Inhibitory Effects of Calycosin Isolated from the Root of *Astragalus membranaceus* on Melanin Biosynthesis

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Tyrosinase is a key enzyme for melanin biosynthesis, and hyperpigmentation disorders are associated with abnormal accumulation of melanin pigments, which can be reduced by treatment with depigmenting agents. A methanol extract of *Astragalus membranaceus* showed inhibitory activity against mushroom tyrosinase. The active compound was purified from the methanol extract of *A. membranaceus* and, following several chromatographic methods, was identified as calycosin *via* spectroscopic analysis. The results showed that calycosin exhibited tyrosinase inhibitory activity with an IC_{50} value of 38.4 μM. Moreover, calycosin showed a melanin biosynthesis inhibition zone in a culture plate of *Streptomyces bikiniensis*, which is commonly used as an indicator organism. Furthermore, calycosin dramatically reduced melanin synthesis of Melan-a cells without any apparent cytotoxicity and reduced expression of melanogenic enzyme, tyrosinase. These results suggest that calycosin may be an effective skin-lightening agent that regulates the expression of melanogenic enzymes.

Key words *Astragalus membranaceus*; calycosin; tyrosinase; melanin biosynthesis

Tyrosinase is a copper-containing enzyme that catalyzes two distinct reactions of melanin synthesis, the hydroxylation of a monophenol and the conversion of an *O*-diphenol to the corresponding *O*-quinone. In these oxidation reactions, three different forms of binuclear copper are involved in the active site.[1)] Tyrosinase is also known as a polyphenol oxidase (PPO); and the browning of some fruits, vegetables and crustaceans due to tyrosinase causes a significant decrease in their nutritional and market values.[2)] In addition, tyrosinase inhibitors have become increasingly important in medicinal and cosmetic products in relation to hyperpigmentation.[3)] A few anti-melanogenic reagents, such as monobenzene and hydroquinone, are clinically useful. Tyrosinase is responsible not only for melanization in animals but also for browning in plants.[4)] The latter case is considered to be deleterious to the color quality of plant derived foods and beverages and results in a loss of nutritional value. Thus, tyrosinase inhibitors have a promising role in medicine, cosmetics and in the food industry, which makes the identification of novel tyrosinase inhibitors extremely important.[5)] Therefore, there is a concerted effort to search for naturally occurring tyrosinase inhibitors from plants, as plants constitute a rich source of bioactive chemicals and many of them are largely free from harmful adverse effects.[6)]

A number of the known natural melanin synthesis inhibitors, including arbutin and kojic acid, have been the focus of research and are currently being utilized as cosmetic additives.[7—10)] However, it is clearly necessary to find safer and more effective skin-whitening agents due to the carcinogenic potential of kojic acid as well as its weak whitening effects. A great deal of attention has focused continuously on the application of natural products in the cosmetics industry.[11—13)] In the course of *in vitro* mushroom tyrosinase inhibitory screening on the various extracts from medicinal herbs, a methanol extract of *Astragalus membranaceus.* was found to show tyrosinase inhibitory activity. *A. membranaceu* is a medicinal herb that is widely used in the treatment of inflammatory diseases, tumors, radical scavenger activity, various cardiovascular diseases, and neuroprotective activity.[14—19)] Its active compound was purified using bioactivity-guided fractionation and identified as calycosin by spectroscopic methods. Calycosin, an isoflavonoid, is the major active component in *Astragali Radix*. Recently, it was chosen as a marker substance for the chemical evaluation or standardization of *Astragali Radix* and its products.[20)] The compound reported shows bioactivities such as anti-tumor, anti-oxidation and neuroprotective acvitities.[19,21)] However, little work has been carried out regarding the effects of active compounds isolated from *A. membranaceu* against mushroom tyrosinase activity, despite its excellent pharmacological action. Therefore, the effects of calycosin were evaluated on Melan-a cells. Calycosin markedly inhibited melanogenesis of Melan-a cells by regulating the expression of the melanogenic enzyme, tyrosinase.

MATERIALS AND METHODS

Materials Mushroom tyrosinase, L-tyrosine, 12-*o*-tetradecanoylphorbol-13-acetate (TPA), and 1-phenyl-2-thiourea (PTU) were all purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The plastics used in the tissue culture were purchased from Falcon (Becton Dickinson, Franklin Lakes, NJ, U.S.A.), and the media and additives were obtained from Gibco (Grand Island, NY, U.S.A.).

Extraction and Isolation Dried *A. membranaceus* (1 kg) were percolated with MeOH at 25 °C for 2 weeks. The residue obtained after the removal of the solvent (43.57 g) was diluted with H_2O (500 ml) and extracted using EtOAc (500 ml×3). The extract left a dark syrup (2.03 g) was loaded onto a Sep-pak C_{18} cartridge (Waters, 5 g). An active fraction (580 mg) was then purified *via* Sephadex LH-20 column chromatography using a solvent system consisting of MeOH and H_2O (6 : 4). The final purification was accomplished *via* HPLC (C_{18} column) with a 30% aqueous acetonitrile (YMC-ODS-AM 250×6 mm, 10 μm; the flow rate, 1.5 ml/min; detection, UV at 220 nm) solvent system. This resulted in pure

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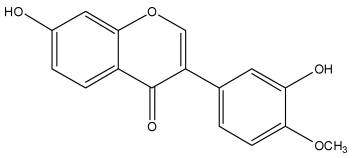

Fig. 1. The Structure of Calycosin

compound **1** (3.6 mg). The structure of the purified substance was determined on the basis of the spectral data (^{1}H- and ^{13}C-NMR, ESI-MS).

Therefore, the chemical structure of this compound was identified as calycosin (Fig. 1) in a comparison of the spectral results of a previous study data with the literature values.[22)]

Mushroom Tyrosinase Inhibitory Activities The reaction mixture for mushroom tyrosinase (E.C. 1.14.18.1, Sigma) activity consisted of 150 μl of 0.1 M phosphate buffer (pH 6.5), 3 μl of sample solution, 8 μl of mushroom tyrosinase (2100 unit/ml, 0.05 M phosphate buffer at pH 6.5), and 36 μl of 1.5 mM L-tyrosine. Tyrosinase activity was determined by reading the optical density at 475 nm on a microplate reader (Bio-Rad 3550, Richmond, CA, U.S.A.) after incubation at 37°C for 20 min. The inhibitory activity of the sample was expressed as the concentration that inhibits 50% of the enzyme activity (IC_{50}).[23)]

Inhibitory Effect of Melanin Production in *Streptomyces bikiniensis* A preserved culture of *S. bikiniensis* NRRL B-1049 was inoculated into a Papavizas' VDYA agar slant containing 200 ml of V-8 juice (Campbell Soup Co., Camden, NJ, U.S.A.), 2 g of glucose, 2 g of yeast extract (Difco), 1 g of $CaCO_3$, 20 g of agar (Difco), and 800 ml of distilled water, adjusting the pH to 7.2. After 2 weeks of incubation at 28 °C, the spore suspension obtained was transferred to a series of sterile microtubes. A volume of 0.4 ml of the suspension of *S. bikiniensis* spores was then added to agar medium ISP No. 7 (40 ml) which had been supplemented with 0.2% Bacto-yeast extract (Difco). This was spread over the surface of the agar uniformly with a glass hockey bar. After the surface of the agar had dried, a paper disc (8 mm diameter) soaked with the sample solution was positioned on the agar plate. The plate was incubated at 28 °C for 48 h and the zone of melanin formation was measured as the diameter in mm from the reverse side of the plate.[24)]

Cell Cultures The Melan-a cell (Murine melan-a melanocyte) is originally derived from C57BL/6 J (black, a/a) mice. It was received as a gift from Prof. Dorothy C. Bennett (St. George's Hospital Medical School, London, U.K.). Melan-a cells are close to melanocytes *in vivo* in character. It is widely used as a suitable substitute for normal primary mouse melanocytes in melanin metabolism tests.[25)] This cell line was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), streptomycin–penicillin (100 μg/ml each) and 200 nM 12-*o*-tetradecanoylphorbol-13-acetate (TPA), a potent tumor promoter, at 37 °C in 5% CO_2. Cells were subcultured every 3 d up to a maximal passage number of 40. Confluent monolayers of melanocytes were harvested with a mixture of 0.05% trypsin and 0.53 mM ethylenediaminetetraacetate (EDTA) (Gibco BRL, Grand Island, NY, U.S.A.).

Cell Viability Assay Cell viability was determined *via* crystal violet staining. After 4 d of incubation with the test substances, the culture medium was removed and replaced with 0.1% crystal violet in 10% ethanol. The cells were then stained for a further 5 min at room temperature and were then rinsed three times. The crystal violet retained by the adherent cells was extracted using 95% ethanol, and absorbance was determined at a wavelength of 590 nm.

Measurement of Melanin Content The cells were seeded in a 24-well plate (Falcon, U.S.A.) at a density of 1×10^5 cells per well and were allowed to attach overnight. They were then incubated in a fresh medium containing various concentrations of compounds for 4 d. After the cells had been washed with phosphate-buffered saline (PBS), they were lysed with 250 μl of 0.85 N KOH and transferred to a 96-well plate. The melanin contents were estimated *via* absorbance measurements at a wavelength of 405 nm.[23)]

Western Blot Analysis The cells were grown in 6-well plates and were treated with each of the test substances. Subsequently, the cells were washed three times in ice-cold PBS, and lysed in cold lysis buffer (0.1 M Tris–HCl, pH 7.2, 1% Nonidet P-40, 0.01% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsufonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml aprotinin). An aliquot of the lysate was used to determine the protein content with a Bradford assay using BSA as a standard. Briefly, 30 μg of proteins was separated *via* 8% SDS-polyacrylamide gel electrophoresis and blotted onto polyvinyl difluoride (PVDF) membranes (Millipore, Bedford, MA, U.S.A.). The membranes were blocked with 5% non-fat skim milk in TBS-T buffer. Tyrosinase and β-actin were all detected with a rabbit polyclonal anti-αPEP7 antibody (a gift from Dr. V. J. Hearing at the National Institutes of Health, Bethesda, MD, U.S.A.) and a mouse monoclonal anti-β-actin antibody (Sigma). The cells were further incubated with horseradish peroxidase-conjugated secondary antibody. All bound antibodies were then detected using an Amersham ECL system (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.).

Statistical Analysis Data are presented as the mean± S.D. Significant differences between results were determined by Student's *t*-test. p values of <0.05 were taken to be significant.

RESULTS

Effect of Calycosin on Tyrosinase Activity and *S. bikiniensis* Melanin Biosynthesis In this study, the inhibitory effect on calycosin on tyrosinase inhibition was examined in the hope of finding a new and effective substance for skin whitening and for the prevention of hyperpigmentation. As shown in Fig. 2, calycosin clearly showed tyrosinase inhibitory activity in a dose-dependent manner. The IC_{50} value for calycosin was 38.4 μM, whereas that of the reference compound, kojic acid, was 51.5 μM. These inhibitory effects on tyrosinase activity and subsequent melanin formation were also confirmed in *S. bikiniensis*. Therefore, the inhibitory effects of calycosin on melanogenesis in *S. bikiniensis* were examined by the paper-disc diffusion method using the inhibition of melanin production. It was observed that calycosin inhibited the biosynthesis of melanin production in comparison with the known melanogenesis inhibitor arbutin

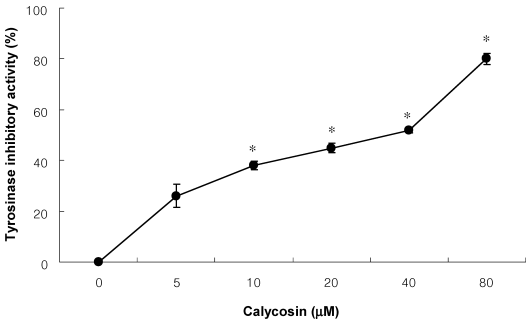

Fig. 2. Tyrosinase Inhibitory Activity of Calycosin

Tyrosinase activity was measured using L-tyrosine as a substrate. The results are averages of triplicate experiments, and the data are expressed as means±S.D. Significant differences were determined by Student's *t*-test; $*p<0.05$ compared to control.

Table 1. Inhibitory Effects on Mushroom Tyrosinase and Melanin Formation in *Streptomyces bikiniensis*

Compound	Mushroom tyrosinase IC_{50} (μM)[a]	*S. bikiniensis* NRRL B-1049 inhibition zone (mm)[b]		
		100 μg[c]	50 μg	25 μg
Calycosin	38.4±0.9	30	22	17
Kojic acid	51.5±1.2	0	0	0
Arbutin	120.9±1.5	0	0	0

a) 50% inhibitory concentration. *b*) *S. bikiniensis* was incubated with a paper disc soaked with the test samples at 28 °C for 48 h. The inhibition zone of melanin formation was measured from the reverse side of the plate. The results were reproduced with three different cultures. *c*) Test sample concentration/paper disc.

(Table 1).

Effect of Calycosin on Melanin Production and Cell Viability Desirable skin-whitening agents should inhibit the synthesis of melanin in melanosomes by acting specifically to reduce the synthesis or activity of tyrosinase. They should also exhibit low cytotoxicity and be nonmutagenic.[26] To investigate whether calycosin exerts cytotoxic effects on Melan-a cells, calycosin was applied to these cells at concentrations of 5—80 μM for 4 d, and cell viability was assessed *via* crystal violet assays. As shown in Fig. 3A, calycosin exerted no cytotoxic effects on the Melan-a cells at the tested concentrations. In addition, to examine the effects of calycosin, the melanin contents of Melan-a cells was measured. We used 1-phenyl-2-thiourea (PTU), kojic acid and arbutin as positive controls in these studies due to their known inhibitory effects on melanin synthesis. The levels of melanin in the Melan-a cells were determined to have been reduced significantly as a result of the calycosin treatment. As shown in Table 2, the 50% inhibitory concentration of melanin biosynthesis in Melan-a cells was about 40 μM by calycosin and 65.5 μM by PTU. However, arbutin and kojic acid had no effect on the melanin production of Melan-a cells. Melanin levels were significantly reduced in a dose-dependent manner by a calycosin treatment in Melan-a cells (Fig. 3B). The effective concentration of calycosin was not cytotoxic to Melan-a cells. This result suggests that the inhibitory effect of calycosin on melanin production is not due to its cytotoxicity, and that calycosin can be a safe skin-lightening agent that does not influence melanocyte growth. Therefore, calycosin is regarded as a promising skin-lightening agent as it inhibits melanin synthesis more strongly compared to other

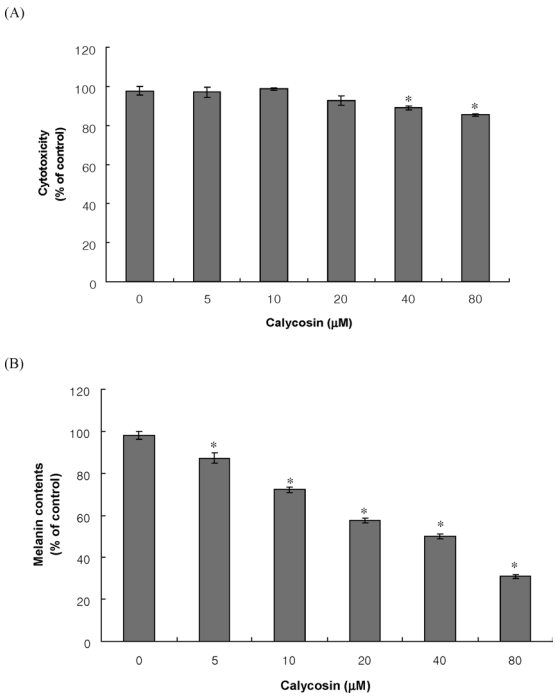

Fig. 3. Effects of Calycosin on Melanogenesis of Melan-a Cells

The cells were cultured with 5—80 μM of calycosin for 4 d. (A) Cytotoxicity, (B) melanin contents were measured in Melan-a cells, as described in Materials and Methods. The results are averages of triplicate experiments, and the data are expressed as means±S.D. Significant differences were determined by Student's *t*-test; $*p<0.05$ compared to control.

Table 2. Effects of Calycosin, PTU, and Kojic Acid on Melanin Production of Melan-a Cells

Compound	Melanin synthesis IC_{50} (μM)[a]	Cytotoxicity LD_{50} (μM)[b]
Calycosin	40.0±1.0	120±0.8
PTU	65.5±2.1	>200
Kojic acid	>200	>200
Arbutin	120±2.2	>200

a) 50% inhibitory concentration. *b*) Dose lethal to 50% of animals tested.

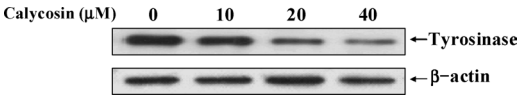

Fig. 4. Effects of Calycosin on Protein Expression of Tyrosinase of Melan-a Cells

Melan-a cells were treated calycosin (10, 20, 40 μM) for 4 d and then harvested. Total protein was extracted and subjected to Western blotting as described in Materials and Methods. Specific detection of tyrosinase was performed using αPEP7 (anti-tyrosinase).

available skin-lightening agents, such as arbutin. Accordingly, it may be a potent potential candidate as a potent potential inhibitor of tyrosinase. This projection will lead our future research into the verification of the efficacy of calycosin and its derivatives on tyrosinase inhibitory activities. Additional studies of the inhibitory mechanism and preclinical animal tests of these compounds are in progress.

Effect of Calycosin on Expression of Tyrosinase To explore the mechanism responsible for the decreased pigmentation, changes in the protein level of the important melanogenic enzyme tyrosinase were examined *via* Western blotting (Fig. 4). Melan-a cells were treated with 10, 20, and

40 μM of calycosin for 4 d and the protein level of the tyrosinase was then examined. When compared with the treated control, the protein levels of the melanogenic enzyme were reduced in a dose dependent manner.

DISCUSSION

Many studies have been performed to screen and develop depigmenting agents. Depigmenting agents can be classified on the basis of their interfering mechanisms that regulate: 1) the expression or activity of tyrosinase, tyrosinase related protein-1 (TRP-1), TRP-2, and/or peroxidase; 2) the uptake and distribution of melanosomes in keratinocytes; and 3) melanin and melanosome degradation and turnover of pigmented keratinocytes.[27)]

Among these, tyrosinase inhibitors are considered to be most useful for treating pigmentation. Thus, they are used as skin-whitening agents in the cosmetics industry.[28)] An ideal depigmenting agent should have a potent and selective skin-lightening effect on undesired pigmented regions such as freckles or chloasma without any side effects.

In this study, in an effort to develop a safe and effective skin-whitening agent, medicinal extracts with a long history of therapeutic use were screened. It was found that *Astragalus membranaceus* has strong mushroom tyrosinase inhibitory activity. The active compound was purified and identified as calycosin.

Additionally, many recent studies have investigated the relationships between the chemical structure and biological activity of flavonoids in several biological systems.[29)] The *ortho*-hydroxylation at the B-ring, the number of free hydroxyl groups, the C2–C3 double bond at the C-ring, and the presence of a 3-hydroxyl group are usually listed as important conditions for high antioxidant activity.[30)] However, few studies have investigated the biological effect of methoxylated flavonoids.

We previously reported that haginin A, a metabolite of *Lespedeza cyrtobotrya*, exhibits a strong hypopigmentary effect in Melan-a cells and inhibits melanin synthesis.[23)] In addition, haginin A and calycosin were both observed to have a methoxy group at the B-ring. These findings reinforce the idea that methoxy groups at the B-ring might enhance the melanin synthesis inhibition of flavonoids.

Melanogenesis is a major function of melanocytes. To investigate the effect of calycosin on melanin production, we treated melanocytes with calycosin and observed a dramatic inhibition of melanin content upon increasing the concentration of calycosin in the culture medium. We then compared the effects of calycosin with those of PTU, kojic acid, and arbutin as positive controls in these trials because of their known inhibitory effects on melanin synthesis.[23)] At a concentration of 40 μM of calycosin, the melanin content of Melan-a cells was reduced by almost 50% compared with an untreated control. Our results show that the inhibitory effect of calycosin on melanin was stronger than that of PTU. Compounded with the fact that calycosin exhibited no cytotoxic activity in our study, it can be surmised that calycosin may prove quite useful as a natural depigmentation agent.

To investigate the mechanism underlying the inhibition of melanogenesis, the effects of calycosin on the expression of tyrosinase protein were examined *via* Western blot analysis with a specific antibody against tyrosinase. As shown in Fig. 4, the levels of tyrosinase protein expression were decreased after a treatement with calycosin.

Several intracellular signaling pathways have been reported to involve the expression of tyrosinase. The activation of microphthalmia-associated transcription factor (MITF), a transcription factor that regulates tyrosinase gene expression, is known to be a critical step during melanogenesis.[23)] Therefore, it is necessary to examine whether calycosin decreases the expression of the melanogenic protein by regulating MITF in a further study.

In conclusion, this study demonstrates that calycosin isolated from the roots of *Astragalus membranaceus* decreases melanin production by regulating tyrosinase. Moreover, these results suggest that calycosin should be viewed as a potential skin-whitening agent.

Acknowledgments This research was supported by a grant (PDM1600526) from Plant Diversity Research Center of the 21st Century Frontier Research Program, which is funded by the Ministry of Science and Technology of the Korean Government and supported by a Grant (Code # 2007030134039) from BioGreen 21 Program, Rural Development Administration, Republic of Korea.

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