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Anti-melanogenesis in B16F0 Melanoma Cells by Extract of Fermented *Cordyceps militaris* Containing High Cordycepin

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To find a novel skin whitening agent, the effect of cordycepin-enriched *Cordyceps militaris* (CMα) extract fermented by fungi on anti-melanogenesis in B16F0 mouse melanoma cells was investigated. Fermented CMα was prepared with fungi, including *Monascus purpureus* (Mp), *Aspergillus oryzae* (Ao), *Aspergillus kawachii* (Ak), and *Rhizopus oryzae* (Ro), respectively. When the content of the phenolics and the flavonoids and the activities of the antioxidant and the mushroom tyrosinase inhibition were measured in the CM fermented by Ak (AkF-CM), the highest content of the phenolics was 46 mg/g dry weight and the highest content of the flavonoids was 0.93 mg/g; the highest activity of the DPPH radical scavenging was 62.74% and the highest activity of the mushroom tyrosinase inhibition was 79.97% CMαCMα. From this result, AkF-CMα exhibited the highest mushroom tyrosinase inhibitory activity and so it was used in subsequent anti-melanogenesis. B16F0 melanoma cells were treated with 1-10 mg/ml concentrations of AkF-CMα and 200 μM arbutin as the positive control. The melanin content and cell viability of the melanoma cells by arbutin treatment decreased to 43% and 92% of the control, respectively. AkF-CMα treatment at 1, 3, and 5 mg/ml concentrations decreased the extracellular melanin release induced by IBMX treatment by 35%, 45%, and 53%, respectively. AkF-CMα showed inhibitory activity against both intracellular tyrosinase in melanoma cells and mushroom tyrosinase. AkF-CMα reduced the protein level of tyrosinase in the IBMX-stimulated cells. These results indicate that AkF-CMα suppressed the activity and protein content of cellular tyrosinase and decreased the total melanin content in cultured B16F0 melanoma cells.

Key words : *Aspergillus kawachii*, B16F0 melanoma cell, cordycepin, *Cordyceps militaris*, tyrosinase

Introduction

Melanin is synthesized in the epidermal melanosomes of melanocytes with UV radiation in human skin and is major factor to determine skin color [23]. Melanin in skin plays important roles in the prevention of sun-induced skin injury, such as age spots, freckles, and melasma [33]. Tyrosinase is the rate-limiting enzyme for melanogenesis and plays an important role in the pathway of melanin synthesis, which is the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the subsequent oxidation of DOPA to dopaquinone [14]. Therefore, the development of tyrosinase inhibitors may be useful as a skin-whitening agent in the cosmetic industry. Recently, attention has been focused on the use of natural products in the development of more safer and effective whitening agents [20]. Hence potent tyrosinase inhibitors have been isolated epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), kaemperol, quercetine, and luteolin from natural plants [12, 27].

In addition, melanin biosynthesis inhibitors were isolated from microbial fermentation products such as terrein from *Penicillium* sp. [21] and aspochalasins from solid-state culture of *Aspergillus flavipes* [9], which potently inhibited melanogenesis in melanocyte cells without cytotoxicity. And potential tyrosinase inhibitors were also produced as secondary metabolites of *Streptomyces roseolilacinus* NBRC 12815 [24], *Aspergillus oryzae* KFRI 00875 and 00888 strains [19]. We have also screened melanin biosynthesis inhibitors from the extracts and fermented products and found strong tyrosinase inhibitors using mushroom tyrosinase inhibitory assay *in vitro* [7, 22]. Moreover, *C. militaris* extract exhibit significant inhibition of tyrosinase activity [8]. *C. militaris* has long been used for nutraceutical and traditional medicines in eastern Asia countries and has been reported to have beneficial effects such as anti-inflammatory, anti-tumor, hypolipidemic,

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immunomodulatory, and antioxidant effect [34, 36]. Since *C. militaris* is widespread throughout the world, this mushroom has attracted much attention from researchers.

C. militaris was also contained the bioactive compounds such as cordycepin (3′-deoxyadenosine) and 2′-deoxycoformycin [18, 25]. Additionally, previous our study newly found that cordycepin content large quantity contained by 7.42 mg/g d.w. in crossbred cordycepin-enriched *C. militaris* JLM 0636 and it was increased by 7-fold compared to the natural *C. militaris* [5]. Dietary supplemented with cordycepin-enriched *C. militaris* protected against orotic aicd-induced hepatotoxicity and oxidative stress in Sprague-Dawley rats [5]. However, there are no reports of inhibitory effects on melanogenesis in B16F0 melanoma cells by the fermented products from solid-state culture of fungi using fruiting bodies of cordycepin-enriched *C. militaris* JLM 0636. Therefore, present study was investigated the inhibitory activity of melanin biosynthesis in B16F0 melanoma cells by the water extract of fermented cordycepin-enriched *C. militaris* JLM 0636 by fungi.

Materials and Methods

Soled-state fermentation using fruiting bodies of cordycepin-enriched *C. militaris*

The dried fruiting body of cordycepin-enriched *C. militaris* JLM0636 (CMα) used in this study were supplied by Chungwonnonsan Co., Ltd. (Gimhae, Republic of Korea). Fungal strains, *Monascus purpureus* KCCM 12002 (Mp), *Aspergillus kawachii* KCCM 32819 (Ak), *Aspergillus oryzae* KCCM 11372 (Ao), and *Rhizopus oryzae* KCCM 11273 (Ro) used for solid-state fermentation products were purchased from the Korean Culture Center of Microorganism (KCCM). Four fermented products made by cultivating these strains on moistened CMα at 30℃ under 95% humidity condition for 12 days according to previous study [6]. The culture was mixed every 12 hr. Fermented CMα was obtained by heat-drying for 6 hr and then extracted with water and stored at 4℃ until analyzed.

Measurement of total phenolic compounds

Total phenolic compound was determined according to a modified Folin-Ciocalteu method [28]. Briefly, 2 ml of Folin-Ciocalteu phenol regent was added to 10 ml of fermented CMα at 1 mg/ml and mixed. After 5 min, 2 ml of saturated sodium carbonate (Na_2CO_3) solution was added and the mixture was shaken. The optimal density of the solution was measured at 725 nm after 60 min using a UV mini 1240 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). Total phenolic content was calculated from a standard curve using various concentrations of chlorogenic acid prepared at the same time. Results were expressed as mg chlorogenic acid per g dry sample weight.

Measurement of flavonoid contents

Flavonoid content was determined by a colorimeter method [17]. Briefly, 0.25 ml of fermented CMαat 1 mg/ml was mixed with 1.25 ml of distilled water in a test tube and 0.75 ml of 5% $NaNO_2$ solution was added. After 5 min, 0.15 ml of 10% $AlCl_3 \cdot 6H_2O$ was added. After 6 min, 0.5 ml of 1 M NaOH and 0.275 ml of distilled water was added to the mixture. The mixture solution was mixed well and the absorbance was read immediately against the blank at 510 nm using a UV mini 1240 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan), and the flavonoid content was calculated from a calibration curve (R_2=0.999) obtained using (+)-catechin hydrate as a standard (20-200 μg/ml) prepared at the same time and expressed as mg catechin equivalents per g of sample weight. All extracts were analyzed in triplicate.

Evaluation of DPPH radical scavenging activity

Antioxidant activity was assayed based on the radical scavenging activity using 1,1-diphenyl-2-picryl hydrazyl (DPPH) of experimental compounds by a colorimeter method [1]. DPPH (16 mg) was dissolved in 100 ml ethanol to produce a 0.2 mM DPPH solution. The solution was filtered with Whatman filter paper No. 2 (Toyo Roshi, Tokyo, Japan). Two milliliters of DPPH solution was added to 1 ml of the fermented CMα solution containing 1 mg/ml. After 30 min of incubation at room temperature, the absorbance at 528 nm was determined using a UV mini 1240 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). The antioxidant activity was expressed as the percentage according to the following calculations:

DPPH radical scavenging activity (%)=[1-(sample absorbance $_{528\ nm}$)/control absorbance $_{528\ nm}$]×100

Melanoma B16F0 cell culture

Melanoma B16F0 cells were purchased from ATCC (Manassas, VA, USA), cultured in DMEM (13.4 mg/ml Dulbecco's modified Eagle's medium, 10 mM HEPES, 100

U/ml penicillin, 100 µg/ml streptomycin, 24 mM $NaHCO_3$, pH 7.2) containing 10% fetal bovine serum (FBS), and incubated at 37℃ under 5% CO_2 atmosphere. The cells were detached from culture dishes using 0.025% trypsin and 0.5 mM ethylenediaminetetra acetic acid (EDTA) in phosphate-buffered saline (PBS).

Cell viability assay

Cell survival was quantified by a colorimetric MTT assay that measures mitochondrial activity in viable cells. This method is based on the conversion of 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) to MTT-formazan crystal by mitochondrial enzyme as previously described [31]. Briefly, cells seeded at a density of 1.1×10^3/cell in a 96-well plate (Corning, NY, USA), were allowed to adhere overnight. The culture medium was then replaced with fresh serum DMEM and AkF-CMα at a various concentration (1-10 mg/ml). After 3 days later, MTT stock solution and phenol-red free DMEM were added to each well, and the plate was incubated at 37°C for 2 hr in an humidified 5% CO_2 incubator. After 2 hr, the medium was removed and 100 µl of DMSO was added to dissolve formazan. After 10 min, the optical density of each well was measured spectrophotometrically with a 560 nm filter. Results are shown from three experiments. Cell viability was expressed as percentages of that of control.

Measurement of melanin concentrations in 96-well plates

Melanoma B16F0 cells were seeded at a density of 2.6×10^3 cells/well in 96-well culture plates and incubated at 37℃ under 5% CO_2 atmosphere for 24 hr. The cells were then treated with AkF-CMα followed by stimulant of IBMX (100 µM) for 3days. Melanin concentrations in the cell-free culture media were spectrophotometrically measured at 400 nm using an ELISA reader. Results are shown from three experiments.

Measurement of extracellular melanin concentrations, and microscopy

Extracellular melanin release was measured as described previously with slight modification [29]. Briefly, B16F0 cells were incubated at a density of 1×10^5 cells in cell culture dish (100×20 mm, Corning Incorporated, NY, USA) overnight. IBMX (100 µM) was then added and cells were treated with various concentrations of AkF-CMα (1-5 mg/ml) in DMEM for 3 days. One hundred µl aliquots of media were then placed in 96-well plates and OD were measured at 400 nm using an ELISA reader. Melanin concentrations were expressed as percentages of that of control. Results are shown from three experiments. Before measuring the melanin content, cells were observed under a light microscope and photographed using a digital camera (Olympus, Tokyo, Japan).

Measurement of cellular melanin concentrations

Cellular melanin concentrations were measured as described previously with slight modification [29]. Briefly, B16F0 melanin cells were treated with AkF-CMα at various concentrations (1-5 mg/ml) for 3 days. After each incubation, cell pellets were washed with cold PBS and then dissolved in 1 ml of 1 N NaOH at 100℃ for 30 min and then centrifuged for 20 min at 16,000× *g*. The optical densities of the supernatants were measured at 400 nm using an ELISA reader. Standard curves of synthetic melanin (0-300 µg/ml) were prepared in triplicate for each experiment. Results are shown from three experiments.

Assay of tyrosinase activity

Tyrosinase activity was determined as described previously with slight modification [4]. Briefly, B16F0 melanoma cells were cultured with AkF-CMα at various concentrations (1-5 mg/ml) for 3 days, the cells were washed with cold PBS and lysed with Ripa buffer. Lysates were clarified by centrifugation at 10,000× *g* for 10 min. Protein concentration of supernatant was determined by the method of Bradford [3] with the Bio-Rad protein assay as specified by the manufacturer. After quantifying protein levels and the cell lysates were adjusted to the same concentration of protein with a lysis buffer. Reaction mixtures containing 90 µl of each lysate (40 µg protein), 10 µl of 10 mM L-DOPA, and 90 µl of lysis buffer were assayed on a 96-well plate. After incubation at 37°C, the absorbance was measured at 475 nm using an ELISA reader every 10 min for at least 1 hr. Arbutin was used as a positive control.

A cell-free assay system was used to test for the direct effects of AkF-CMα on mushroom tyrosinase activity. Seventy µl of PBS containing 1 mg/ml AkF-CMα were mixed with 20 µl of 200 U/ml mushroom tyrosinase and incubated at room temperature. After 10 min incubation, 10 µl of 10 mM L-DOPA was added to each reaction well. Following incubation for another 10 min at 37°C, absorbance was then measured at 475 nm using an ELISA reader. Kojic acid was

used as a positive control in cell-free assay system. The inhibitory effect of mushroom tyrosinase activity was calculated as follows: % inhibition = [(OD at 475 nm without test substance and with mushroom tyrosinase - OD at 475 nm without test substance and mushroom tyrosinase)-(OD at 475 nm with test substance and mushroom tyrosinase - OD at 475 nm with test substance and without mushroom tyrosinase)]×100

Western blot analysis

Changes of melanin synthesis related tyrosinase protein in B16F0 melanoma cells after treated with AkF-CMα at 3 and 5 mg/ml were evaluated by western blot. B16F0 melanoma cells were cultured with AkF-CMα for 3 days, the cells lysed with Ripa buffer. Lysates were clarified by centrifugation at 10,000× *g* for 10 min. After quantifying protein levels and the cell lysates were adjusted to the same concentration of protein with a lysis buffer. Proteins were separated by 8% SDS-polyacryamide gel electrophoresis, as described previously [32]. After the gels were rinsed 3 times with TBST (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20), the separated proteins were transferred electrophoretically to a nitrocellulose membrane at 4℃ for 16 hr at a constant current of 300/240 mA/cm^2. After blocking with TBST containing 10% nonfat milk for 1 hr at room temperature, the membranes were incubated with primary goat tyrosinase antibody (C-19, Santa Cruz, USA) at room temperature for 1hr and then incubated with the horseradish peroxidase-conjugated anti-goat (tyrosinase, Trp-1 and Trp-2) secondary antibodies. The equivalent amount of protein was loaded and reacted with the goat polyclonal β-actin conjugated HRP antibody (I-19 SantaCruz USA), which was used as a control for protein loading. Blotted antibodies were detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). The relative density in each reaction was quantified with a densitometer (Lumi-Imager F1, Roche, Switzerland) and a software (Lumianalyst 3.1.0).

Statistical analysis

The experimental data are presented as the mean ± SD, and were analyzed using one way analysis of variance (ANOVA).

Results and Discussion

Contents of phenolics and flavonoids and the activities of antioxidant and tyrosinase

Recently phytochemical studies have been identified flavonoids and phenolics as antioxidant ingredients in medicinal mushrooms, such as *C. militaris*, *C. sinesis*, *P. japonica*, and their fermentation preparations [16, 35]. These antioxidants are good inhibitors of tyrosinase activity and melanin production in melanoma cells [8, 15]. These effects may be related to their antioxidant activity. Thus, the contents of total phenolics and flavonoids and the antioxidant activity in fermented CMα were also evaluated in this study. According to our results, phenolics contents of fermented CMα by Mp, Ao, Ak, and Ro strains were 35.37, 32.51, 46.00, and 25.63 mg/g dry weight basis, respectively and these contents were higher than the water extract of crossbred cordycepin-enriched *C. militaris* (Table 1). Flavonoid contents of fermented CMα by Mp, Ao, Ak, and Ro strains were 0.47, 0.82, 0.93, and 0.73 mg/g dry weight, respectively (Table 1). Fermented CMα by AK (AkF-CMα) showed the highest contents of phenolics and flavonoids, but presented a similar antioxidant activity in DPPH radical scavenging. The highest tyrosinase inhibitory activity was also observed in AkF-CM α. The antioxidant activity of fermented CMα did not agree directly with their phenolic compound contents. Gu *et al.*

Table 1. The contents of phenolics and flavonoids and the activities of tyrosinase inhibition and DPPH radical scavenging of fermented cordycepin-enriched *Cordyceps militaris* extract with fungi

Fungi	Phenolic content (mg/g)	Flavonoid content (mg/g)	Tyrosinase inhibitory activity (%)	DPPH radical scavenging activity (%)
CMα	14.30±0.09	0.99±0.16	13.67±0.86	42.46±0.79
MpF-CMα	35.37±3.05	0.47±0.06^b	57.99±0.16	60.42±1.10^a
AoF-CMα	32.51±2.15	0.82±0.03^c	44.80±1.40	58.90±1.21^c
AkF-CMα	46.00±2.00	0.93±0.03^c	79.97±1.62	62.74±1.17^d
RoF-CMα	25.63±3.63	0.73±0.03^a	44.17±1.34	61.06±2.02^c

MpF-CMα: fermented cordycepin-enriched *Cordyceps militaris* (CMα) with *Monascus purpureus*, AoF-CMα: fermented cordycepin-enriched *Cordyceps militaris* (CMα) with *Aspergillus oryzae*, AkF-CMα: fermented cordycepin-enriched *Cordyceps militaris* (CMα) with *Aspergillus kawachii*, RoF-CMα: fermented cordycepin-enriched *Cordyceps militaris* (CMα) with *Rhizopus oryzae*.

reported that the water extract of fermented *C. militaris* has a strong free radical scavenging activity, indicating its antioxidant activity [15]. Chien *et al.* reported that mushroom tyrosinase activity showed less than 25% inhibitory effect in the extracts treatment (1 mg/ml) of ethanol and water from *C. militaris* mycelia and this effect was more pronounced in water extract than in ethanol extract [8]. However, the water extract of AkF-CMα showed higher inhibition effects (44.80-79.97%) on the mushroom tyrosinase than that of water extract from *C. militaris* mycelia above mentioned [8], which is acceptable to conclude that other factors may be involved in these properties. The fermented process affected the tyrosinase inhibition activity of CMα, these activities of both CMα and fermented CMα were significantly related with the quantity of phenolics.

Cordycepin, known as 3'-deoxyadenosine, is a bioactive compound present in species of fungi belonging to the genus *Cordyceps* [18]. Cordycepin has a variety of biological effects such as antioxidant activity and tyrosinase inhibition in Cloudman S-91 mouse melanoma cells [5, 13, 25]. Previous our study found that cordycepin contained large quantity in crossbred *C. militaris* JLM 0636 by 742 mg/100 g d.w. and it was higher 1.7-7-fold than in other *C. militaris* [16, 25]. Steinberg and Whittaker reported that the melanotic stimulation by theophylline in cultures of RPMI 3460 hamster melanoma cells was prevented by cordycepin [30]. Thus, the potential anti-melanogenesis effect by AkF-CMα is probably based on the antioxidant and tyrosinase inhibitory activities by cordycepin. From these results, it is concluded that AkF-CMα containing relatively higher cordycepin has more potential antioxidative and tyrosinase inhibition effects compared to the other *Cordyceps* species containing relatively lower cordycepin.

Aspergillus species such as *A. kawachii* and *A. oryzae*, have been utilized in the traditional fermented products in Korea and Japan for more than 1,000 years [2]. Futhermore, a potent antioxidant substance has recently been produced from solid-state culture silkworm and soybean fermented with *A. kawachii* and *A. oryzae* [6, 11]. Thus, another antioxidant and tyrosinase inhibitory properties of *C. militaris* may be due to the increased content of phenolic compounds through solid-state fermentation process. AkF-CMα may play an important role in inhibiting tyrosinase activity and melanin production. Therefore, AkF-CMα was further tested by melanin production and cellular tyrosinase assay in melanoma cells.

Effect of AkF-CMα on cell viability and melanin production

The effect of AkF-CMα on cell viability of B16F0 melanoma cells is shown in Fig. 1, which was determined using an MTT assay. B16F0 melanoma cells were treated with AkF-CMα of 1-10 mg/ml concentrations and arbutin of 200 μM in culture for cell viability for 3 days. Melanoma cell viability was expressed as the percentage value and was calculated with respect to that in the control cells. Arbutin as positive control showed no toxicity on cell viability by 91%, as previously reported [26]. AkF-CMα was not cytotoxicity to B16F0 melanoma cells at low concentration range from 1-5 mg/ml, but somewhat cytotoxicity showed at higher concentrations (8 and 10 mg/ml).

The effect of AkF-CMα on melanogenesis in B16F0 melanoma cells was evaluated by measurement of the melanin formation after 3 days of incubation which is shown in percentage value (Fig. 1). The melanin contents in B16F0 melanoma cells by arbutin decreased to 43% of the control. AkF-CMα showed a significant inhibitory effect on melanin content with concentration-dependent manner.

Effect of AkF-CMα on melanin synthesis

We determined the effect of AkF-CMα on the IBMX-mediated stimulation of melanogenesis in the B16F0 melanoma cells, and found that 100 μM IBMX treatments for 3 days increased the amount of melanin detected in the extracellular media compared with negative control value (Fig.

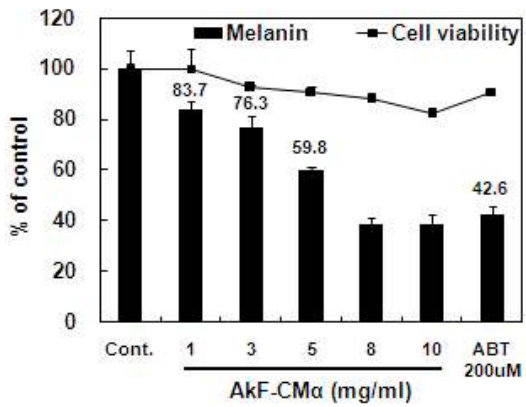

Fig. 1. Effects of AkF-CMα and arbutin on the cell viability and melanin content of B16F0 melanoma cells. AkF-CMα: the water extract of cordycepin-enriched *Cordyceps militaris* fermented by *Aspergillus kawachii*. B16F0 melanoma cells were treated with 1-10 mg/ml AkF-CMα in the presence of 100 μM IBMX for 3 days. Results are means ± SD of triplicate data.

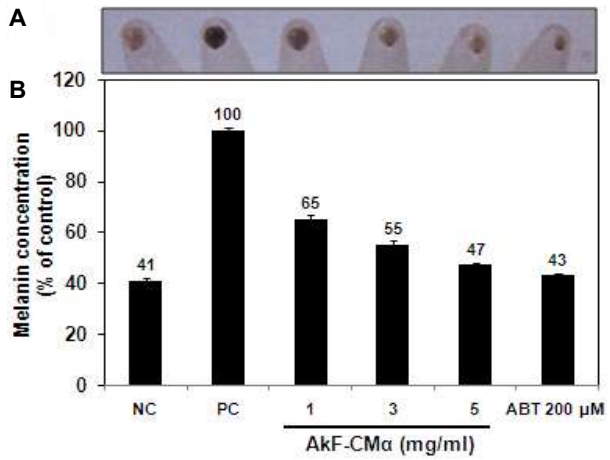

Fig. 2. Effect of AkF-CMα on melanin production in B16F0 melanoma cells. The photograph of melanin in cell pellet (A) and the melanin content in cell-free media (B) of B16F0 melanoma cells treated with 1, 3, and 5 mg/ml AkF-CMα in the presence of 100 μM IBMX for 3 days. Results are means±SD of triplicate data.

2B). AkF-CMα decreased the amount of melanin secreted into the media, reducing it to 35, 45, and 53% at 1, 3 and 5 mg/ml concentrations, respectively. Moreover, the colors of the cell pellets changed corresponding to the tested AkF-CMα (Fig. 2A). As shown in the morphological analysis of B16F0 melanoma cells under a light microscope (Fig. 3), AkF-CMα treated cells were also showed much less pigmented than positive control cells by 100 μM IBMX treatment.

Effect of AkF-CMα on the activities of mushroom and cellular tyrosinase

Tyrosinase is the key enzyme in the pathway of melanogenesis and plays a regulatory role in melanin production [14]. Because melanin synthesis is ultimately regulated by tyrosinase, the direct inhibitory effect of AkF-CMα on tyrosinase was examined by mushroom tyrosinase and cellular tyrosinase in cultured B16F0 melanoma cells. As expected, kojic acid (200 μM), which is well known as a direct tyrosinase inhibitor, showed a significant inhibition effect on the mushroom tyrosinase by 70% (Fig. 4). AkF-CMα showed potent inhibitory effect on the mushroom tyrosinase in a concentration-dependent manner and was more potent than kojic acid. Chien *et al* also reported that the 1 mg/ml water extract of *C. militaris* mycelia exerted a direct inhibitory effect on mushroom tyrosinase activity [8].

As shown in Fig. 5, AkF-CMα inhibited the cellular tyrosinase activity, which was some reduced by 1, 3, and 5 mg/ml of AkF-CMα to 15, 40, and 43% of that in the positive control cells and this effect was more potent than arbutin. Additionally, cordycepin inhibited the melanin biosynthesis and the tyrosinase activity stimulated with theophylline, an inhibitor of cAMP-degrading phosphodiesterase [30]. This result strongly indicated that the depigmenting effect of AkF-CMα can down-regulate the expression of tyrosinase.

Western Blotting of B16F0 melanoma proteins treated with AkF-CMα

Recently new developed depigmenting agents, such as fermented *Viola mandshurica* and fermented rice bran extract were shown to inhibit melanogenesis by down-regulation of melanogenic proteins such as tyrosinase and MITF [10, 22]. Thus, to investigate the inhibition mechanism of melanin

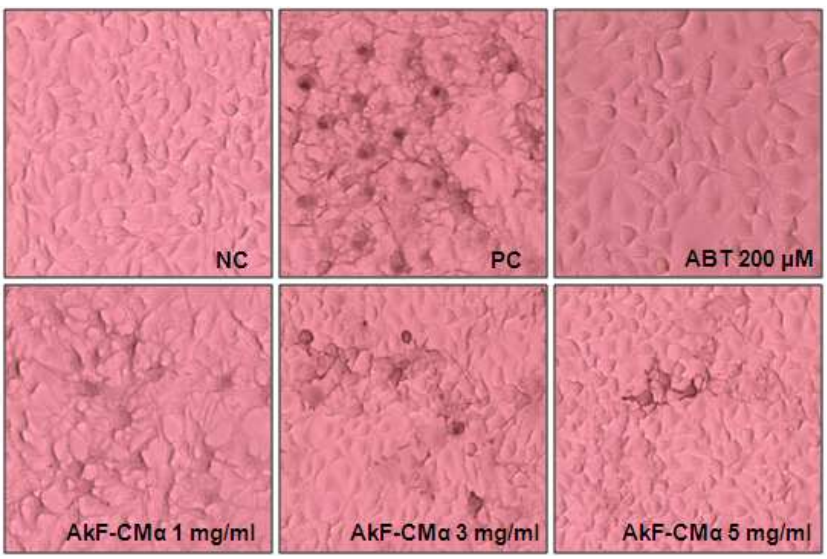

Fig. 3. Effect of AkF-CMα on cellular morphology of B16F0 melanoma cells. AkF-CMα: the water extract of cordycepin-enriched *Cordyceps militaris* fermented by *Aspergillus kawachii*. B16F0 melanoma cells were treated with 1, 3, and 5 mg/ml AkF-CMα in the presence of 100 μM IBMX for 3 days.

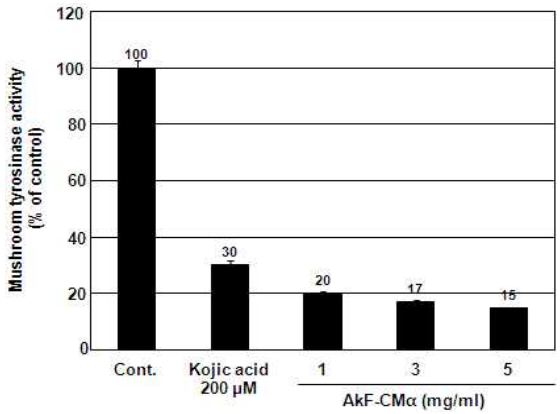

Fig. 4. Effects of AkF-CMα and kojic acid on mushroom tyrosinase inhibitory activity. AkF-CMα: The water extract of cordycepin-enriched *Cordyceps militaris* fermented by *Aspergillus kawachii*. Results are means ± SD of triplicate data.

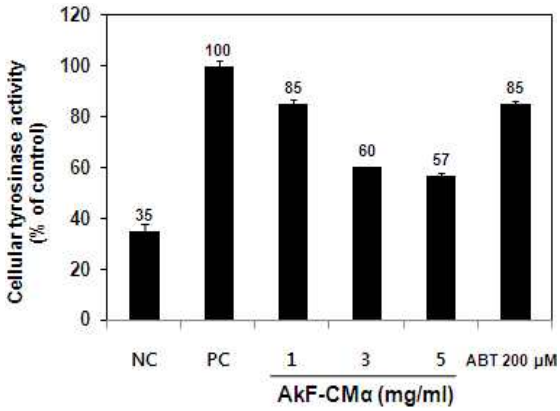

Fig. 5. Effect of AkF-CMα on cellular tyrosinase activity in B16F0 melanoma cells. AkF-CMα: The water extract of cordycepin-enriched *Cordyceps militaris* fermented by *Aspergillus kawachii*. B16F0 melanoma cells were treated with 1, 3, and 5 mg/ml AkF-CMα in the presence of 100 μM IBMX for 3 days. Results are means ± SD of triplicate data.

biosynthesis by AkF-CMα, we performed western blotting against the cell protein obtained from AkF-CMα treated B16F0 melanoma cells. The protein content of tyrosinase was significantly elevated in B16 melanoma cells stimulated with IBMX as positive control, whereas AkF-CMα treatment was significantly decreased this protein content (Fig. 6), suggesting that AkF-CMα inhibits melanogenesis primarily via tyrosinase inhibition. However, the more detailed mechanism for tyrosinase inhibition in B16F0 melanoma cells remained to be clarified.

In conclusion, present study was first demonstrated that AkF-CMα suppressed cellular tyrosinase activity and protein content and melanin production in cultured B16F0 melanoma cells.

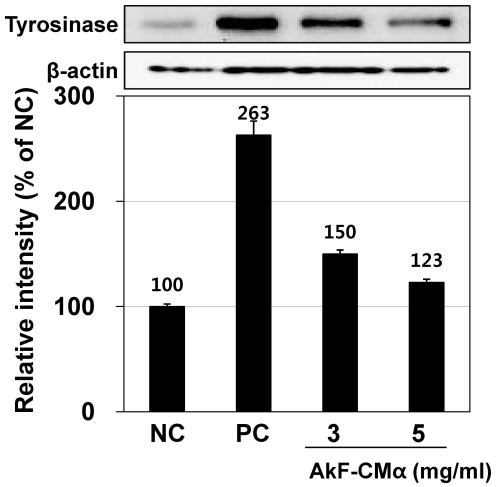

Fig. 6. Effect of AkF-CMα on the protein content of tyrosinase in B16F0 melanoma cells. AkF-CMα: The water extract of cordycepin-enriched *Cordyceps militaris* fermented by *Aspergillus kawachii*. B16F0 melanoma cells were treated with 3 and 5 mg/ml AkF-CMα in the presence of 100 μM IBMX for 3 days and protein contents were determined by western blotting.

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초록 : Cordycepin-고함유 동충하초(*Cordyceps militaris*) 발효 추출물의 미백효과

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본 연구는 *Monascus purpureus* (Mp), *Aspergillus oryzae* (Ao), *Aspergillus kawachii* (Ak) 및 *Rhizopus oryzae* (Ro) 균주로 Cordycepin-고함유 동충하초(*Cordyceps militaris*)(CMα)를 발효시켜 수용성 추출물을 얻어 페놀화합물 및 플라보노이드 농도와 항산화 및 티로시나제 저해 활성을 측정한 결과 Ak로 발효시킨 CMα (AkF-CMα)에서 각각 46 mg/g 및 093 mg/g과 6274% 및 7997%로 가장 우수한 효과를 나타내었다. 이러한 결과로부터 AkF-CMα를 선택하여 멜라닌 세포(B16F0 mouse melanoma cell)에서 미백효과를 검토하였다. 양성 대조구 arbutin 처리 B16F10 melanoma 세포는 92% 이상의 세포 생육과 43%의 멜라닌 생성 억제 효능을 보였고, AkF-CMα 1, 3 및 5 mg/ml 처리 시 멜라닌 생성은 각각 35, 45 및 53% 억제되었다. 또한 AkF-CMα은 멜라닌 세포 내 tyrosinase 활성과 mushroom tyrosinase 활성 모두를 저해시켰고, 멜라닌 생성 관련 tyrosinase 단백질 발현량도 무첨가군에 비해 처리 농도 의존적으로 억제되었다. 이상의 결과에 따라 *Aspergillus kawachii* 균주로 발효시킨 Cordycepin-고함유 동충하초(*Cordyceps militaris*)의 수용성 추출물은 미백 화장품 소재로 개발 가능성이 높은 것으로 사료된다.