is a tyrosine−agar medium (*21*) containing the same antibiotics, in addition to 0, 15, and 30 μL of trilinolein. After the tyrosine−agar medium became firm, the LB soft agar medium, which was supplemented with the recombinant *E. coli* culture solution (50 μL) grown for 12 h, was overlaid. After the agar surface dried up, a paper disk soaked with 50 μL of 125 mM IPTG or distilled water was placed on the agar plate and incubated at 37 °C. The formation of a brownish color on the plate was observed.

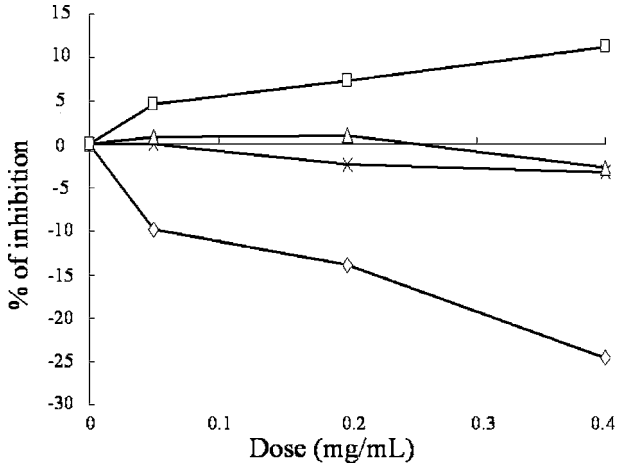

Figure 1. Inhibitory effects of the freeze-dried sake lees extracted with the given solvent on the oxidase activity of mushroom tyrosinase: (□) *n*-hexane; (◇) chloroform; (△) ethyl acetate; (×) water.

RESULTS

Purification and Identification of a Tyrosinase Inhibitor in Freeze-Dried Sake Lees. We examined whether some tyrosinase inhibitors are present in sake lees, which are byproducts of sake production. The freeze-dried sake lees were first extracted with methanol. The resulting crude extracts were partitioned between *n*-hexane and water. The water-soluble fraction was further extracted with chloroform and then with ethyl acetate. However, the inhibition of the oxidase activity on mushroom tyrosinase was observed in only the *n*-hexane-soluble fraction (**Figure 1**). Therefore, the *n*-hexane extract of sake lees was used for further purification of the tyrosinase inhibitor. Finally, we obtained a mixture of triacylglycerols from the *n*-hexane extract, which displays the tyrosinase inhibitory effect (**Figure 2**). The IC_{50} values of the triacylglycerol mixtures for the oxidase activity on mushroom and *S. castaneoglobisporus* tyrosinases were 20 and 0.14 μg/mL, respectively.

The chemical shifts of ^1H and ^{13}C are shown in **Tables 1** and **2**, respectively. These data were compared with spectral

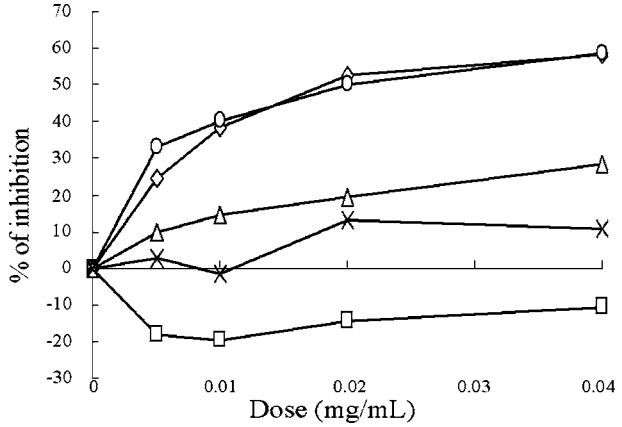

Figure 2. Inhibitory effects of purified fractions developed with hexane/ethyl acetate = 8:1 and the mixture of triacylglycerols isolated from freeze-dried sake lees on the oxidase activity of mushroom tyrosinase: (□) fraction 1; (◇) fraction 2; (△) fraction 3; (×) fraction 4; (○) mixture of triacylglycerols isolated from freeze-dried sake lees.

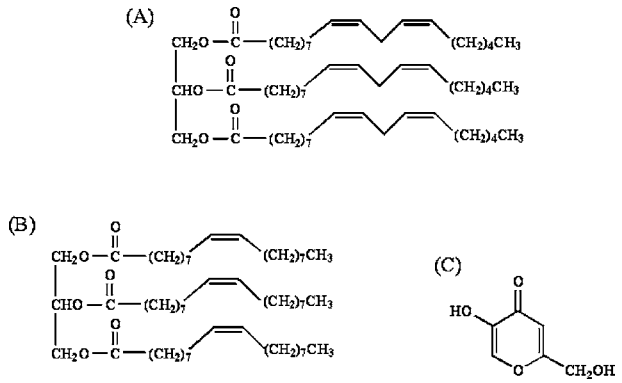

Figure 3. Structures of compounds: (**A**) trilinolein; (**B**) triolein; (**C**) kojic acid.

data reported previously (*22*−*25*). The absorption band at 1747 cm^{-1} indicated the carbonyl group, and that at 760 cm^{-1} indicated the cis stereochemistry of the double bond. The mass spectra were obtained by both the EI and CI modes. The profile of GC-MS showed the mixture containing some kinds of triacylglycerols. The triacylglycerols contained in the mixture are listed in **Table 3**. We examined the tyrosinase inhibitory effects of triolein and trilinolein, which are contained in the freeze-dried sake lees and commercially available, together with kojic acid as a positive control (**Figure 3**).

Inhibition of Oxidase Activity on Mushroom Tyrosinase. **Figure 4** shows the inhibitory ability of triolein, trilinolein, and kojic acid on the oxidase activity on the mushroom tyrosinase. Trilinolein at 5, 10, 25, and 50 μM inhibited the activity by 43, 55, 68, and 71%, respectively. The IC_{50} value of trilinolein for the oxidase activity on mushroom tyrosinase was 8.4 μM. Because triolein is hardly soluble in a water-based solution, the IC_{50} value was not determined. The IC_{50} value of kojic acid was 14 μM, and the value was about 1.5-fold higher than that of trilinolein.

Inhibition of the Oxidase Activity on *S. castaneoglobisporus* Tyrosinase. **Figure 5** shows the inhibitory effects of triolein and trilinolein on the oxidase activity of *S. castaneoglobisporus* tyrosinase. The inhibition by trilinolein was also higher than that by kojic acid. Trilinolein at 5 μM inhibited the oxidase activity by 86%. On the other hand, triolein at 5, 10, 25, and 50 μM inhibited the oxidase activity on *S. castaneoglobisporus* tyrosinase by 17, 34, 46, and 67%, respectively.

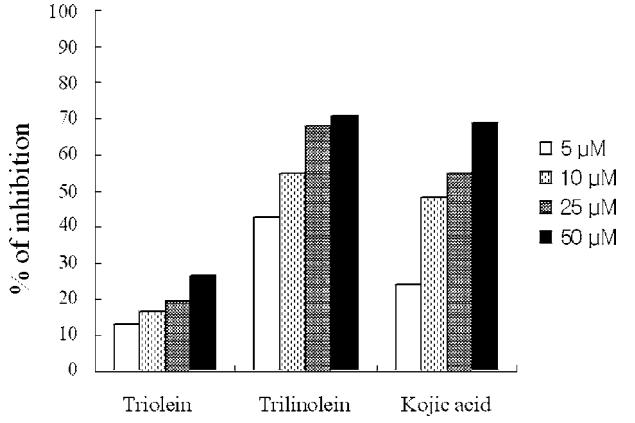

Figure 4. Inhibitory effects of triolein, trilinolein, and kojic acid for the oxidase activity on mushroom tyrosinase at different concentrations. The concentration of L-DOPA was 5 mM.

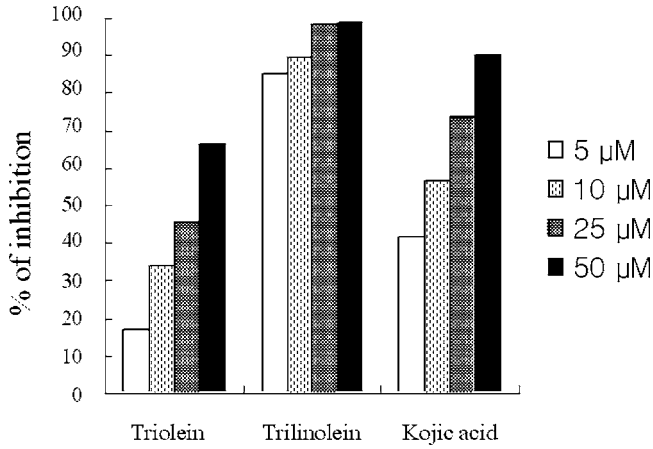

Figure 5. Inhibitory effects of triolein, trilinolein, and kojic acid for the oxidase activity on *Streptomyces castaneoglobisporus* tyrosinase at different concentrations. The concentration of L-DOPA was 5 mM.

The IC_{50} values of triolein, trilinolein, and kojic acid for the oxidase activity on *Streptomyces* tyrosinase were 30, 1.0, and 7.8 μM, respectively. Thus, trilinolein inhibits the tyrosinase activity more strongly than kojic acid.

Inhibition Pattern of the Oxidase Activity of Tyrosinase by Trilinolein. The kinetic behaviors of the oxidation of L-DOPA catalyzed by the mushroom and *S. castaneoglobisporus* tyrosinases were investigated using various concentrations of trilinolein and L-DOPA. The inhibition patterns of the mushroom and *S. castaneoglobisporus* tyrosinases are shown in **Figures 6** and **7**, respectively. In **Figures 6** and **7**, the abscissa 1/[L-DOPA] is the reciprocal of the concentrations of L-DOPA, whereas the ordinate $1/V$ is the reciprocal of the change of the absorbance with time, which reflects the reciprocal of the oxidase activity on tyrosinase. Judging from the results of Lineweaver−Burk plots of $1/V$ versus 1/[L-DOPA], which are shown in **Figures 6** and **7**, the V_{max} value to the oxidase reaction was dose-dependently decreased, whereas the K_m value was not quite altered. Therefore, we conclude that trilinolein is a noncompetitive inhibitor. The dissociation equilibrium constants (K_i) of trilinolein on the tyrosinase inhibitory effect were 282 and 52 μM on mushroom and *S. castaneoglobisporus* tyrosinase, respectively.

The inhibition kinetics for trilinolein was also analyzed by Dixon plot analysis, which is obtained by plotting $1/V$ versus [I] with various concentrations of a substrate using mushroom and *S. castaneoglobisporus* tyrosinases (data not shown). The

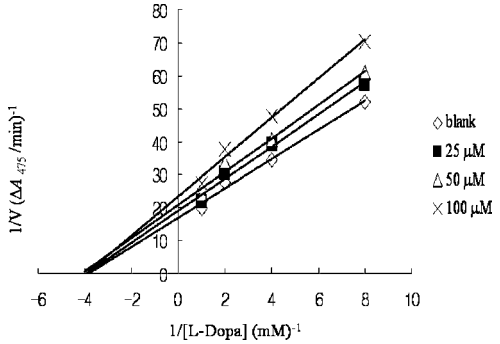

Figure 6. Lineweaver–Burk plots of the oxidase activity on mushroom tyrosinase treated with several concentrations of trilinolein and without trilinolein. The concentrations of L-DOPA were 0.125, 0.25, 0.5, and 1 mM. The concentrations of trilinolein were 0, 25, 50, and 100 μM.

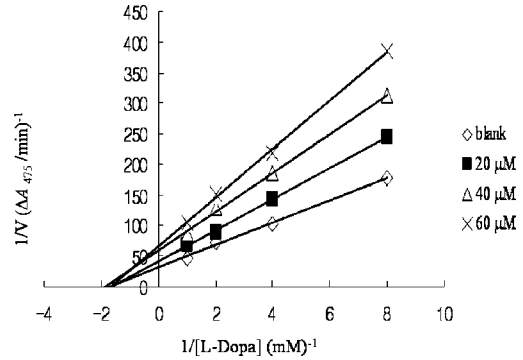

Figure 7. Lineweaver–Burk plots of the oxidase activity on *Streptomyces castaneoglobisporus* tyrosinase treated with several concentrations of trilinolein and without triolein. The concentrations of L-DOPA were 0.125, 0.25, 0.5, and 1 mM. The concentrations of trilinolein were 0, 20, 40, and 60 μM.

Dixon plot analysis also shows that trilinolein functions as a noncompetitive inhibitor of the oxidase activity of the two tyrosinases.

Trilinolein Inhibits the Melanin-Synthesizing Ability of *E. coli* Harboring the *Streptomyces* Tyrosinase Gene. Figure 8 shows plates overlaid with *E. coli* harboring pET-mel3. The plates shown in panels **A**, **B**, and **C** of **Figure 8**, respectively, contain trilinolein at 0, 15, and 30 μL and were incubated for 0, 24, and 28 h, respectively. The paper disks located on the surface of the agar were soaked with diluted water or a 125 μM IPTG/mL solution. The brownish color appeared around the paper disk soaked with the IPTG solution. However, the brownish color formation of *E. coli* grown on a medium supplemented with trilinolein was dose-dependently repressed. These results suggest that trilinolein inhibits the melanin-synthesizing ability of *E. coli* harboring the *Streptomyces* tyrosinase gene.

DISCUSSION

In this study, we showed that triacylglycerols, contained in sake lees, function as a tyrosinase inhibitor. However, it was suggested that some constituent in sake lees stimulates the catalytic activity of the tyrosinase, as shown in **Figure 1**. The constituent might be a saturated fatty acid or an oxidizer. Practically, when experiments were performed with palmitic acid or stearic acid as a saturated fatty acid, we confirmed that the saturated fatty acids activate the catalytic activity of tyrosinase (data not shown). It has been also reported the palmitic acid retarded the proteolysis of tyrosinase (*30*).

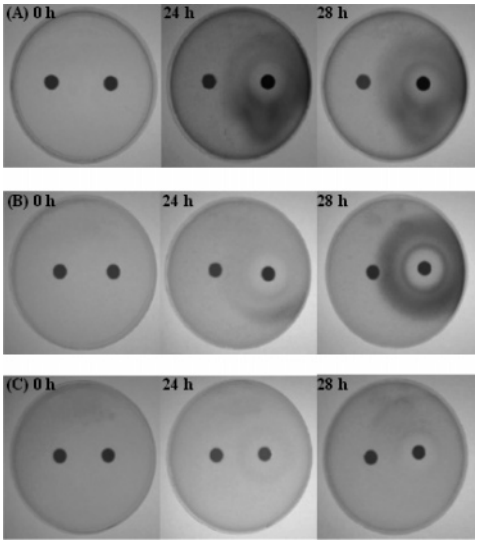

Figure 8. Assay of the melanin-synthesizing ability of *Escherichia coli* BL21(DE3)-pLysS harboring pET-mel3. The recombinant *E. coli* cells were grown on an assay medium supplemented with or without trilinolein at the given concentrations: (**A**) without trilinolein; (**B**) 15 μL of trilinolein; (**C**) 30 μL of trilinolein. The paper disks shown on the left and the right were soaked with 50 μL of distilled water and 50 μL of 125 μM IPTG/mL, respectively.

We performed three purifications by using a silica gel column for the identification of the constituents, whereas the inhibitory activities did not nearly change after the work of first column. The results indicate that the fraction developed with a solvent system (hexane/EtOAc = 32:1) was almost constituted with tricaylglycerols, which is enough to use practically. The ^{1}H NMR spectra of triacylglycerols originated from the freeze-dried sake lees showed that the ratio of olefinic protons (−CH═CH−) to the sum of protons on the 1 and 3 carbons of the glyceryl group (−CH_2OCOR) was 2 to 1. One triacylglycerol molecule contains four protons, which is the sum of two protons on the 1 carbon of the glyceryl group and two protons on the 3 carbon of the glyceryl group, whereas one double bond contains two protons. Judging from the ratio of the proton numbers, the triacylglycerol from the freeze-dried sake lees is assumed to have four double bonds in one triacylglycerol on average. Therefore, we investigated the inhibitory effects of trilinolein, which was found in the freeze-dried sake lees. In addition, the tyrosinase inhibitory effect of triolein, which is a commercially available triacylglycerol, was also analyzed. These compounds have three or six double bonds.

As shown in **Figure 4**, trilinolein exhibits a higher inhibitory effect than both triolein and kojic acid on mushroom tyrosinase, and the IC_{50} value was 8.4 μM, suggesting that the compound is sufficient as a tyrosinase inhibitor. **Figure 5** also shows that trilinolein and triolein inhibit the oxidase activity on *S. castaneoglobisporus* tyrosinase. The inhibition intensity of trilinolein against the *S. castaneoglobisporus* tyrosinase is significantly higher than that of kojic acid.

By Lineweaver−Burk plots and Dixon plot analyses, trilinolein was identified as a noncompetitive inhibitor. Furthermore, triolein also showed an inhibitory mode as a noncompetitive inhibitor to mushroom tyrosinase (data not shown). In other words, triacylglycerols may function as noncompetitive inhibitors. The noncompetitive inhibitory mode suggests that triacylglycerol may bind to some site other than the catalytic site of tyrosinase and combine with either the free enzyme or the enzyme−substrate complex.

Furthermore, we examined whether trilinolein and triolein exhibit a chelating ability with copper(II) ion. However, no bathochromic shift was observed (data not shown), suggesting that triacylglycerols do not form a chelate with the copper(II) ion. The observation is in agreement with the results obtained from the Lineweaver−Burk plots and Dixon plot analyses, which show that both trilinolein and triolein are noncompetitive inhibitors. If trilinolein and triolein form a chelate with a copper-(II) ion, the inhibition mode will be a mixed-type or a competitive-type inhibition.

Radicals are generated by ultraviolet rays or environmental toxins and stimulate tyrosinase in human skin. However, the compounds, which have a radical scavenging ability, can prevent the activation of tyrosinase or autoxidative processes. Therefore, we also evaluated the DPPH radical scavenging ability of trilinolein and triolein; however, these triacylglycerols lacked this ability (data not shown). The result suggests that the tyrosinase inhibitory effects of trilinolein and triolein do not come from their antioxidant ability to scavenge a radical or proton.

From these results it may be concluded that the tyrosinase inhibitory effects of triacylglycerols may be caused by binding of the compounds to some site of tyrosinase, except the catalytic site. The inhibitory activities and dissociation equilibrium constants were different between the sources of tyrosinase, suggesting that the ability of tyrosinase inhibitors may be affected by the primary structure of tyrosinases. The differences between trilinolein and triolein are the number of double bonds and the bending degree by one or two cis double bonds. However, triacylglycerols have long and flexible alkyl chains. The long and flexible alkyl chains probably overcome a steric difference between trilinolein and triolein. Therefore, the inhibitory effect may be associated with the number of double bonds, and the binding ability of triacylglycerols to tyrosinase is influenced by the double bond. That is, the number of double bonds is also an important factor in the exhibition of tyrosinase inhibitory effect.

In general, α-methylenic protons with two double bonds of fatty acids react with reactive oxygen to generate a peroxide of a fatty acid. The amount of dissolved reactive oxygen decreases by the generation of peroxide, which induces the inactivation of tyrosinase. Trilinolein is also expected to react with reactive oxygen and exhibit inhibitory effect. However, the result remains unclear because evidence of an interaction between tyrosinase and its inhibitor has not yet been observed. Our laboratory has recently determined the crystal structure of *S. castaneoglobisporus* tyrosinase (*31*). As an approach to make clear the interaction between tyrosinase and tryacylglycerol, the crystallographic analysis of this complex may be necessary.

E. coli transformed with a plasmid carrying the *Streptomyces* tyrosinase gene produced the melanin pigment near a paper disk soaked with IPTG. However, this did not happen when distilled water was used instead of IPTG. Thus, IPTG induced the expression of tyrosinase in the transformed *E. coli* cells. Under this condition, the recombinant *E. coli* cells grown on a medium containing trilinolein do not produce the melanin pigment even in the presence of IPTG. Unsaturated fatty acids, such as linoleic acid and α-linolenic acid, have been reported to reduce hyperpigmentation of the skin. In addition, the compounds have been found to positively regulate the degradation of tyrosinase (*29*, *30*). Because trilinolein is easy to synthesize chemically by the conjugation of linoleic acid, it might be expected to reduce the hyperpigmentation and to degrade tyrosinase, as would linoleic acid.

The freeze-dried sake lees contain many triacylglycerols. Because one of the triacylglycerols, that is, trilinolein, is a safe tyrosinase inhibitor from food material, it will be very useful for application in the cosmetic field.

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LITERATURE CITED

(1) Oetting, W. S. The tyrosinase gene and oculocutaneous albinism type 1 (OCA1): a model for understanding the molecular biology of melanin formation. *Pigment Cell. Res.* **2000**, *13*, 320−325.
(2) Priestly, G. C. *Molecular Aspects of Dermatology*; Wiley: Chichester, U.K., 1993.
(3) Sanchez-Ferrer, A.; Rodriguez-Lopez, J. N.; Garcia-Ganovas, F.; Garcia-Carmona, F. Tyrosinase: a comprehensive review of its mechanism. *Biochim. Biophys. Acta* **1995**, *1247*, 1−11.
(4) Hearing, V. J.; Tsukamoto, K. Enzymatic control of pigmentation in mammals. *FASEB J.* **1991**, *5*, 2902−2909.
(5) Aroca, P.; Urabe, K.; Kobayashi, T.; Tsukamoto, K.; Hearing, V. J. Melanin biosynthesis patterns following hormonal stimulation. *J. Biol. Chem.* **1993**, *268*, 25650−25655.
(6) Mayer, A. M. Polyphenol oxidase in plants—recent progress. *Phytochemistry* **1987**, *26*, 11−20.
(7) Martinez, M. V.; Whitaker, J. R. The biochemistry and control of enzymatic browning. *Trends Food Sci. Technol.* **1995**, *6*, 195−200.
(8) Friedman, M. Food browning and its prevention: an overview. *J. Agric. Food Chem.* **1996**, *44*, 631−653.
(9) Xu, Y.; Stokes, A. H.; Freeman, W. M.; Kumer, S. C.; Vogt, B. A.; Vrana, K. E. Tyrosinase mRNA is expressed in human substantia nigra. *Mol. Brain Res.* **1997**, *45*, 159−162.
(10) Mosher, D. B.; Pathak, M. A.; Fitzpartrick, T. B. Vitiligo, etiology, pathogenesis, diagnosis, and treatment. In *Update: Dermatology in General Medicine*; Fitzpatrick, T. B., Eisen, A. Z., Wolff, K., Freedberg, I. M., Austen, K. F., Eds.; McGraw-Hill: New York, 1983; pp 205−225.
(11) Maeda, K.; Fukuda, M. In vitro effectiveness of several whitening cosmetic components in human melanocytes. *J. Soc. Cosmet. Chem.* **1991**, *42*, 361−368.
(12) van Gelder, C. W. G.; Flurkey, W. H.; Wichers, H. J. Sequence and structural features of plant and fungal tyrosinases. *Phytochemistry* **1997**, *45*, 1309−1323.
(13) Lerch, K. Protein and active-site structure of tyrosinase. *Prog. Clin. Biol. Res.* **1988**, *256*, 85−98.
(14) Betancourt, A. M.; Bernan, V.; Herber, W.; Katz, E. Analysis of tyrosinase synthesis in *Streptomyces antibioticus*. *J. Gen. Microbiol.* **1992**, *138*, 787−794.
(15) McEvily, J. A.; Iyengar, R.; Otwell, W. S. Inhibition of enzymatic browning in foods and beverages. *Crit. Rev. Food Sci. Nutr.* **1992**, *32*, 253−273.
(16) Seo, S. Y.; Sharma, V. K.; Sharma, N. Mushroom tyrosinase: recent prospects. *J. Agric. Food Chem.* **2003**, *51*, 2837−2853.
(17) Kubo, I.; Yokokawa, U.; Kinst-Hori, I. Tyrosinase inhibitors from Bolivian medicinal plants. *J. Nat. Prod.* **1995**, *58*, 739−743.
(18) Lee, G. G.; Lee, C. Y. Inhibitory effect of caramelization products on enzyme browning. *Food Chem.* **1997**, *60*, 231−235.
(19) Adachi, A.; Hamamoto, H.; Okano, T. Use of lees materials as an adsorbent for removal of organochlorine compounds or benzene from wastewater. *Chemosphere* **2005**, *58*, 817−822.
(20) Saito, Y.; Wanezaki, K.; Kawamoto, A.; Imayasu, S. Structure and activity of angiotensin I converting enzyme inhibitory peptides from sake and sake lees. *Biosci., Biotechnol., Biochem.* **1994**, *58*, 1767−1771.

(21) Ikeda, K.; Masujima, T.; Suzuki, K.; Sugiyama, M. Cloning and sequence analysis of the highly expressed melanin synthesizing gene operon from *Streptomyces castaneoglobisporus*. *Appl. Microbiol. Biotechnol*. **1996**, *45*, 80−85.

(22) Guillen, M. D.; Ruiz, A. ^{1}H nuclear magnetic resonance as a fast tool for determining the composition of acyl chains in acylglycerol mixtures. *Eur. J. Lipid Sci. Technol*. **2003**, *105*, 502−507.

(23) Mannina, L.; Luchinat, C.; Emanuele, M. C.; Segre, A. Acyl positional distribution of glycerol tri-esters in vegetable oils: a ^{13}C NMR study. *Chem. Phys. Lipids* **1999**, *103*, 47−55.

(24) Holcapke, M.; Jandera, P.; Zderadicka, P.; Hruba, L. Characterization of triacylglycerol and diacylglycerol composition of plant oils using high-performance liquid chromatography atmospheric pressure chemical ionization mass spectrometry. *J. Chromatogr. A* **2003**, *1010*, 195−215.

(25) Lin, J. T.; Woodruff, C. L.; McKeon, T. A. Non-aqueous reversed-phase high-performance liquid chromatography of synthetic triacylglycerols and diacylglycerols. *J. Chromatogr. A* **1997**, *782*, 41−48.

(26) Masamoto, Y.; Iida, S.; Kubo, M. Inhibitory effect of Chinese crude drugs on tyrosinase. *Planta Med*. **1980**, *40*, 361−365.

(27) Yamaguchi, T.; Takamura, H.; Matoba, T.; Terao, J. HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1-diphenyl-2-picrylhydrazyl. *Biosci., Biotechnol., Biochem.* **1998**, *62*, 1201−1204.

(28) Kohashi, P. Y.; Kumagai, T.; Matoba, Y.; Yamamoto, A.; Maruyama, M.; Sugiyama, M. An efficient method for the overexpression and purification of active tyrosinase from *Streptomyces castaneoglobisporus*. *Protein Expr. Purif.* **2004**, *34*, 202−207.

(29) Ando, H.; Ryo, A.; Hashimoto, A.; Oka, M.; Ichihashi, M. Linoleic acid and α-linolenic acid lightens ultraviolet-induced hyperpigmentation of the skin. *Arch. Dermatol. Res.* **1998**, *290*, 375−381.

(30) Ando, H.; Watabe, H.; Valencia, J. C.; Yasumoto, K.; Furumura, M.; Funasaka, Y.; Oka, M.; Ichihashi, M.; Hearing, V. J. Fatty acids regulate pigmentation via proteasomal degradation of tyrosinase: a new aspect of ubiquitin-proteasome function. *J. Biol. Chem*. **2004**, *15*, 15427−15433.

(31) Matoba, Y.; Kumagai, T.; Yamamoto, A.; Yoshitsu, H.; Sugiyama, M. Crystallographic evidence that dinuclear copper center of tyrosinase is flexible during catalysis. *J. Biol. Chem.* **2006**, *281*, 8981−8990.

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