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Synthesis and Evaluation of Coumaroyl Dipeptide Amide as Potential Whitening Agents

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Coumaroyl dipeptide amide, Coumaric acid-LG-NH_2, was prepared successfully using the solid-phase method, and its efficacy as a skin whitening agent was studied. Coumaric acid-LG-NH_2 was prepared with Rink-amide resin, and 96.354% of purity was obtained. Using MTT assay and LDH release assay, we found that it exhibited very low cytotoxicity. And, we found that Coumaric acid-LG-NH_2 inhibited tyrosinase activity dose-dependently and showed superior tyrosinase inhibitory activity to well-known whitening agent, arbutin. IC_{50} value of Coumaric acid-LG-NH_2 was 182.4 μM, and IC_{50} value of arbutin was 384.6 μM. Also, in measurement of melanin contents using B16F1 melanoma cell lines, Coumaric acid-LG-NH_2 reduced melanin production induced by α-MSH statistically significant, and showed superior melanin inhibitory activity to *p*-coumaric acid or arbutin. In addition, Coumaric acid-LG-NH_2 reduced MC1R mRNA expression level. Thus, we concluded that MC1R pathway is the significant pathway of Coumaric acid-LG-NH_2, and Coumaric acid-LG-NH_2 has great potential to be used as novel whitening agents.

Key Words : Coumaroyl dipeptide amide, Tyrosinase, Melanin, Whitening agent

Introduction

Melanin is very important in protecting skin against UV light. Also, it determines skin color and several aspects of phenotypic appearance. However, hyperpigmentation which usually presents as age spots, uneven color, freckles and sometimes melasma has become an object of public concern.[1] Thus, skin whitening agents, such as retinoic acid, hydroquinone, arbutin, and so on, are developed for the prevention and treatment of irregular hyperpigmentation.[2]

Tyrosinase is the rate-limiting enzyme which control melanin biosynthesis. Melanogenesis is initiated by tyrosine that is metabolized into DOPA and then dopaquinone by tyrosinase.[3,4] So, the majority of whitening agents are tyrosinase inhibitors. But recently, various mechanisms or approaches for skin whitening agents are studying such as inhibition of melanosome transfers, acceleration of epidermal turnover and desquamation, antioxidants, and so on.

Melanocortin receptor 1 (MC1R) is a G-protein-coupled receptor (GPCR) with seven trans-membrane domains. MC1R is the high affinity receptor for alpha-melanocyte stimulating hormone (α-MSH) that is expressed specifically on melanocytes.[5] α-MSH is the major melanocortin for skin pigmentation. It is well known that á-MSH stimulates de novo melanin synthesis in mammalian melanocytes, and induces eumelanin synthesis.

We synthesized Coumaroyl dipeptide amide, Coumaric acid-LG-NH_2, using the solid-phase method. Peptides generally have biocompatibility and high activity, but also have relatively low stability due to its fast biodegradation rate. Therefore, C-terminal amidation or N-terminal capping using small organic material was used to decrease peptide biodegradation rate.[6] *p*-Coumaric acid was used as a small organic material for N-terminal capping of peptide. *p*-Coumaric acid is a hydroxycinnamic acid, an organic compound that is a hydroxy derivative of cinnamic acid. Dipeptide sequences of Coumaric acid-LG-NH_2 were originated from Melanostatin. Melanostatin (MSH release-inhibiting hormone or MIF-1) is an endogenous peptide fragment derived from cleavage of the hormone oxytocin. Melanostatin is a chain of three amino acids linked by peptide bonds. Its structure has been shown to be L-prolyl-L-leucyl-glycine amide (Pro-Leu-Gly-NH_2).[7] It is known to inhibit the release of α-MSH.[8] In our previous study,[9] various types of 2 to 3 amino acid sequences originated from Melanostatin sequences were used for small molecules-peptide conjugates synthesis. Their melanogenesis inhibitory activities were investigated, and we found that Leu-Gly sequences showed melanin inhibitory activity.

In this study, efficacy of Coumaric acid-LG-NH_2 as skin whitening agent was studied. Melanogenesis was induced by α-MSH in melanocytes. Also, B16F1 murine melanoma cells were used because these cells naturally express MC1R.[10]

Experimental

Materials. Fmoc-amino acid was purchased from Bead-Tech (Seoul, Korea). Benzotriazole-1-yl-oxy-tris-(dimethyl-amino)-phosphonium hexafluorophosphate (BOP reagent), hydroxybenzotriazole (HOBt), diisopropylethylamine (DIEA), *p*-coumaric acid were purchased from TCI (Tokyo, Japan). Rink amide resin and 2-Chlorotrityl (CTC) resin were obtained from GLS (shanghai, China). Mushroom tyrosinase (EC 1.14.18.1), L-tyrosine, α-melanocyte stimulating hormone

(α-MSH), synthetic melanin, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and albumin from bovine serum (BSA) were purchased from Sigma (St. Louis, MO, USA). Protein assay reagent was obtained from Cytoskeleton (Denver, USA). Non-radioactive LDH assay kit was purchased from Promega (Madison, WI, USA).

Coumaroyl Dipeptide Amide Synthesis. Fmoc-Gly-OH (1.5 equiv.), BOP (1.5 equiv.), HOBt (1.5 equiv.) and DIPEA (1.5 equiv.) were added to a swollen Rink amide resin (0.5 mmol of NH_2/g, 1 g, 0.5 mmol) in NMP (10 mL) and the reaction mixtures were stirred for 3 h. The resins were filtered, washed, and dried under high vacuum. The loading levels of the resulting resins were 0.45-0.47 mmol/g, which were confirmed by Fmoc titration. After deprotection of Fmoc with 20% piperidine in NMP for 30 min, the next amino acid (Fmoc-Leu-OH) were coupled to the resin by the general BOP-mediated solid-phase peptide synthesis protocol. Then, *p*-coumaric acid (2 equiv.) was added to the resin-bound dipeptide in NMP with BOP (2 equiv.), DIPEA (2 equiv.) and the mixture was stirred for 3 h. The resins were filtered, washed, and dried under high vacuum. Finally, the resin was treated with reagent K (82.5% TFA : 5% phenol : 5% H_2O : 5% thioanisole : 2.5% EDT) for 10 min. The resin was filtered and washed. The collected filtrate was evaporated and precipitated with cold ether to give a product. The product was filtered, washed with diethyl ether, and dried under a high vacuum to give the desired coumaroyl dipeptide amide. The purity and molecular weight of Coumaric acid-Leu-Gly-NH_2 were analyzed using HPLC (Shimadzu LC-10Avp system, Shimadzu, Japan) and MALDI-TOF-MS (Kratos Kompact matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Shimadzu, Japan).

Cell Culture. B16F1 murine melanoma cell lines used in this study were purchased from Korea Cell Line Bank (KCLB). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Bio Whittaker, Walkersville, MA) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37 °C under 5% CO_2. 80% confluent monolayer cells were harvested with 0.05% trypsin-EDTA (Gibco BRL, Grand Island, NY). HaCaT cell lines (human immortalized keratinocytes) were cultured in DMEM supplemented with 5% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37 °C under 5% CO_2.

MTT Assay. HaCaT cells cultured in 96-well plates were treated with or without sample for 24 h. HaCaT cells were treated with 10 μL MTT solution (5 mg/mL in PBS) for 4 h. The resultant formazan crystals were dissolved in 150 μL DMSO. Absorbance was measured at 540 nm using microplate reader (SpectraMax 340PC, Molecular Devices, USA).

LDH Release Assay. HaCaT cells cultured in 96-well plates were treated with or without sample for 24 h. Lactate dehydrogenase (LDH) is released into the cell culture medium upon damage of the plasma membrane. CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega, USA) was used. Absorbance was measured at 490 nm using microplate reader (SpectraMax 340PC, Molecular Devices, USA).

Mushroom Tyrosinase Assay. Tyrosinase activity was determined using Jang's method with some modification.[11,12] 100 μL of 10 mM L-tyrosine and 50 μL of test sample solution were mixed. Then 50 μL of mushroom tyrosinase (2400 units/mL) was added. Control was replaced 50 μL of L-tyrosine with 50 μL of 0.1 M sodium phosphate buffer. The test mixture was incubated for 10 min at 37 °C, and the amount of dopachrome produced in the reaction was measured at 475 nm using microplate reader (SpectraMax 340PC, Molecular Devices, USA). IC_{50} represents the concentration of test sample that is required for 50% tyrosinase activity inhibition.

$$\text{Tyrosinase inhibition rate (\%)} = 100 - (b-b')/(a-a') \times 100$$

a: absorbance of blank solution after reaction.

b: absorbance of test solution after reaction.

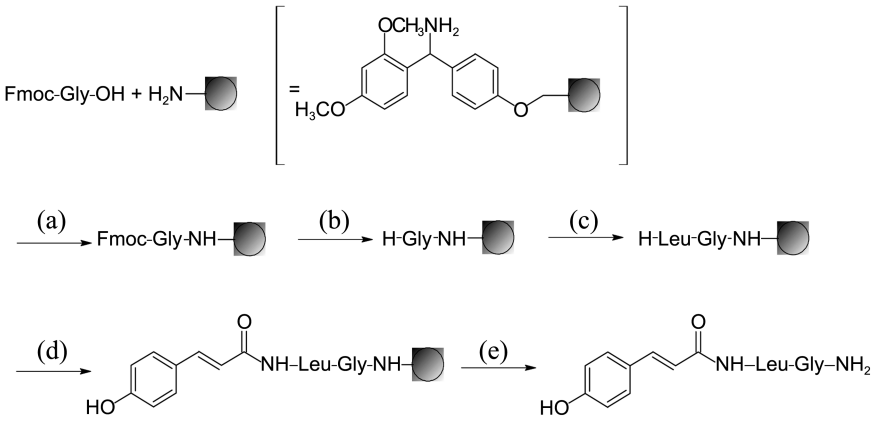
a' and b': absorbance when tested with the addition of buffer instead of tyrosinase.

Measurement of Melanin Contents. B16F1 murine melanoma cells were seeded into each well of 6-well plate at the concentration of 1×10^5 cells/well. Culture media were changed after 24 h, and samples and 1 μM of α-MSH were treated in each well. After 3 days of culture, cells were washed using PBS buffer and obtained using 0.25 M trypsin-EDTA solution. Cell mixtures were centrifuged in the condition of 2500 rpm, 4 °C for 10 min. The supernatant solution was removed, and pellets were dried under 60 °C oven. Dried pellets were solubilized in 200 μL of 1 N NaOH + 10% DMSO solution, and then sonicated at 60 °C for 1 h to dissolve melanins. The relative quantity of melanin was estimated by the absorbance at 490 nm and correlated to the quantity of proteins. The quantity of proteins was estimated by the standard curve of bovine serum albumin (BSA). Protein assay reagent was utilized to assay and absorbance was measured at 595 nm using microplate reader (SpectraMax 340PC, Molecular Devices, USA).

Quantitative Real-time PCR Analysis. Total RNA was extracted from B16F1 murine melanoma cells using RNA-spin™ total RNA extraction kit (iNtRON, Korea). Complementary (cDNA) was synthesized from 1 μg of total RNA using Maxime™ RT Premix (oligo dT_{15} Primer) (iNtRON, Korea). Gene expression levels were measured using ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Inc., CA, USA). The primer sequences were as follow: MC1R (melanocortin receptor 1) forward 5'-TGACCTGAT GGTAAGTGTCAGC-3' and reverse 5'-ATGAGCACGTCA ATGAGGTTG-3'; tyrosinase forward 5'-GGCCAGCTTTC AGGCAGAGGT-3' and reverse 5'-TGGTGCTTCATGGGC AAAATC-3'; and β-actin forward 5-TGTACCCAGGCATT GCTGAC-3' and reverse 5'-CTGCTGGAAGGTGGACAG TG-3'.

Results and Discussion

Coumaroyl dipeptide amide was prepared with Rink-amide resin (Scheme 1). In Scheme 1, Fmoc-Gly-OH was quantitatively introduced to the Rink amid (0.3-0.5 mmol/g) resin



Scheme 1. Coumaroyl dipeptide amide synthesis. Reagents and conditions: (a) BOP (benzotriazole-1-yloxy-tris(dimethylamino)-phosphonium hexafluorophosphate), 1-hydroxybenzotriazole (HOBt), DIPEA, NMP; (b) 20% piperidine in NMP; (c) repeat (a) and (b) with Fmoc Leu-OH; (d) *p*-coumaric acid, BOP, HOBt, DIPEA; (e) Reagent K (82.5% TFA : 5% phenol : 5% H_2O : 5% thioanisole : 2.5% EDT).

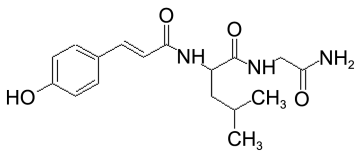


Figure 1. Chemical structure of Coumaric acid-LG-NH_2.

using the general protocol of BOP-mediated coupling method, and then introducing Fmoc-Leu-OH with same method afforded resin-bound dipeptides. *p*-Coumaric acid was introduced to free amine of above resin-bound peptide, and coumaroyl dipeptide amide, Coumaric acid-Leu-Gly-NH_2, was obtained by cleavage solution (reagent K). The product was purified with cold ether precipitation method. Figure 1 shows the chemical structure of Coumaric acid-Leu-Gly-NH_2 (Coumaric acid-LG-NH_2). HPLC and MALDI-TOF-MS were used to analyze the compound. Figure 2(a) shows HPLC chromatogram. 10 μL (0.5 mg/mL) of sample was loaded onto the C18 analytical column (Shimadzu, Japan). Solvent A (0.1% TFA/H_2O) and Solvent B (0.1% TFA/Acetonitrile) were used to make 35 min gradient. The gradient profile was 35 min gradient from 5% to 65% buffer B. Flow rate was 1 mL/min, and the wavelength of UV detector was 230 nm. Major peak was obtained at RT 18.258 min and peak area % was 96.354%. Figure 2(b) shows MALDI-TOF-MS spectrum. Coumaric acid-LG-NH_2 was observed at *m/z* 333.6.

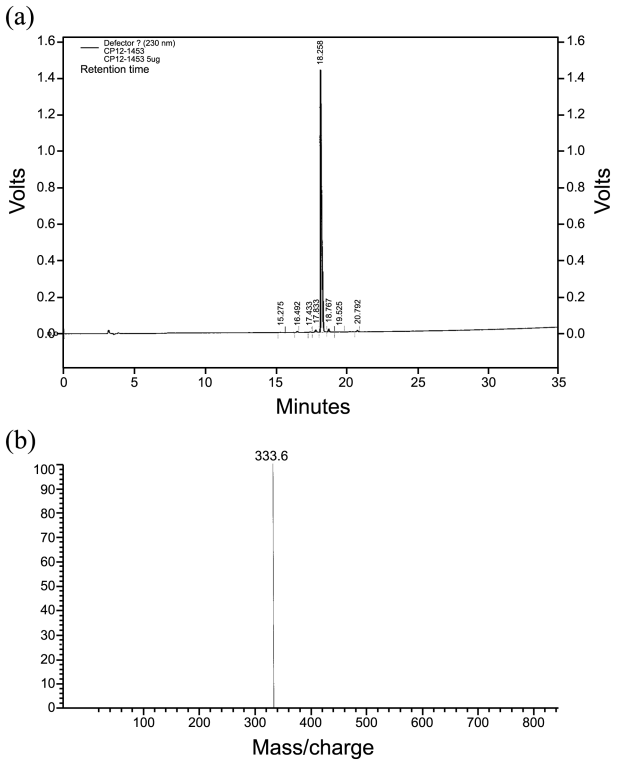


Figure 2. HPLC and MALDI-TOF-MS data of Coumaric acid-LG-NH_2. (a) HPLC chromatogram. Solvent A (0.1% TFA/H_2O) and Solvent B (0.1% TFA/Acetonitrile) were used to make 35 min gradient. (b) MALDI-TOF-MS spectrum.

To investigate cytotoxic effect of Coumaric acid-LG-NH_2, MTT assay and LDH release assay were performed. MTT assay detects living, but not dead cells and the signal generated is dependent on the degree of activation of the cells.[13] As shown in Figure 3, although cytotoxicity of Coumaric acid-LG-NH_2 was higher than *p*-coumaric acid and arbutin, it exhibited very low cytotoxicity at concentrations of up to 156 μM. Cell viability at concentration of 156 μM was 84.4%. Figure 4 shows the result of LDH release assay. LDH is an enzyme which is released into the cell culture medium upon damage of the plasma membrane. In this assay, Coumaric acid-LG-NH_2 exhibited very low cytotoxicity at concentrations of up to 313 μM, and cytotoxicity at concentration of 313 μM was 12.3%. Up to 313 μM, cytotoxicity of Coumaric acid-LG-NH_2 was similar to *p*-coumaric acid or arbutin.

Tyrosinase is key enzyme in melanogenesis Tyrosinase is the rate-limiting enzyme which control melanin biosynthesis. Melanogenesis is initiated by tyrosine that is metabolized into DOPA and then dopaquinone by tyrosinase. The method used to evaluate the degree of tyrosinase inhibition is widely

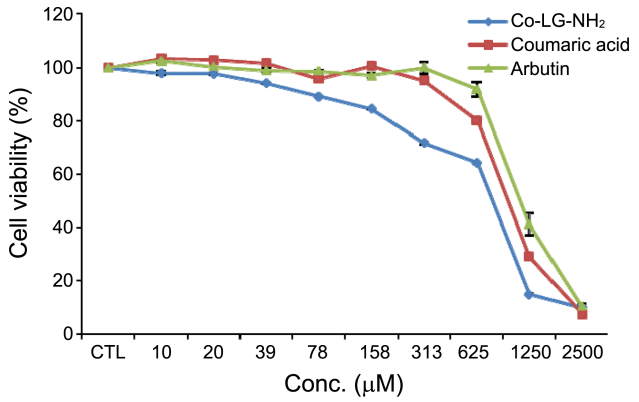


Figure 3. Cytotoxicity by MTT assay. Absorbance was measured at 540 nm using microplate reader. The error bars indicate S.E.M. (n = 2).

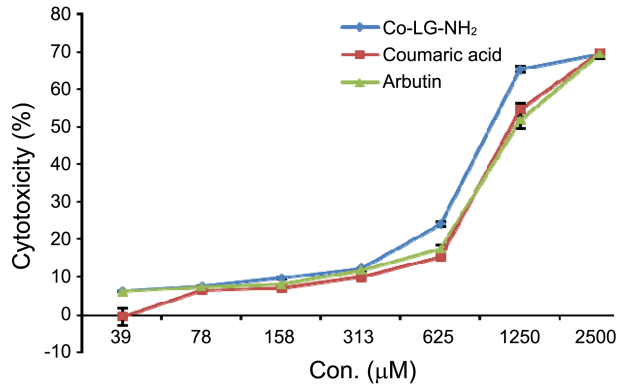


Figure 4. Cytotoxicity by LDH release assay. Absorbance was measured at 490 nm using microplate reader. The error bars indicate S.E.M. (n = 2).

used in the screening of whitening agents. To investigate tyrosinase inhibitory activity of Coumaric acid-LG-NH_2, mushroom tyrosinase (enzyme) and L-tyrosine (substrate) were used. Tyrosinase inhibitory activity of Coumaric acid-LG-NH_2 was compared with it of well-known tyrosinase inhibitor, arbutin. Figure 5 shows the result of mushroom tyrosinase assay. Both Coumaric acid-LG-NH_2 and arbutin inhibited tyrosinase activity dose-dependently. However, Coumaric acid-LG-NH_2 showed superior tyrosinase inhibitory activity to arbutin. At concentrations of 500 μM, Coumaric acid-LG-NH_2 inhibited tyrosinase activity 78.7% whereas arbutin inhibited tyrosinase activity 57.9%. IC_{50} value of Coumaric acid-LG-NH_2 was 182.4 μM, and IC_{50} value of arbutin was 384.6 μM.

Evaluation of melanogenesis inhibitory activity using melanocytes was also performed. It can be performed by measuring the amount of melanin produced. In this study, B16F1 melanoma cell lines were used, and α-MSH was used as a stimulant of melanogenesis. After treated with 1, 10, 50, 100 μM of test compound and 1 μM of α-MSH for 3 days, melanins from cell pellets were obtained. Because α-MSH showed the most melanin production at 1 μM (data not shown), 1 μM of α-MSH was used to stimulate melanogenesis in the experiment. The relative quantity of melanin

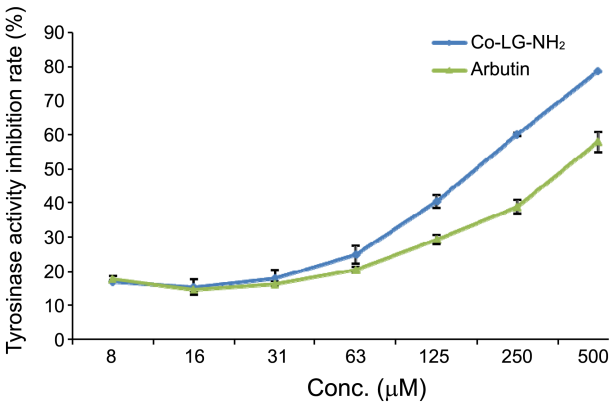


Figure 5. Results of mushroom tyrosinase assay. Tyrosinase inhibitory activity of Coumaric acid-LG-NH_2 was compared with arbutin. IC_{50} represents the concentration of test sample that is required for 50% tyrosinase activity inhibition. The error bars indicate S.E.M. (n = 2).

was estimated by the absorbance at 490 nm and correlated to the quantity of proteins. Table 1 shows the effect of Coumaric acid-LG-NH_2, *p*-coumaric acid and arbutin on melanin contents in cultured B16F1 murine melanoma cells. Lower melanin contents (%) means higher melanin inhibitory activity. Melanin inhibitory activities of Coumaric acid-LG-NH_2 were compared with *p*-coumaric acid and arbutin. All compounds decreased melanin contents dose-dependently. However, Coumaric acid-LG-NH_2 showed superior melanin inhibitory activity to *p*-coumaric acid or arbutin. At concentrations of 100 μM, melanin contents of Coumaric acid-LG-NH_2 was 70.99% whereas melanin contents of *p*-coumaric acid and arbutin were 75.99% and 79.91%, respectively. Also, at concentrations of 1 μM, melanin contents of Coumaric acid-LG-NH_2 was 83.64% whereas melanin con-

Table 1. Effect of Coumaric acid-LG-NH_2, *p*-coumaric acid and arbutin on melanin contents in cultured B16F1 murine melanoma cells. The relative quantity of melanin was estimated by the absorbance at 490 nm and correlated to the quantity of proteins. All experiments were performed in duplicate. An asterisk indicates values significantly different from the α-MSH(+) group as determined by t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Sample	Concentration (μM)	Melanin contents (% of α-MSH(+))
Coumaric acid-LG-NH_2	100	70.99**
	50	75.09***
	10	79.41***
	1	83.64*
p-Coumaric acid	100	75.99*
	50	80.15***
	10	85.99***
	1	96.50**
Arbutin	100	79.91***
	50	83.73**
	10	84.56**
	1	89.84*

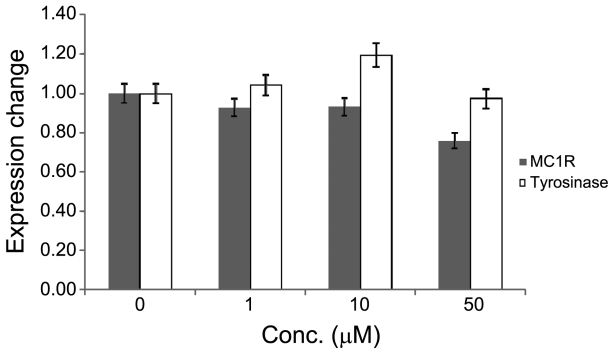


Figure 6. Effect of Coumaric acid-LG-NH_2 on the expression of MC1R and tyrosinase mRNA. B16F1 melanoma cells were cultured in the presence or absence of different concentrations of Coumaric acid-LG-NH_2 for 3 h. The relative expressions of target genes were normalized to that of β-actin.

tents of *p*-coumaric acid and arbutin were 96.50% and 89.84%, respectively. We could estimate that Coumaric acid-LG-NH_2 has more improved melanin inhibitory activity than *p*-coumaric acid. In addition, towards lower concentrations the effect was greater.

Using real-time PCR analysis, expression changes of mRNAs related to melanin biosynthesis were studied. B16F1 melanoma cells were cultured in the presence or absence of different concentrations of Coumaric acid-LG-NH_2 for 3 h. The relative expressions of target genes were normalized to that of β-actin. Figure 6 shows the effect of Coumaric acid-LG-NH_2 on the expression of MC1R and tyrosinase mRNA. Although Coumaric acid-LG-NH_2 showed tyrosinase inhibitory activity, it didn't inhibit tyrosinase mRNA expression directly. We found that Coumaric acid-LG-NH_2 reduced MC1R mRNA expression level. At a concentration of 50 μM, inhibition rate of MC1R mRNA was about 24%. MC1R is the high affinity receptor for α-MSH that is expressed specifically on melanocytes. It is well known that α-MSH stimulates de novo melanin synthesis in mammalian melanocytes, and induces eumelanin synthesis. We could estimate that Coumaric acid-LG-NH_2 play a role as MC1R antagonist, and further efforts to find mechanism are underway.

Conclusion

Coumaroyl dipeptide amide, Coumaric acid-LG-NH_2, was prepared successfully using the solid-phase method, and its efficacy as a skin whitening agent was studied. Coumaric acid-LG-NH_2 was prepared with Rink-amide resin, and 96.354% of purity was obtained. In MTT assay, it exhibited very low cytotoxicity at concentrations of up to 156 μM. Also, in LDH release assay, it exhibited very low cytotoxicity at concentrations of up to 313 μM. Therefore, cell-based experiments were performed at concentrations of less than 156 μM.

Melanogenesis inhibitory effects of Coumaric acid-LG-NH_2 have been studied. Through the results of cell-free mushroom tyrosinase assay, we found that Coumaric acid-LG-NH_2 showed superior tyrosinase inhibitory activity to well-known whitening agent, arbutin. IC_{50} value of Coumaric acid-LG-NH_2 was 182.4 μM, and IC_{50} value of arbutin was 384.6 μM. Also, in measurement of melanin contents using B16F1 melanoma cell lines, Coumaric acid-LG-NH_2 reduced melanin production induced by α-MSH statistically significant, and showed superior melanin inhibitory activity to *p*-coumaric acid or arbutin. Additionally, in study of expression changes of mRNAs related to melanin biosynthesis, we found that Coumaric acid-LG-NH_2 reduced MC1R mRNA expression level. Because MC1R is the receptor of α-MSH that stimulates de novo melanin synthesis in mammalian melanocytes and induces eumelanin synthesis, we could estimate that reduction of MC1R mRNA expression level is related to inhibition of melanogenesis. And, we concluded that MC1R pathway is the significant pathway of Coumaric acid-LG-NH_2, and further efforts to find mechanism are underway. From these studies, we concluded that Coumaric acid-LG-NH_2 has great potential to be used as novel whitening agents. Further studies to investigate whitening effect of Coumaric acid-LG-NH_2 on UV-B induced hyperpigmentation of human skin are underway.

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