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Regulation of Melanin Synthesis by Selenium-Containing Carbohydrates

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This study reports depigmenting potency of selenium-containing carbohydrates, which would be based upon the finding of direct inhibition to mushroom tyrosinase. Two selenoglycosiede, SG-3 (bis(2,3,4-tri-*O*-acetyl-*β*-D-arabinopyranosyl) selenide) and SG-8 (4′-methylbenzoyl 2,3,4,6-tetra-*O*-acetyl-D-selenomanopyranoside) among eleven selenium-containing compounds examined, were discovered to be effective depigmenting compounds on a mushroom tyrosinase inhibitory assay. SG-3 exhibited a competitive inhibition effect that was similar to kojic acid, well-known tyrosinase inhibitor. At 100 μM and 150 μM, SG-8 had an uncompetitive inhibitory effect that was higher than kojic acid. A study of a melan-a cell originated-tyrosinase inhibition assay showed that SG-8 had a lower inhibitory effect than kojic acid. SG-3 showed a similar inhibition effect to kojic acid on the melan-a cell-originated tyrosinase inhibitory assay. SG-8 showed dose-dependently cytotoxicity in a study of inhibition melanin synthesis by melan-a cells. Most melan-a cells did not survive after being treated with 20 μM of SG-8. At 10 μM, SG-3 inhibited melanin synthesis in the melan-a cells, and the effect was similar to phenylthiourea, which is a well-known inhibitor of melanin synthesis. Therefore, SG-3 is a new candidate for depigmenting reagents.

Key words selenium; carbohydrate; melanin; mushroom tyrosinase

Melanins are the skin pigments in humans, play a major role in photoprotection.[1] Skin pigmentaion from melanin delivered to keratinocyte and stored in the epidermis, increasing melanin production in melanocyte.[2] Melanocytes have a unique intracytoplasmic organelle, such as melanosome, which is the site of melanin biosynthesis. The activity of melanocytes depends on the melanization and diameter of melanosomes. As melanin is deposited in the melanocytes, premelanosomes containing melanin are transferred from the melanocyte to keratinocytes in the epidermis.[3] The major factor of skin pigmentation is the melanin synthesis in melanosomes and its transfer to keratinocytes.[4] Although melanin plays a crucial role in the absorption of free radicals from the cytoplasm and protecting from UV light,[5] the overproduction and accumulation of melanin in skin could be a serious pathological source resulting in numbers of skin disorders such as freckles, chloasoma dermatitis and geriatric pigment spots. For the control of melanogenesis, many whitening cosmetics and medicines are being developed.

Since the 1980s, the melanin biosynthesis pathways were studied by skin cancer researchers,[6,7] who have led to the development of whitening cosmetics and medicines. Arbutin, kojic acid and their derivatives were developed in 1980s.[8] However, the clinical efficacy of these materials is unsatisfactory.[9] The hydroquinone compounds have been used as depigmenting agents for skin pigmentation, but they are strongly irritable to the skin and exhibit cell toxicity.[9,10] Therefore, there are many needs for more safe whitening agents.

Melanogenesis is mainly regulated by tyrosinase and dopachrome tautomerase. The primary material of melanin biosynthesis is the tyrosine, and the oxidation of tyrosine is catalyzed by copper-containing enzyme tyrosinase to dopaquinone. Tyrosinase is one of the important enzymes in melanogenesis of mammalian skin.[11,12] Thus, inhibition of tyrosinase can regulate melanin synthesis.

Selenium is a biological trace essential element of several enzymes, and has been used as a nutritional supplement and medicine due to its potential effect in low concentrations as an antioxidant and in higher concentrations as an anticancer agent.[13,14] Some heterocyclic compounds containing selenium have been demonstrated to possess anticancer, antifungal, antibacterial and antiviral efficacy.[15—18] And we previously reported that selenazole derivatives can inhibit the mushroom tyrosinase.[19] Selenoglycoside (SG) series have been recently developed as a series of selenium-containing carbohydrates[20] (Fig. 1). This study shows inhibitory effect of SG series on tyrosinase in the melanin synthesis. Eleven SGs were examined for their tyrosinase inhibitory effect using mushroom tyrosinase and melan-a cell tyrosinase. Among them, two compounds, SG-3 (bis(2,3,4-tri-*O*-acetyl-*β*-D-arabinopyranosyl) selenide) and SG-8 (4′-methylbenzoyl 2,3,4,6-tetra-*O*-acetyl-D-selenomanopyranoside), showed strongest inhibitory effects on tyrosinase.

Experimental

Reagents The SG series were prepared according to the reference[20] (Fig. 1). All chemicals were analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) and TCI (Tokyo, Japan).

Cell Lines The melan-a cells were kind gift from Dr. Byeong Gon Lee at the Skin Research Institute, Amore-Pacific Co., Yongin city Kyunggi, Korea.

Inhibition of Mushroom Tyrosinase Activity Each concentration (1 μM, 10 μM, 100 μM, 150 μM) of the test substance was dissolved in methanol. 120 ml of L-dopa (8.3 mM, dissolved in 80 mM phosphate buffer, pH 6.8) and 40 μl of each SG series solution were added to a 96-well microplate, and then mixed with 40 μl of mushroom tyrosinase (125 U, dissolved in 80 mM phosphate buffer, pH 6.8). After 30 min incubation at 37 °C, the amount of dopachrome in the reaction mixture was determined. The UV spectra were obtained with the E max microplate reader (Molecular Devices, U.S.A.). The inhibitory activity of the sample was determined from its optical density at 490 nm (OD 490). Kojic acid was used as the reference. The type of inhibition of the test substance was determined by Lineweaver-Burk's plot using various L-dopa concentrations.[21]

Cell Culture The melan-a cells were cultured in RPMI1640 medium with 10% FBS and 200 nM TPA (phobol 12-myristate 13-acetate) conditions.[22] Ten milliliters of the medium was added to a 100 mm culture dish, and seeded with approximately 5×10^5 cells.[23]
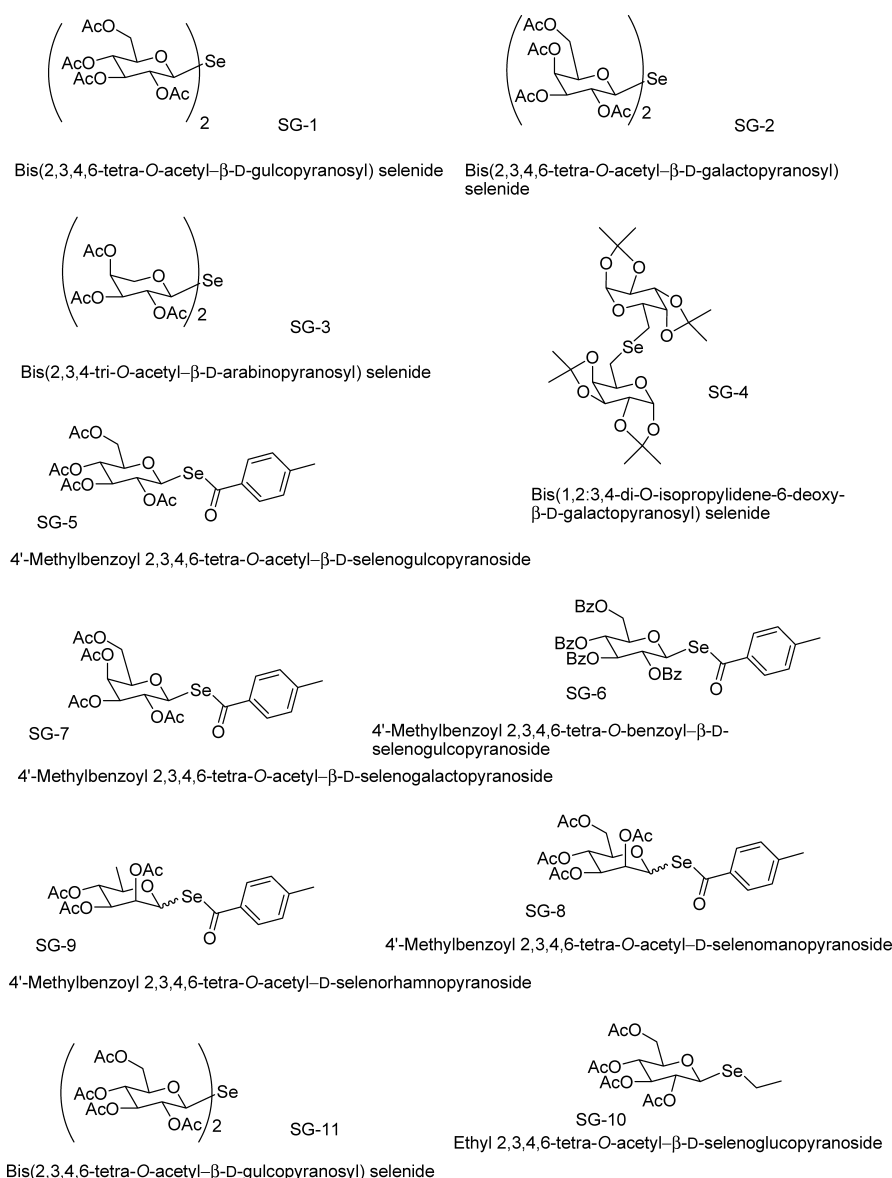
Fig. 1.   Structures of Selenoglycosides and SG Series

Tyrosinase Extraction from Melan-a Cell   The melan-a cells were disrupted by resuspending them in a tyrosinase buffer (80 mM PO$_4$ buffer+1% Triton-X 100+100 μg/ml PMSF) followed by sonication in an ice bath. After centrifugation at 12500 rpm for 15 min, the supernatant was used for the enzyme assay. 150 μg of proteins were required for each reaction.[24]

Melanin Contents and Cell Viability in Cultured Melan-a Cells   The cells were grown to confluence after four days under 37 °C and a 5% CO$_2$ atmosphere. They were seeded with 10^5 cells/well in the 24-well plate and incubated for 24 h. The medium in each well was changed with 990 μl of medium everyday and treated with 10 μl of various concentrations of test sample for 3 d (solvent system; propylene glycol : EtOH : H$_2$O=3 : 2 : 1). The cells were then incubated for 24 h.

Determination of Cell Viability   The percentage of viable cells was determined by staining the cell population with crystal violet. The cells were washed with PBS after removing the medium from each well. The 200 μl of crystal violet (0.1% CV, 10% EtOH, with the remainder being PBS) was added. The cells were incubated at room temperature for 5 min, and washed twice with water. After adding 1 ml of ethanol, the cells were shaken at room temperature for 10 min. The crystal violet absorption was measured at 590 nm.

Determination of the Melanin Level   The melanin content was measured using a modification of the methods reported by Hosoi.[25] After removing the media from each well, the wells were washed with PBS. This was followed by adding 1 ml of 1 N NaOH to dissolve the melanin. The absorbance was measured at 400 nm, and the melanin contents per well were calculated, and were expressed as a percentage of the control. Phenylthiourea (PTU), which is an inhibitor of melanogenesis acting on tyrosinase, was used as a positive standard control.[26,27]

Statistical Analysis   The data is presented as a mean±S.E. The statistical comparisons between the different treatments were carried out using Student's t-test.

Results

Two Compounds of SG Series Inhibit Mushroom Tyrosinase Activity   Eleven compounds of the SG series and kojic acid as the positive control were examined for their mushroom tyrosinase inhibitory activity (Fig. 2). Each concentration sample of SG series and kojic acid in MeOH were dissolved to 1 μM, 10 μM, 100 μM and 150 μM. Among them, SG-3 and SG-8 had a significant inhibitory effect on mushroom tyrosinase. In addition, the inhibitory effects of SG-3 and SG-8 on mushroom tyrosinase were dose-dependent.

Comparison of Inhibitory Activities between Compounds SG-3, SG-8 and Kojic Acid   The inhibitory activity of SG-3 and SG-8 against mushroom tyrosinase was de-
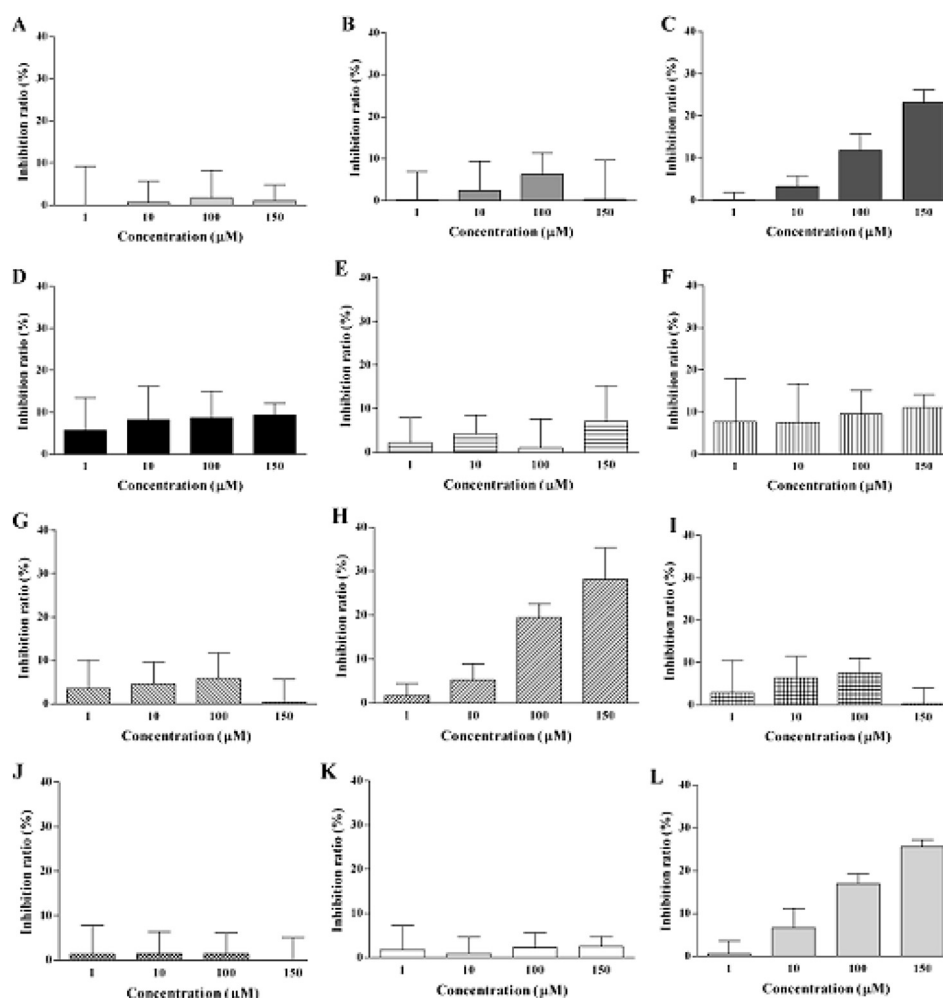
Fig. 2. Inhibition Assay of the SG Series on Mushroom Tyrosinase

(A) SG-1, (B) SG-2, (C) SG-3, (D) SG-4, (E) SG-5, (F) SG-6, (G) SG-7, (H) SG-8, (I) SG-9, (J) SG-10, (K) SG-11, (L) Kojic acid. (C) and (H) shows inhibitory effects on mushroom tyrosinase. (A), (B), (D)—(G), (I)—(K) show that those do not inhibit mushroom tyrosinase. (L) kojic acid was used as positive control. Each value represents the mean±S.D. of five experiments. ∗$p<0.05$, ∗∗$p<0.01$, compared with the control.
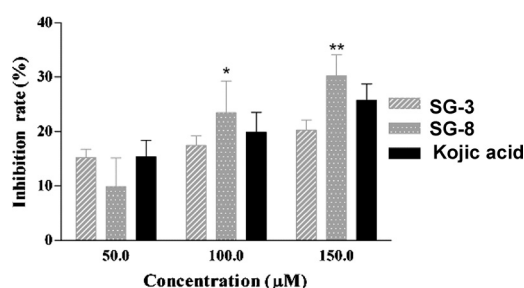

Fig. 3. SG-3 and SG-8 Inhibits Mushroom Tyrosinase to a Similar Level to Kojic Acid

Samples, SG-3 and SG-8, were dissolved to 50, 100 and 150 μM in MeOH. 2.5 U/ml of mushroom tyrosinase was reacted with 5 mM of L-DOPA or without 5 mM of L-DOPA and each samples at 37 °C for 20 min. After reaction, dopachrome content was measured at 490 nm using an ELISA reader. At 100 μM and 150 μM, SG-8 showed a stronger effect on mushroom tyrosinase than kojic acid. Each value represents the mean±S.D. of five experiments. ∗$p<0.05$, ∗∗$p<0.005$, compared with control.

termined in the 50—150 μM concentration range (Fig. 3) because SG-8 could not inhibit mushroom tyrosinase at concentrations over 200 μM (data not shown). Kojic acid is a copper ion-chelating reagent. The copper ions play a key role in tyrosinase activity.[28]

The patterns of the inhibitory effects between SG-3, SG-8 and kojic acid had a similar as sigmoidal shape in 50—150 μM concentration range. The SG-8 showed stronger inhibitory effects on tyrosinase than kojic acid at 100 μM and 150 μM.

SG-3 Compound Is a Competitive Inhibitor of Mushroom Tyrosinase The enzyme kinetics was examined to determine show SG-3 inhibited the mushroom tyrosinase. 50 and 100 μM of SG-3 was dissolved in MeOH. 125 μM to 1 mM of the substrate, L-DOPA, was dissolved from in 80 mM phosphate buffer at pH 6.8. Mixed samples and substrate with mushroom tyrosinase, were incubated for 20 min at 37 °C. A Lineweaver-Burk's plot was made from the optical density at 490 nm (Fig. 4).

The graph shows crosses at Y-axis pattern. It shows, SG-3 seemed to be a competitive inhibitor. The competitive inhibitor binds to active site of the enzyme by structural similarity of substrate.[29]

SG-8 Compound Is an Uncompetitive Inhibitor of Mushroom Tyrosinase The same assay was used to determine how SG-8 inhibits mushroom tyrosinase (Fig. 5). The graph shows a parallel pattern. It means that SG-8 is an uncompetitive inhibitor. For an uncompetitive inhibitor to work, the enzyme must combine with the substrate first followed by
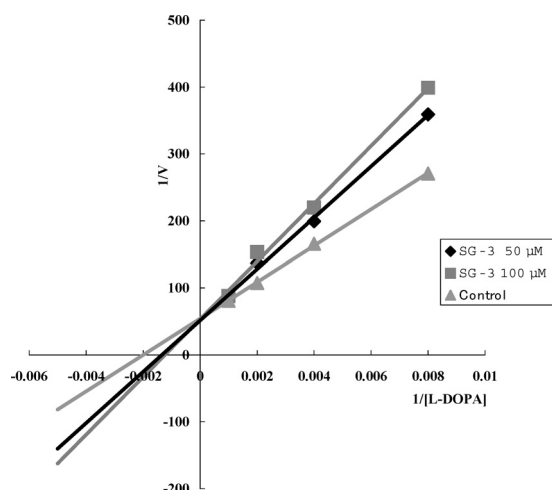
Fig. 4. SG-3 Has a Competitive Inhibitory Effect on Mushroom Tyrosinase

SG-3 dissolved to 50 and 100 μM in MeOH. 2.5 U/ml of mushroom tyrosinase was reacted with each 125 μM, 250 μM, 500 μM and 1 mM of L-DOPA and each concentration of SG-3 at 37 °C for 20 min. The dopachrome content was measured at 490 nm using an ELISA reader. Lineweaver–Burk curves show the reciprocal of the velocity (20/A490) of the mushroom tyrosinase reaction $vs.$ the reciprocal of the substrate concentration (1/S) with L-DOPA as the substrate.
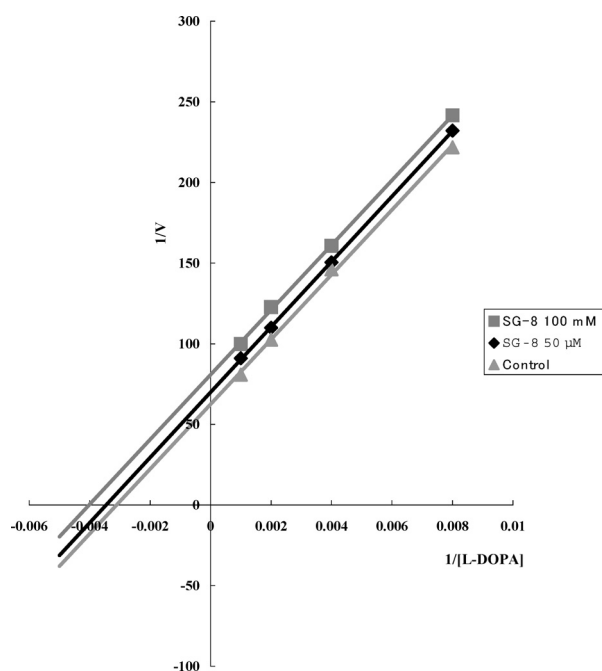

Fig. 5. SG-8 Has an Uncompetitive Inhibitory Effect on Mushroom Tyrosinase

SG-8 dissolved to 50 and 100 μM in MeOH. 2.5 U/ml of mushroom tyrosinase reacted with each 125 μM, 250 μM, 500 μM and 1 mM of L-DOPA and each concentration of SG-8 at 37 °C for 20 min. The dopachrome contents were measured at 490 nm using an ELISA reader. Lineweaver–Burk curves show the reciprocal of the velocity (20/A490) of the mushroom tyrosinase reaction $vs.$ the reciprocal of the substrate concentration (1/S) with L-DOPA as the substrate.

a combination of the inhibitor with the enzyme-substrate complex. A combination of the inhibitor with the free enzyme is not possible.[30]

SG-3 and SG-8 Compounds Inhibit Melan-a Cell-Originated Tyrosinase Activity It was found that SG-3 and SG-8 had inhibitory effect on not only mushroom tyrosinase but also the animal cell-originated tyrosinase.

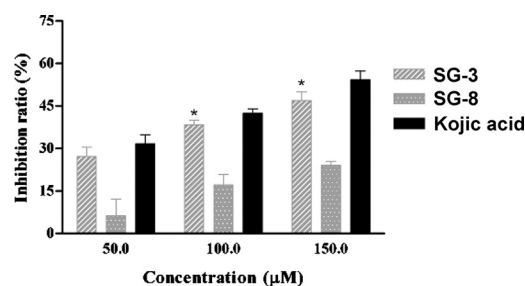Melan-a cells are synergetic with a B16 melanoma and its

Fig. 6. Melan-a Cell Tyrosinase Inhibitory Effects of SG-3 and SG-8

SG-3 and SG-8, were dissolved to 50, 100 and 150 μM in MeOH. 750 mg/ml of the melan-a cell extracts were reacted with or without each samples at 37 °C for 20 min. After the reaction, the dopachrome contents were measured at 490 nm using an ELISA reader. SG-3 showed similar inhibition effects on melan-a cell tyrosinase. However, SG-8 showed a considerably lower inhibition effect on melan-a cell tyrosinase than mushroom tyrosinase. Each value represents the mean±S.D. of five experiments. ∗$p < 0.05$, compared with control.

sublines, and provides an excellent parallel non-tumorigenic line for examining a melanoma.[21] A tyrosinase inhibition test was performed with the SG-3 and SG-8 using extracted melan-a cell tyrosinase (Fig. 6).

The activity of melan-a cell tyrosinase was inhibited by SG-3 and SG-8. SG-3 showed similar inhibition effects to that observed with mushroom tyrosinase. However, the effects of SG-8 on melan-a cell tyrosinase were lower than that on mushroom tyrosinase.

Melanin Production and Cell Viability in Cultured Melan-a Cells Melan-a cells were used to investigate the inhibition of melanin production in melanocytes by SG-3 and SG-8. This study measured the level of cell toxicity and melanin production from melanocytes for the SG-3 and SG-8 using the melan-a cell line. The melan-a cells were treated with 2 μM, 3 μM, 4 μM, 5 μM, and 10 μM of SG-3 and of SG-8 as well as 20 μM SG-3 for 72 h (Fig. 7). At 10 μM, SG-3 inhibited melanin biosynthesis as much as PTU which was used as the positive control, without any significant cytotoxicity. Over 20 μM, SG-3 exhibited cytotoxicity. However, SG-8 exhibited cytotoxicity in a dose-dependent manner.

Discussion

Melanin is an important skin pigment. Skin pigmentation is caused by melanin storage in keratinocyte and the epidermis through the melanosomes in the melanocytes. Melanosomes are unique intracytoplasmic organelles in melanocytes. Melanogenesis is mainly regulated by both enzymes, tyrosinase and dopachrome tautomerase. The starting material for the biosynthesis of melanin is amino acid, tyrosine, which is oxidized to dopaquinone catalyzed by copper-containing enzyme tyrosinase.[29] Therefore, tyrosinase is one of the key enzymes in melanin synthesis, and a tyrosinase inhibitor can regulate the melanin synthesis. Selenium is essential element in various biological activities. It has been reported to be an antioxidant.[13,14] L-DOPA is oxidized to dopachrome by tyrosinase.[31] Selenium-containing compounds might decrease L-DOPA oxidation of tyrosinase.

Two selenoglycosides inhibited mushroom tyrosinase (Fig. 2). SG-3 and SG-8 had inhibitory effects on mushroom tyrosinase in a dose-dependent manner. SG-3 and SG-8 had similar inhibitory effects on mushroom tyrosinase to kojic acid. In addition, the SG-8 showed stronger inhibitory effects on mushroom tyrosinase than kojic acid at 100 μM and
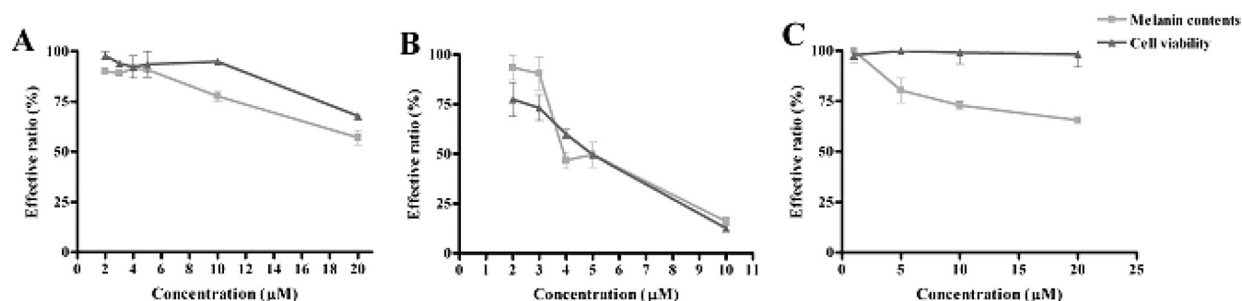
Fig. 7.   Melanin Contents and Cell Viability in Cultured Melan-a Cells Treated with SG-3 and SG-8

A: SG-3, B: SG-8, and C: PTU. (A) shows the effects of SG-3. At 10 μM SG-3 shows an effective decrease in the melanin contents without any cytotoxicity. Citotoxicity was observed at concentration >20 μM. (B) Dose-dependent increase in cell cytotoxicity. Over 10 μM SG-8, most cells did not survive. (C) PTU is a positive control. Each value represents the mean±S.D. of five experiments.

150 μM. Kojic acid is a copper ion chelater. Copper ions are important co-factor of tyrosinase. Therefore, kojic acid appears to show a competitive inhibition pattern. In the enzyme kinetic assay, SG-3 was found to be a competitive inhibitor and SG-8 was found to be an uncompetitive inhibitor.

In contrast to a competitive inhibitor, which binds to the active site of the enzyme, an uncompetitive inhibitor can only bind to the enzyme-substrate complex. It means that SG-8 may bind to any other sites, except for the active sites of mushroom tyrosinase. Because the structure of mushroom tyrosinase has not been established,[32] it is difficult to understand why the uncompetitive inhibition of SG-8 is more effective on mushroom tyrosinase than melan-a cell tyrosinase. It seems that the structure of SG-8 binding site in Melan-a cell tyrosinase may be less similar with that in mushroom tyrosinase. Therefore, SG-3 shows similar inhibition effects to animal cell tyrosinase, but SG-8 has less inhibition effects to melan-a cell tyrosinase. Melan-a cells are synergetic with the B16 melanoma and its sublines, and provide an excellent parallel non-tumorigenic line for studying the melanoma malignancy. So melan-a cell tyrosinase is useful for the identification of inhibitory effect of animal cell tyrosinase. SG-3 showed effective decrease of melanin contents without significant cytotoxicity at 10 μM on melan-a cells. Over 20 μM, SG-3 showed its cytotoxicity. But, SG-8 was highly toxic. The SG-8 showed an increase of cytotoxicity dose-dependently.

In the view of structure and activity relationship, among the selenide compounds, SG-3 showed the strongest efficacy in the inhibition of mushroom tyrosinase. Among the selenide compounds, SG-1, SG-2, and SG-11 have 6-*O*-acetyl group except SG-3. We assume that 6-*O*-acetyl group of selenoglycosides might hinder the inhibitory activity. Therefore, the most effective inhibition of SG-3 on mushroom tyrosinase may be caused by not having acetyl group at 6 position. Among the acyl-selenoglycoside compounds, SG-8 showed the strongest efficacy in the inhibition of mushroom tyrosinase. If the functional group of acyl-selenoglycoside compound is O=C–Se–CH–O, the more steric hindrance to the –CH–O side oxygen might have the more effective inhibitory activity. Unlike the selenide compounds, acyl-selenoglycoside compounds are not symmetry structure, so direct binding site may be O=C side oxygen because of its more electronegative force than CH–O side oxygen and less steric hindrance than CH–O side oxygen. Thus we assume that steric hindrance of –CH–O side oxygen make enhance

the function of O=C side oxygen. Among the acyl-selenoglycoside compounds, SG-5, SG-6, SG-7, and SG-9 have the less steric hindrance to CH–O side oxygen than SG-8. And further we assume that SG-3, selenide compound, is a competitive inhibitor of mushroom tyrosinase due to its stereochemical symmetry structure and SG-8, acyl-selenoglycoside compound, is an uncompetitive inhibitor of mushroom tyrosinase due to its stereochemical non-symmetry structure.

In 1980s, lots of whitening cosmetics and medicines, such as hydroquinones, kojic acid and its derivatives, were developed. However, the clinical effect of these materials is unsatisfactory. For example, the kojic acid is used widely to prevent the enzymatic browning of food and as cosmetic whitening reagent. Recently, Takizawa *et al.* reported that kojic acid cause hepatocarcinogenesis.[33] Therefore, kojic acid is not used widely as a food additive or in cosmetic reagents. Hydroquinones and their derivatives are widely used to treat hyperpigmentation in many countries but, they are unstable and easily oxidized.[34] Therefore a new tyrosinase inhibitor is needed. SG-8 showed so much cytotoxicity that it could not be considered as a melanin regulator. On the other hand, SG-3 showed effective regulation without cell toxicity. Accordingly, SG-3 may be a candidate for future research into its use as a potent inhibitor on tyrosinase activity.

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