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Anti-skin-aging benefits of exopolymers from *Aureobasidium pullulans* SM2001

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Synopsis

Background: There have been many attempts to search for affordable and effective functional cosmetic ingredients, especially from natural sources. Objectives: As research into developing a functional cosmetic ingredient, we investigated whether exopolymers from *Aureobasidium pullulans* SM2001 (E-*AP*-SM2001) exert antioxidant, antiwrinkle, whitening, and skin moisturizing effects. Methods: Antioxidant effects of E-*AP*-SM2001 were determined by measuring free radical scavenging capacity and superoxide dismutase (SOD)-like activity. Antiwrinkle effects were assessed through the inhibition of hyaluronidase, elastase, collagenase, and matrix metalloproteinase (MMP)-1. Whitening effects were measured by tyrosinase inhibition assay, and by melanin formation test in B16/F10 melanoma cells. Skin moisturizing effects were detected by mouse skin water content test. Results: E-*AP*-SM2001 showed potent DPPH radical scavenging activity and SOD-like effects. Additionally, hyaluronidase, elastase, collagenase, and MMP-1 activities were significantly inhibited by E-*AP*-SM2001. We also observed that E-*AP*-SM2001 effectively reduced melanin production by B16/F10 melanoma cells and mushroom tyrosinase activities. Furthermore, significant increases in skin water content were detected in E-*AP*-SM2001-treated mouse skin, as compared with vehicle-treated control skin. Notably, a mask pack containing E-*AP*-SM2001 showed a >twofold more extensive moisturizing effect compared with one containing *Saccharomycopsis* ferment filtrate. Conclusions: Our results suggest that E-*AP*-SM2001 has adequate antiaging, antiwrinkle, and whitening benefits and skin moisturizing effect. These effects involve reducing hyaluronidase, elastase, collagenase, and MMP-1 activities, as well as inhibition of melanin production and tyrosinase activities. Therefore, the antioxidant E-*AP*-SM2001 may serve as a predictable functional ingredient.

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INTRODUCTION

Skin aging is characterized by clinical signs including wrinkles, irregular dryness, dyspigmentation, sallowness, deep furrows or severe atrophy, dehydration, telangiectases, premalignant lesions, laxity and a leathery appearance of skin (1). Skin aging is a complex biological process involving intrinsic factors (genetic factors, hormonal status, and metabolic reactions, such as oxidative stress) and extrinsic factors (chronic light exposure, pollution, ionizing radiation, chemicals, and toxins). A combination of these factors causes physiological alterations and progressive changes in each skin layer, and concomitant changes in skin appearance (2).

Oxidative stress caused by reactive oxygen species (ROS) plays a pivotal role in the process of skin aging at the cellular level (3,4). ROS can block the formation of collagen, disrupt cellular renewal cycles, damage DNA, and stimulate the release of proinflammatory mediators (cytokines), which cause inflammatory skin diseases (5–8). Additionally, ROS causes the depletion of antioxidant enzymes and destroy the cytoprotective defense mechanism by weakening antioxidant systems, thus rendering the skin susceptible to oxidative injury (9–11).

To prevent and reduce skin aging, many people have used functional cosmetics that have a potent skin protective pharmacological effect (antiaging, whitening, antiwrinkle, moisturizing, and skin protective effects) (12). Currently, there are various available ingredients for functional cosmetics in the market. However, they have a number of limitations; they are too expensive and have side effects, and their exact pharmacological mechanisms are not fully understood (13). Because of these factors, various investigations have continuously attempted to search for affordable and effective functional ingredients, with fewer side effects, especially from natural sources (12,14).

Purified exopolymers from *Aureobasidium pullulans* SM2001 (E-*AP*-SM2001) comprise mostly β-1,3/1,6-glucan and other organic materials [amino acids, mono- or di-unsaturated fatty acids (linoleic and linolenic acids) and fibrous polysaccharides] (15). Recently, our team demonstrated that E-*AP*-SM2001 shows antiosteoporotic (16), anti-inflammatory (17,18), and immunomodulatory effects (19). This finding prompted us to examine the protective effects of E-*AP*-SM2001 against skin aging *in vitro* and *in vivo*. The murine B16F10 cell line was used in this study, as it can produce melanin in response to α-melanocyte stimulating hormone (α-MSH) activation (20,21).

The antioxidant effects of E-*AP*-SM2001 were determined by DPPH assay and by measuring superoxide dismutase (SOD)-like activity. Antiwrinkle effects were evaluated through the inhibition of hyaluronidase, elastase, collagenase, and matrix metalloproteinase (MMP)-1, because there is much evidence of close correlation between wrinkle formation and the loss of elasticity, collagenase, and MMP-1. Whitening effects were measured by tyrosinase inhibition assay and by measuring melanin formation in B16/F10 melanoma cells. To assess the skin moisturizing effects of E-*AP*-SM2001, skin water content was measured in Imprinting Control Region mice.

METHODS AND MATERIALS

CHEMICALS

The solution and a viscous mask pack containing E-*AP*-SM2001 were supplied by Ari-Med Therapeutics (Daegu, Korea). Based on a previously reported analysis (15), the exopolymers of E-*AP*-SM2001 are known to consist of β-1,3/1,6-glucan (17%), β-1,4-glucan (18%),

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α-(1,4)-(1,6)-glucan (8%), glucose (37.7%), galactose (0.8%), mannose (1.5%), protein (3.1%), and ash (7.2%). The standard references ascorbic acid, oleanolic acid, and kojic acid were purchased from Sigma (St. Louis, MO), and a facial treatment mask containing *Saccharomycopsis* ferment filtrate (SFF) (P&G, Japan) was obtained from a local cosmetics shop.

DPPH RADICAL SCAVENGING ACTIVITY TEST

The assay for free radical scavenging capacity was carried out according to the method reported previously by Blois *et al*. (22). The DPPH radical shows a deep violet color due to its unpaired electron, and radical scavenging capacity can be followed spectrophotometrically by the loss of absorbance at 525 nm. Briefly, 0.2 mM DPPH (Sigma, Steinheim, Germany) in a 95% ethanol solution (1 ml) was added to a sample of the stock (2 ml). Each sample solution was diluted with distilled water to final E-*AP*-SM2001 concentrations of 12.5, 25, 50, 100, 200, and 400 μg/ml or final ascorbic acid concentrations of 6.25, 12.5, 25, 50, 100, and 200 μg/ml, and the samples were then agitated. The optical density (OD) at 525 nm was measured after 10 min with a UV/V is spectrophotometer (Beckman, Munich, Germany). The free radical scavenging activity of each sample was calculated using equation (1): DPPH radical scavenging activity (%) = $100 - [(OD_s/OD_c) \times 100]$, where OD_s and OD_c are, respectively, the absorbances of the experimental sample and the vehicle-treated control at 525 nm. The results are reported in terms of IC_{50} (the concentration needed to reduce 50% of DPPH). Ascorbic acid, a representative antioxidant, was used as a control.

SOD-LIKE ACTIVITY TEST

The assay for free radical scavenging capacity was carried out according to the method reported previously by Marklund and Marklund (23). Each sample solution was diluted with distilled water to final E-*AP*-SM2001concentrations of 12.5, 25, 50, 100, 200, and 400 μg/ml or final ascorbic acid concentrations of 6.25, 12.5, 25, 50, 100, and 200 μg/ml, and the samples (0.2 ml) were then agitated with Tris-HCl buffer [50 mM Tris (hydroxymethyl)aminomethane (Sigma, St. Louis, MO) and 10 mM EDTA (Sigma), pH 8.5] (3 ml) and 7.2 mM pyrogallol (Merck, Rahway, NJ) (0.2 ml) for 10 min at 25°C. After agitation, the reactions were stopped by adding 1N HCl (Daejung, Siheung-si, Korea). Among reacted solutions, the oxidized pyrogallol was detected by measuring the absorbance at 420 nm after 10 min with a UV/V is spectrophotometer (Beckman). The SOD-like activity of each sample was calculated using equation (2): SOD-like activity (%) = $100 - [(OD_s/OD_c) \times 100]$, where OD_s and OD_c are, respectively, the absorbances of the experimental sample and the vehicle-treated control at 420 nm. The results are reported in terms of IC_{50} (the concentration needed to reduce pyrogallol oxidation by 50%). Ascorbic acid, a representative antioxidant, was used as a control.

HYALURONIDASE INHIBITION ASSAY

The assay was performed according to a method reported previously (24). Hyaluronidase reacts with the substrate hyaluronic acid to release *N*-acetyl glucosamine. In the presence of an inhibitor, the release of *N*-acetyl glucosamine, which is monitored by measuring the

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absorbance at 600 nm, is reduced. The inhibitory activity of E-*AP*-SM2001 (12.5, 25, 50, 100, 200, and 400 μg/ml) was compared with that of oleanolic acid (6.25, 12.5, 25, 50, 100, and 200 μg/ml) as a standard under exactly the same experimental conditions. The 600 nm value of intact undigested hyaluronic acid was set at 100%. The OD at 600 nm was measured after 15 min with a 96-well microplate reader (Tecan Sunrise, Männedorf, Switzerland) and the hyaluronidase inhibitory activity of each sample was calculated using equation (3): Hyaluronidase inhibitory activity (%) = $100 - \{[(OD_s + OD_c)/OD_c] \times 100\}$, where OD_s and OD_c are, respectively, the absorbances of the experimental sample and the vehicle-treated control at 600 nm. The results are reported in terms of IC_{50} (the concentration at which the percentage inhibition of hyaluronidase activity was 50%).

ELASTASE INHIBITION ASSAY

The elastase inhibition assay was performed by measuring the release of *p*-nitroaniline due to proteolysis of *N*-succinyl-(Ala)3-*p*-nitroanilide by human leucocyte elastase (Sigma) (25) in the presence or absence of E-*AP*-SM2001 (12.5, 25, 50, 100, 200, and 400 μg/ml) or oleanolic acid (6.25, 12.5, 25, 50, 100, and 200 μg/ml) as a standard under exactly the same experimental conditions. The absorbance was measured at 410 nm with a 96-well microplate reader and the elastase inhibitory activity of each sample was calculated using equation (4): Elastase inhibitory activity (%) = $100 - [(OD_s/OD_c) \times 100]$, where OD_s and OD_c are, respectively, the absorbances of the experimental sample and the vehicle-treated control at 410 nm. The results are reported in terms of IC_{50} (the concentration at which the percentage inhibition of elastase activity was 50%).

COLLAGENASE INHIBITION ASSAY

The collagenase inhibition assay was performed according to the method reported previously by Niemann (26). Accordingly, 0.15 ml of collagenase (1 mg/ml; Sigma) was added to mixed solutions consisting of 0.25 ml of 2 mM 4-phenylazobenzyloxycarbonyl-pro-leu-gly-pro-d-ar (Sigma) and 0.1 ml of E-*AP*-SM2001 (12.5, 25, 50, 100, 200, and 400 μg/ml) in 0.1 M Tris-HCl buffer (pH 7.5) and then reacted for 20 min at 37°C. After that, the reactions were stopped by adding 0.5 ml of 6% citric acid (Daejung). The absorbance was measured at 320 nm with a UV/Vis spectrophotometer after addition of 1.5 ml of ethyl acetate (Sigma) and the collagenase inhibitory activity of each sample was calculated using equation (5): Collagenase inhibitory activity (%) = $100 - [(OD_s/OD_c) \times 100]$, where OD_s and OD_c are, respectively, the absorbances of the experimental sample and the vehicle-treated control at 320 nm. The results are reported in terms of IC_{50} (the concentration at which the percentage inhibition of collagenase activity was 50%). Oleanolic acid (6.25, 12.5, 25, 50, 100, and 200 μg/ml) was used as a standard under exactly the same experimental conditions.

MMP-1 INHIBITION ASSAY

The assay was performed using a fluorescence microplate according to a previous report (27) with slight modifications. Briefly, 20 μl of type I collagen (substrate; Sigma) was

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mixed with 80 μl of diluted E-*AP*-SM2001 (12.5, 25, 50, 100, 200, and 400 μg/ml) or oleanolic acid (6.25, 12.5, 25, 50, 100, and 200 μg/ml) as a standard under exactly the same experimental conditions. Then 100 μl of diluted MMP-1 (0.2 U/ml; Sigma) was added to each well and the plate was incubated at room temperature for 1–2 h protected from light. Fluorescence was measured at an excitation maximum of 495 nm and an emission maximum of 515 nm. All the dilutions were made with reaction buffer containing 0.5 M Tris-HCl, 1.5 M NaCl, 50 mM $CaCl_2$, and 2 mM sodium azide (pH 7.6). The control used for this experiment was buffer with the substrate and the inhibitors but without MMP-1. The MMP-1 inhibitory activity of each sample was calculated using equation (6): MMP-1 inhibitory activity (%) = $100 - [(OD_s/OD_c) \times 100]$, where OD_s and OD_c are, respectively, the absorbances of the experimental sample and the vehicle-treated control at 515 nm. The results are reported in terms of IC_{50} (the concentration at which the percentage inhibition of MMP-1 activity was 50%).

TYROSINASE INHIBITION ASSAY

Tyrosinase inhibition was assayed according to the method of Masamoto (28). Briefly, aliquots (0.05 ml) of E-*AP*-SM2001 (12.5, 25, 50, 100, 200, and 400 μg/ml) were mixed with 0.5 ml of L-DOPA (Sigma) solution (1.25 mM) and 0.9 ml of sodium acetate buffer solution (0.05 M, pH 6.8), and preincubated at 25°C for 10 min. Then, 0.05 ml of an aqueous solution of mushroom tyrosinase (333 U/mL; Sigma) was added to the mixture. This solution was immediately monitored for the formation of dopachrome by measuring the linear increase in OD at 475 nm with a UV/Vis spectrophotometer, and the tyrosinase inhibitory activity of each sample was calculated using equation (7): Tyrosinase inhibitory activity (%) = $100 - [(OD_s/OD_c) \times 100]$, where OD_s and OD_c are, respectively, the absorbances of the experimental sample and the vehicle-treated control at 475 nm. The results are reported in terms of IC_{50} (the concentration at which the percentage inhibition of tyrosinase activity was 50%). Kojic acid (1.25, 2.5, 5, 10, 20, and 40 μg/ml) was used as a standard under exactly the same experimental conditions.

MELANIN FORMATION TEST IN B16/F10 MELANOMA CELLS

B16F10 murine melanoma cells (CRL-6475) were purchased from the American Type Culture Collection (ATCC; Manassas, VA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) containing 2 mM L-glutamine (Sigma), supplemented with 10% fetal bovine serum (Gibco, Irvington, NJ), 100 U/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma), in culture flasks in a CO_2 incubator with a humidified atmosphere containing 5% CO_2 in air at 37°C. The culture medium was changed every 2 days. The cells were harvested by trypsinization when they were about 70% confluent, counted with a hemocytometer and seeded at appropriate numbers into wells of cell culture plates for further experiments. Melanin content was measured as described previously (29) with slight modifications. The B16F10 melanoma cells were seeded (2×10^5 cells/well) in 3 ml of medium in 6-well culture plates and incubated overnight to allow them to adhere. The cells were exposed to various concentrations of E-*AP*-SM2001 (50, 100, 200, 400, 800, and 1,600 μg/ml) for 72 h in the presence or absence of 100 nM α-MSH (Sigma). At the end of the treatment, the cells were washed

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with PBS and lysed with 800 μl of 1 N NaOH (Merck, Darmstadt, Germany) containing 10% DMSO (Sigma) for 1 h at 80°C. The absorbance at 400 nm was measured using a microplate reader. The inhibitory activity of each sample against melanin production was calculated using equation (8): Inhibitory activity (%) = $100 - [(OD_s/OD_c) \times 100]$, where OD_s OD_c are, respectively, the absorbances of the experimental sample and α-MSH-treated control at 400 nm. The results are reported in terms of IC_{50} (the concentration at which the percentage inhibition of melanin production was 50%). Kojic acid (25, 50, 100, 200, 400, and 800 μg/ml) was used as a standard under exactly the same experimental conditions.

IN VIVO SKIN MOISTURIZING MEASUREMENT

The assay for skin moisturizing measurement was carried out according to the method reported previously by Hou *et al* (30). In this study, 192 male ICR mice (6-weeks old on receipt; SLC, Shizuoka, Japan) were used after 7 days of acclimatization. Animals were housed four per polycarbonate cage in a temperature-controlled (20–25°C) and humidity-controlled (40–45%) room. The light:dark cycle was 12 h:12 h and normal rodent pellet diet and water were supplied freely during acclimatization. After acclimatization, mice were divided into four groups for each time point (30 min and 1, 2, 4, 8, and 24 h; eight mice per time point) based on body weight. Then, 100 μl of vehicle (distilled water) or E-*AP*-SM2001 was directly applied to shaved dorsal skin. A gel mask pack (area 2 × 3 cm) containing E-*AP*-SM2001 or SFF (area 2 × 3 cm) was patched on the shaved dorsal skin. Then, 30 min after application of test materials, all remaining samples were eliminated using cotton balls. Subsequently, 0.5, 1, 2, 4, 8, and 24 h after the end of exposure to the test materials, 2 × 3-cm skin samples were removed and skin water content (%) was measured using an automated moisture balance analyzer (MB23; Ohaus, Parsippany, NJ). In addition, percentage change compared to the vehicle control was calculated to help determine the efficacy of test materials using equation (9): Change compared with vehicle control (%) = {[(Data for test material-treated group – Data for vehicle-treated control)/Data for vehicle-treated control] × 100}.

STATISTICAL ANALYSES

All *in vitro* data are expressed as the mean ± S.D. of five independent experiments, and skin water content was calculated as the mean ± S.D. of eight mouse skins at each time point. Multiple comparison tests for different dose groups were conducted. Variance homogeneity was examined using the Levene test (31). If the Levene test indicated no significant deviations from variance homogeneity, the obtained data were analyzed by one-way ANOVA followed by a least-significant differences (LSD) multicomparison test to determine which pairs in the group comparison were significantly different. If the Leven test showed significant deviation from variance homogeneity, a nonparametric comparison test, the Kruskal–Wallis H test, was conducted. When a significant difference was observed in the Kruskal–Wallis H test, the Mann–Whitney U (MW) test was conducted to identify the specific pairs in the group comparison that were significantly different. IC_{50} values for each in vitro assay were calculated by probit methods. Statistical analyses were conducted using SPSS for Windows (release 14.0K; SPSS, Chicago, IL) (32).

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RESULTS

ANTIOXIDANT EFFECT OF E-AP-SM2001

Significant ($p < 0.01$) increases in DPPH radical scavenging activities were detected in samples treated with ascorbic acid and E-*AP*-SM2001 at concentrations from 6.25 and 12.5 μg/ml, respectively. The IC_{50} values for the DPPH radical scavenging activity of ascorbic acid and E-*AP*-SM2001 were calculated as 6.84 ± 1.03 and 46.48 ± 10.76 μg/ml, respectively (Figure 1A and B).

SOD-like activity showed significant increases in samples treated with ascorbic acid and E-*AP*-SM2001 at concentrations from 12.5 and 25 μg/ml, respectively. The IC_{50} values for the SOD-like activity of ascorbic acid and E-*AP*-SM2001 were calculated as 65.17 ± 12.15 and 132.95 ± 45.53 μg/ml, respectively (Figure 1C and D).

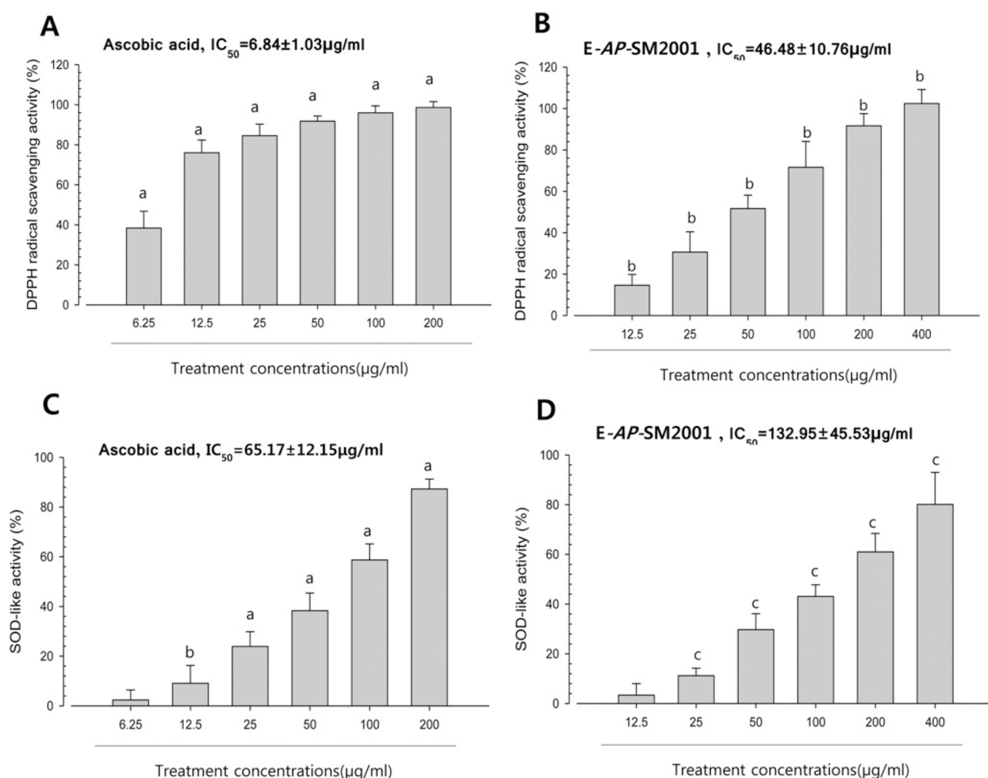

Figure 1. Antioxidant effects of exopolymers from *Aureobasidium pullulans* SM2001 (E-*AP*-SM2001). DPPH radical scavenging activity (A and B) and SOD-like activity (C and D) of ascorbic acid and E-*AP*-SM2001. Values are expressed as the mean ± SD of five independent experiments. The vehicle control was set to 0%. DPPH: 1,1-diphenyl-2-picrylhydrazyl. IC_{50} indicates the concentration needed to reduce 50% of DPPH. SOD: superoxide dismutase. IC_{50} indicates the concentration needed to reduce pyrogallol oxidation by 50%. [a]$p < 0.01$ compared with control (LSD test); [b]$p < 0.01$ compared with control (MW test).

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ANTI-WRINKLE BENEFIT OF E-*AP*-SM2001

Significant increases in inhibitory activity against hyaluronidase were found in samples treated with oleanolic acid and E-*AP*-SM2001 at concentrations from 6.25 and 12.5 μg/ml, respectively. The IC_{50} values for the effects of oleanolic acid and E-*AP*-SM2001 on hyaluronidase activity were calculated as 33.34 ± 3.16 and 50.88 ± 16.89 μg/ml, respectively (Figure 2A and B).

Elastase activity was significantly inhibited by treatment with oleanolic acid and E-*AP*-SM2001 at concentrations from 6.25 and 12.5 μg/ml, respectively. The IC_{50} values for

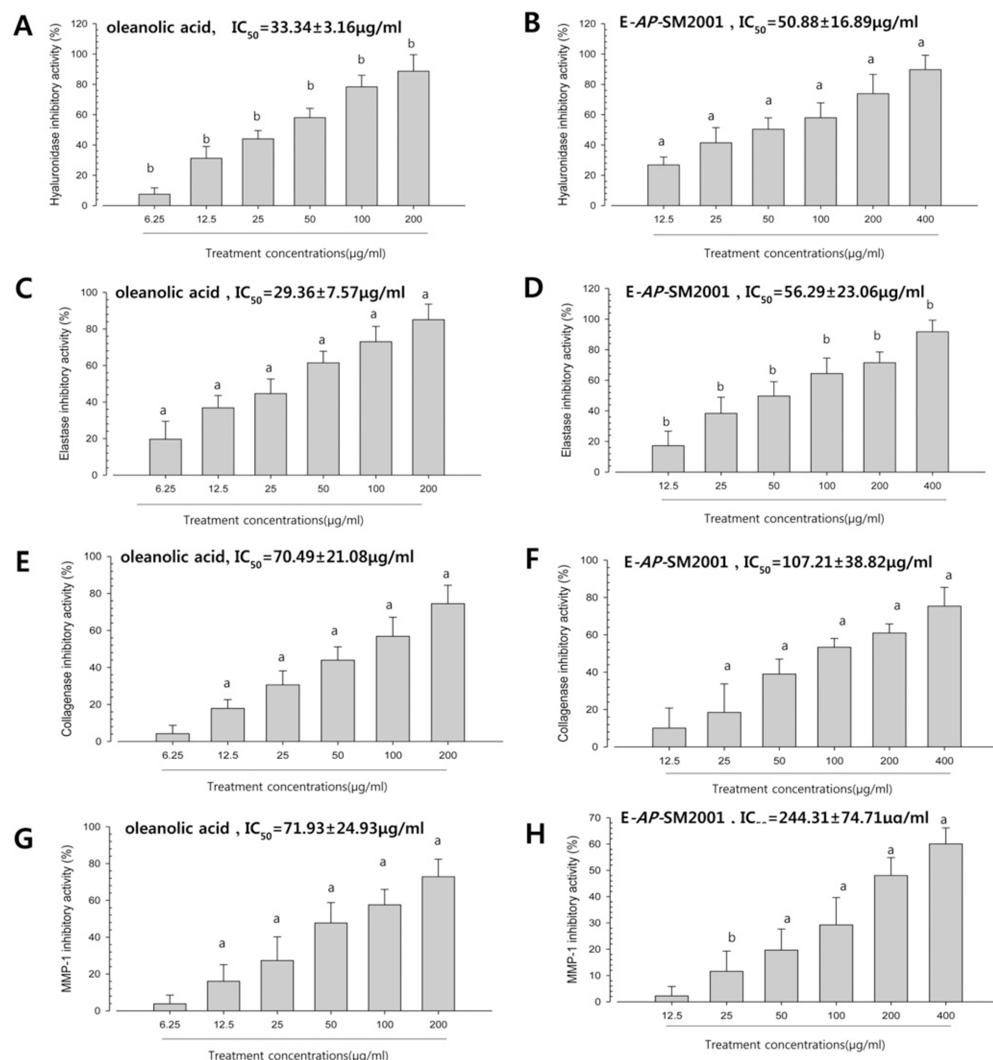

Figure 2. Anti-wrinkle benefits of exopolymers from *Aureobasidium pullulans* SM2001 (E-*AP*-SM2001). Inhibitory activity of oleanolic acid and E-*AP*-SM2001against hyaluronidase (A and B), elastase (C and D), collagenase (E and F) and MMP-1 (G and F). Values are expressed as the mean ± SD of five independent experiments. The vehicle control was set to 0%. MMP-1: matrix metalloproteinase 1. IC_{50} indicates the concentration at which the percentage inhibition of hyaluronidase, elastase, collagenase, and MMP-1 activity was 50%. $^{a}p < 0.01$ and $^{b}p < 0.05$ compared with control (LSD test).

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the effects of oleanolic acid and E-*AP*-SM2001 on elastase activity were calculated as 29.36 ± 7.57 and 56.29 ± 23.06 μg/ml, respectively (Figure 2C and D).

Collagenase activity was significantly inhibited by treatment with oleanolic acid and E-*AP*-SM2001 at concentrations from 12.5 and 25 μg/ml, respectively. The IC_{50} values for the effects of oleanolic acid and E-*AP*-SM2001 on collagenase activity were calculated as 70.49 ± 21.08 and 107.21 ± 38.82 μg/ml, respectively (Figure 2E and F).

MMP-1 activity was significantly ($p < 0.01$) inhibited by treatment with oleanolic acid and E-*AP*-SM2001 at concentrations from 12.5 and 25 μg/ml, respectively. The IC_{50} values for the effects of oleanolic acid and E-*AP*-SM2001 on collagenase activity were calculated as 71.93 ± 24.93 and 244.31 ± 74.71 μg/ml, respectively (Figure 2G and H).

WHITENING EFFECTS OF E-*AP*-SM2001

Kojic acid and E-*AP*-SM2001 significantly ($p < 0.01$) inhibited mushroom tyrosinase activity at concentrations from 1.25 and 12.5 μg/ml, respectively. The IC_{50} values for the effects of kojic acid and E-*AP*-SM2001 on tyrosinase activity were calculated as 2.94 ± 1.04 and 53.17 ± 24.17 μg/ml, respectively (Figure 3A and B).

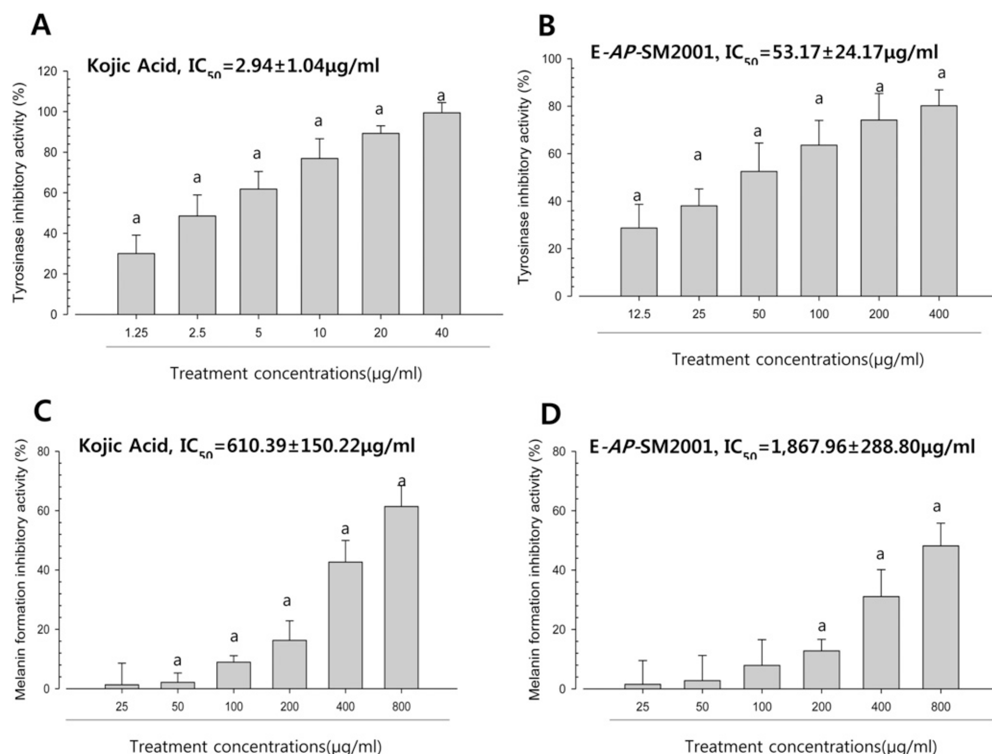

Figure 3. Whitening effects of exopolymers from *Aureobasidium pullulans* SM2001 (E-*AP*-SM2001). Inhibitory activity of kojic acid and E-*AP*-SM2001 against tyrosinase (A and B) and melanin formation (C and D). Values are expressed as the mean ± SD of five independent experiments. The vehicle control was set to 0%. IC_{50} indicates the concentration at which the percentage inhibition of tyrosinase activity and melanin formation was 50%. [a]$p < 0.01$ compared with control (MW test).

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Kojic acid and E-*AP*-SM2001 significantly ($p < 0.01$) inhibited B16/F10 melanoma cell melanin production at concentrations from 100 and 400 μg/ml, respectively. The IC_{50} values for the effects of kojic acid and E-*AP*-SM2001 on melanin production were calculated as 610.39 ± 150.22 and 1,867.96 ± 288.80 μg/ml, respectively (Figure 3C and D).

SKIN MOISTURIZING EFFECTS OF E-AP-SM2001

Compared with vehicle control skins, E-*AP*-SM2001 and SFF mask-treated mice skin showed an increase in water content from 30 min to 4 h after the end of application. The E-*AP*-SM2001-containing mask had a >twofold more extensive moisturizing effect compared with the SFF-containing mask, and showed significant ($p < 0.01$ or $p < 0.05$) increases in skin water content from 30 min to 8 h after the end of application (Table I).

Compared with vehicle-treated control skins, the skin water contents of E-*AP*-SM2001-treated skins showed 26.82%, 32.79%, 24.76%, 14.28%, 6.62%, and 3.36% changes at 30 min and 1, 2, 4, 8, and 24 h after the end of application. Changes of 41%, 63%, 53.78%, 26.74%, 30.27%, 20.23%, and 4.21% were observed in E-*AP*-SM2001 mask-treated skins. Additionally, 25.69%, 28.50%, 21.93%, 13.12%, 3.63%, and 0.64% changes were detected in SFF mask-treated skins.

DISCUSSION

In the present study, we observed that E-*AP*-SM2001 appeared to be a suitable ingredient for functional cosmetics, exerting antiaging, antiwrinkle, whitening benefits and skin moisturizing effect. E-*AP*-SM2001 being a natural antioxidant, extracted from *Aureobasidium pullulans* SM2001, constitutes an affordable ingredient. We suggest that E-*AP*-SM2001 is an attractive ingredient for anti-skin-aging benefits.

E-*AP*-SM2001 showed potent DPPH radical scavenging effects and SOD-like activity. It is well documented that ROS causes oxidative damage of biological macromolecules,

Table I
Changes in Skin Water Content (%)

	Time after the end of application (hours)					
	0.5	1	2	4	8	24
Control	59.71 ± 3.23	52.26 ± 5.11	52.23 ± 3.40	52.88 ± 4.64	53.43 ± 4.31	53.13 ± 5.30
E-*AP*-SM2001	75.72 ± 5.00[a]	69.40 ± 7.60[a]	65.16 ± 5.00[c]	60.43 ± 6.63[b]	56.97 ± 4.04	54.92 ± 6.43
Mask 1	84.57 ± 4.94[a]	80.36 ± 7.22[a]	71.42 ± 8.75[c]	68.89 ± 7.76[a]	64.25 ± 6.34[a]	55.37 ± 6.34
Mask 2	75.05 ± 5.87[a]	67.15 ± 7.58[a]	63.68 ± 5.72[c]	59.82 ± 4.68[b]	55.37 ± 4.23	52.79 ± 3.70

Values are expressed as the mean ± SD. of eight mouse skins.
Exopolymers from *Aureobasidium pullulans* SM2001 (E-*AP*-SM2001).
Mask 1: mask pack containing *E-AP-SM2001.*
Mask 2: mask pack containing *Saccharomycopsis* Ferment Filtrate (SFF).
[a] $p < 0.01$ compared with control (LSD test).
[b] $p < 0.05$ compared with control (LSD test).
[c] $p < 0.01$ compared with control (MW test).

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including lipids, proteins, and DNA, and accelerate skin-aging processes (3,4). In normal healthy conditions, antioxidant defense systems detoxify ROS and prevent damage to cellular biomolecules. However, when endogenous antioxidants fail to defend against oxidative stresses, due to overproduction of ROS (14), the balance between the generation of ROS and antioxidant capacity is broken. This leads to oxidative damage that contributes to the progression of skin aging (33,34). Thus, antioxidants play an important role in counteracting the damaging effect of ROS. SOD is an endogenous antioxidant enzyme that contributes to enzymatic defense mechanisms (35). Increased SOD activity, or SOD-like activities, is implicated in increased resistance to oxidative stress-induced damage (36). Therefore, effective antioxidants can protect cells against ROS-mediated skin aging, and various antioxidants have been used as antiaging agents in functional cosmetics (12,37).

We further examined the whitening benefits of E-*AP*-SM2001 by tyrosinase inhibition assay and by melanin formation test in B16/F10 melanoma cells. Our results indicated that E-*AP*-SM2001 can inhibit the activation of tyrosinase and reduce melanin formation in a concentration-dependent manner. These results suppose that E-*AP*-SM2001 may be have a potential effect for skin whitening. Melanin is the main component determining the color of skin, and up to 10% of cells in the innermost layer of the epidermis produce melanin pigments (38,39). Melanin is synthesized in melanocytes and transported to keratinocytes to protect cells. Melanin biosynthesis is regulated by tyrosinase, tyrosinase-related protein (TRP)-1, and TRP-2 (39,40). The activity of tyrosinase is important in the control of melanogenesis as it functions as the catalyst in the rate-limiting reaction of the melanogenic pathway. It is accepted that the inhibition of tyrosinase is the most common approach to achieve skin whiteness (39,41).

To determine the skin moisturizing effect, a mask containing E-*AP*-SM2001 was compared with an SFF-containing mask. E-*AP*-SM2001 showed concentration-dependent inhibition of hyaluronidase, elastase, collagenase, and MMP-1. Skin wrinkling, the appearance of visible signs on the surface of the skin (42), is a complex inevitable process of skin aging and involves an age-dependent decline in skin cell function. It is closely associated with harmful proteolytic degradation of the extracellular matrix. Evidence suggests that there is a strong connection between the activity of dermal enzymes (hyaluronidase, collagenase, elastase, and MMP-1) and wrinkle formation (27,42). For this reason, an antiwrinkle agent should have the potential to inhibit these enzymes (1,18).

Skin water content was significantly increased in E-*AP*-SM2001-treated mouse skin, as compared to vehicle control mouse skin. In addition, a mask pack containing E-*AP*-SM2001 showed >twofold more extensive moisturizing effects, as compared to an SFF-containing mask. These observations indicated that E-*AP*-SM2001 have skin moisturizing effects. Skin moisturizing effects can be easily detected by measuring water contents, in animal skins or human pilot studies (30,43,44). Healthy human keratin layers maintain a water content of 10–20%. However, sunlight and other factors decrease moisture content, resulting in the formation of wrinkles. For these reasons, keratin layers must contain enough moisture to maintain healthy elastic skin, even in dry conditions (45).

Collectively, these results suggest that E-*AP*-SM2001 have adequate antiaging, antiwrinkle, and whitening benefits and skin moisturizing effect as an ingredient of functional cosmetics. Interestingly, a mask pack containing E-*AP*-SM2001 had more extensive

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moisturizing effects than an SFF-containing mask. These effects involve the inhibition of hyaluronidase, elastase, collagenase, and MMP-1, as well as the inhibition of melanin production and tyrosinase activities. These results suggest that skin aging can be prevented and reduced by the antioxidant effect of E-*AP*-SM2001.

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