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Skin Anti-Aging Activities of Bacteriochlorophyll *a* from Photosynthetic Bacteria, *Rhodobacter sphaeroides*

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In this work, the anti-aging skin effects of bacteriochlorophyll *a* isolated from *Rhodobacter sphaeroides* are first reported, with notably low cytotoxicity in the range of 1% to 14% in adding 0.00078 (% (w/w)) of the extracts, compared with the normal growth of both human dermal fibroblast and keratinocyte cells without any treatment as a control. The highest production of procollagen from human fibroblast cells (CCD-986sk) was observed as 221.7 ng/ml with 0.001 (% (w/w)) of bacteriochlorophyll *a*, whereas 150 and 200 ng/ml of procollagen production resulted from addition of 0.001 (% (w/w)) of the photosynthetic bacteria. The bacteriochlorophyll-*a*-induced TNF-α production increased to 63.8%, which was lower secretion from HaCaT cells than that from addition of 0.00005 (% (w/w)) of bacteriochlorophyll *a*. Additionally, bacteriochlorophyll *a* upregulated the expression of genes related to skin anti-aging (*i.e.*, keratin 10, involucrin, transglutaminase-1, and MMPs), by up to 4–15 times those of the control. However, crude extracts from *R. sphaeroides* did not enhance the expression level of these genes. Bacteriochlorophyll *a* showed higher antioxidant activity of 63.8% in DPPH free radical scavenging than those of water, ethanol, and 70% ethanol extracts (14.0%, 57.2%, and 12.6%, respectively). It was also shown that the high antioxidant activity could be attributed to the skin anti-aging effect of bacteriochlorophyll *a*, although *R. sphaeroides* itself would not exhibit significant anti-aging activities.

Keywords: Bacteriochlorophyll *a*, *Rhodobacter sphaeroides*, antioxidation, skin anti-aging activity

Introduction

Photosynthetic bacteria are photosynthesizing phototrophs that are distributed in various land and ocean environments. Photosynthetic bacteria are also gram-negative bacilli. Among this group, *Rhodobacter sphaeroides* are non-sulfur purple photosynthetic bacteria that photosynthesize under microaerophilic conditions without generating oxygen [1]. Together with *Rhodobacter capsulatus*, *Rhodobacter sphaeroides* are thus far the most extensively studied among the photosynthetic bacteria. Photosynthetic bacteria convert CO_2 into organic substances, generate hydrogen from organic waste resources (for effective treatment of wastewater), are effectively utilized in environmental cleanup, and are studied in the area of alternative energy. In addition, because the bacterial bodies are rich in nutrients, they are used as feed additives in the livestock industry to show such effects as prevention of infectious livestock diseases due to drinking water and reduction of bad odors from excrement [17]. In the area of agriculture, these bacteria are used to increase the yields of agricultural products, enhance their quality (sugar contents and aromas), improve soil, and leverage antibacterial and antiviral effects against plant pathogenic bacteria and pathogenic viruses.

Skin aging includes endogenous aging that occurs naturally over time and photo-aging that occurs from the effects of ultraviolet rays [20]. Aging is primarily expressed by the occurrence of skin wrinkles, and skin wrinkles are greatly affected by decreases in collagen, which is a protein that maintains elasticity. As a leading cause of collagen

decrease, active oxygen is generated when ultraviolet rays infiltrate the skin, and active oxygen causes not only skin aging but also inflammation, cell death, and cancers in the skin [25]. In particular, active oxygen induces the generation of collagenase MMP (matrix metalloproteinase) and promotes collagen decomposition that results in a decrease in skin elasticity [26].

In addition to the areas studied thus far and based on the function of photosynthetic bacteria as electron donors, this study attempted to identify for the first time the value of photosynthetic bacteria and most photosynthetic bacteria that contain pigments, mainly known as bacteriochlorophyll *a* that primarily works for converting light energy into chemical energy within the cells. Several biological activities of bacteriochlorophyll *a*, such as phospholipid transfer activity and NADH oxidase activity, were also found elsewhere [6, 23, 28]. To this end, this study identified the photosynthetic bacteria's antioxidant activity according to extracts and the resultant anti-skin aging activity and measured the activity of the bacteriochlorophyll *a* pigment, which also showed the redox effects, and the ability of photosynthetic bacteria to reduce DPPH free radicals.

Materials and Methods

Sample Preparation

Photosynthetic bacteria were used *Rhodobacter sphaeroides* EBN-BL6 supported from Doosan ecobiznet (Chuncheon, Korea). For mass culture of the photosynthetic bacteria *Rhodobacter sphaeroides*, a pure high-concentration culture was implemented using a 700 L fermenter. The initial working volume was 480 L, and a 12 L (2.5% (v/v)) volume of an activated flask culture medium was inoculated and cultured for 4 days. For the culture, the temperature was set at 30°C under light conditions controlled by halogen lamps. The agitation speed and the amount of aeration were flexibly adjusted according to the growth rates, and the pH was maintained at a constant value of 6.80 using acetic acid. For high-concentration culture of *Rhodobacter sphaeroides*, additional media were added on the third day of culture. After culture, the cells of photosynthetic bacteria were crushed by heat treatment and ultrasonication and subsequently extracted for 12 h at 60°C using distilled water, ethanol, and a 70% ethanol solvent. The extracted liquid was filtered and vacuum dried for 72 h in a lyophilizer (PVTFA 10AT; ILSHINBioBase, Dongducheon, Korea) to obtain a powder. The powder obtained from drying was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) and used in the experiments. In the case of bacteriochlorophyll *a*, the photosynthetic bacteria culture solution was subjected to centrifugation for 10 min at 6,000 rpm and the supernatant was removed. The photosynthetic microorganism pellet that remained after removal of the supernatant was collected, and the cells in the pellet were crushed using a sonicator with a 25 mm solid probe for 10 min; the sonicator was operated for 5 sec at a frequency of 20 kHz and an amplitude of 25% and halted for 2 sec before repetition. The crushed cells were reacted for 30 min using a solvent consisting of methyl alcohol:acetone = 7:2 under light blocking. After reaction, the crushed cells of the photosynthetic microorganisms were subjected to centrifugation for 10 min at 12,000 rpm (Supra 25K, Korea), and the supernatant was collected. Experiments were conducted with the collected supernatant [14].

Cells and Reagents

The human dermal fibroblasts used for toxicity evaluation were CCD-986sk cells purchased from the Korea Cell Line Bank (KCLB, Seoul, Korea) in a frozen state. HaCaT cells were also purchased from the Korea Cell Line Bank. The purchased cells were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) under 37°C, 5% CO_2 prior to use.

Measurement of Cytotoxicity Against Human Skin Cells

Cell cytotoxicity was estimated by adding the extracts of photosynthetic bacteria and their pigment, bacteriochlorophyll *a*, into both human skin fibroblast cells (CCD-986) and keratinocytes (HaCaT) as follows: First, the cells were injected into a 96-well plate at a concentration of 7.0×10^5 cells/ml and cultured for 24 h in an incubator under 5% CO_2, 37°C conditions. The medium was removed after 24 h and 200 µl of each of the extract and bacteriochlorophyll *a* at different concentrations was injected into the 96-well plate and cultured for another 24 h under the same conditions. The treated cells were removed 24 h later, and then 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) solution (Sigma-Aldrich, USA) at a concentration of 0.33 g/l was added to the 96-well plate. The plate was left unattended for 90 min in a 37°C darkroom. The MTT solution was removed after the 90 min, and the 96-well plate was washed two times with PBS [22]. After washing, 100 µl of DMSO was injected and allowed to react for 30 min, and the optical density was measured using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 570 nm to evaluate the cytotoxicity. The cytotoxicity was calculated as the ratio of the cytotoxicity of the experimental groups to that of an untreated group, as shown in the following equation [15].

$$\text{Cytotoxicity (\%)} = \left(1 - \frac{\text{Sample group}}{\text{Untreated group}}\right) \times 100 \quad (1)$$

where, Sample group is viable cell numbers/ml after adding the extracts into the cell cultures, and Untreated group is viable cell numbers/ml without adding any sample as a control.

Measurement of Skin Irritation by Artificial Skin Test

Skin irritation was tested using a 3D tissue model of artificial skin (EPI-200; Mattek Corporation, Ashland, MA, USA). The test was conducted using the same protocol as the MTT assay used in the cytotoxicity evaluation, and the resultant values were shown

in terms of cell viability (%). The No treatment group's cell viability reached 100%, and samples were compared with the notreatment group to indicate the cell viability of each sample using the relevant ratio [22].

Production of Procollagen from Human Skin Fibroblast Cells

To investigate the wrinkle improvement effects of photosynthetic bacteria and bacteriochlorophyll *a*, we measured the generation of procollagen, which is a precursor of collagen, a skin elasticity maintenance protein. Skin fibroblasts CCD-986sk were inoculated into a 6-well plate at a concentration of 2×10^5 cell/ml and cultivated for 24 h. Thereafter, the medium was removed, and the cells were treated with serum-free medium and left to starve for 24 h. After 24 h, the serum-free medium was removed, and the 6-well plate was washed with PBS and irradiated by UV at a wavelength of 312 nm at a rate of 12.5 mJ. Next, the samples were treated with phenol-red-free DMEM at different concentrations and additionally cultured for 48 h. A group treated with ascorbic acid (50 μM) was used as a positive control, a UV(+) group was used as a negative control, and a group treated with medium only was used as an additional control, and the results from the samples were compared with results from the controls. After 48 h of culture, the medium and cell lysate were separately collected. An RIPA buffer mixed with a protease inhibitor was placed into each well on ice, and cell lysate was collected using a cell scraper. The cell lysate was subjected to centrifugation for 10 min at 14,000 rpm, the supernatant was collected, and the amount of proteins was corrected using the Bradford assay.

The amount of collagen in the collected medium was measured using a Procollagen Type-I C-Peptide (PIP) EIA kit (Takara Bio, Otsu, Japan). Antibody-POD conjugate solution in the amount of 100 μl was added to each well, 20 μl of each of the sample or standard was added into each well, and the contents were mixed well and left unattended for 3 h at 37°C. When the reactions had completed, the contents were removed, and the well plate was washed four times with 400 μl of PBS. After removal, 100 μl of the substrate solution was added to each well, and the well plate was held at room temperature for 15 min for reaction of the solutions. Next, 100 μl of the stop solution was added and gently mixed with the solution. The optical density was measured at a wavelength of 450 nm, and the amount of collagen was calculated using the standard curve.

Measurement of Tumor Necrosis Factor-Alpha (TNF-α) production

To evaluate the wrinkle-improvement effects of the photosynthetic bacteria and pigment substance, experiments were conducted to evaluate TNF-α generation. The amounts of TNF-α generated were assessed *via* quantitative measurement of TNF-α. The quantity of TNF-α was measured using a TNF-α kit (Invitrogen, Waltham, MA, USA). HaCaT cells at a concentration of 1.0×10^5 cells/ml were inoculated into a 12-well plate and cultured for 24 h. Next, with the intent to induce TNF-α, the samples were treated with the medium at different concentrations together with UV irradiation and additionally cultured for 24 h. A group that did not receive UV and a group treated with UV and with 1 μM of dexamethasone were used as controls for comparison with the results of the samples.

Measurement of Skin-Aging-Related Gene Expression Levels

Human fibroblast cell line CCD-986sk and human keratinocyte cell line HaCaT were prepared at a concentration of 2×10^5 and injected into a 12-well plate. The cell lines were cultured with 10% FBS and DMEM high-glucose media, the media was replaced with the same media with 1% FBS, and the cell lines were treated with photosynthetic bacteria samples. After 12 h of treatment, the total RNA was separated using TRIzol reagent (Invitrogen), and cDNA was synthesized using 2 μg of the separated RNA, random primer, and a PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio). Using SYBR Green Realtime PCR Master Mix and a AB7500 real-time PCR machine, the expression levels of cell-differentiation-related genes in the synthesized cDNA were examined. The relevant primers are described in Table 1. Gene amplification conditions used in the examination were 30 sec at 94°C, 30 sec at 61°C, and 30 sec at 72°C [3, 7, 24, 27].

Measurement of α,α-Diphenyl-β-Picrylhydrazyl Free Radical Scavenging Activity

A DPPH (α,α-diphenyl-β-picrylhydrazyl) free-radical scavenging activity experiment was conducted using Dietz's method [5]. The prepared photosynthetic bacteria sample (150 μl) was injected into a 96-well plate at a certain concentration and mixed with a 0.2 mM DPPH solution (150 μl) in methanol as a solvent. The mixture was held at room temperature for 30 min in the dark to allow the solutions to react, and the optical density was measured at a wavelength of 570 nm. Assuming the optical density value of the positive control ascorbic acid as 100%, the ratios of the measured optical density values relative to the foregoing value were shown in %.

Statistical Analysis

All experimental data were statistically processed three times by two-way ANOVA using the SAS (Statistical Analysis System) program. The minimum difference in significance levels in the processing interval was set to $p < 0.05$.

Results

Measurement of Cytotoxicity Against Human Skin Cells

The cell cytoxicity of the samples against human skin fibroblasts (CCD-986sk cells) and keratinocytes (HaCaT cells) is shown in Fig. 1. The cytotoxicity of the extracts from photosynthetic bacteria were from 1% to 14% for all the ranges of the sample concentrations, which tells that the cytotoxicity of all of the extracts should be less than 14%. However, for the case of the pigment, bacteriochlorophyll *a*,

Table 1. List of RT-PCR primers related to skin-aging genes.

Gene	Direction	Primer sequence
Keratin 10	Forward	hKRT10-F GGTGGGAGTTATGGAGGCAG
	Reverse	hKRT10-R CGAACTTTGTCCAAGTAGGAAGC
Involucrin	Forward	hIVL-F TCCTCCAGTCAATACCCATCAG
	Reverse	hIVL-R CAGCAGTCATGTGCTTTTCCT
Transglutaminase-1	Forward	hTGM1-F CATCAAGAATGGCCTGGTCT
	Reverse	hTGM1-R CAATCTTGAAGCTGCCATCA
Laminin 5	Forward	hLAMA5-F CCCACCGAGGACCTTTACTG
	Reverse	hLAMA5-R GGTGTGCCTTGTTGCTGTT
MMP1	Forward	hMMP1-F CAACTCTGGAGTAATGTCACACC
	Reverse	hMMP1-R TGTTGGTCCACCTTTCATCTTC
MMP2	Forward	hMMP2-F GATACCCCTTTGACGGTAAGGA
	Reverse	hMMP2-R CCTTCTCCCAAGGTCCATAGC
MMP3	Forward	hMMP3-F CTGGACTCCGACACTCTGGA
	Reverse	hMMP3-R CCAGGAAAGGTTCTGAAGTGACC
MMP7	Forward	hMMP7-F GAGTGAGCTACAGTGGGAACA
	Reverse	hMMP7-R CTATGACGCGGGAGTTTAACAT
MMP8	Forward	hMMP8-F TGCTCTTACTCCATGTGCAGA
	Reverse	hMMP8-R TCCAGGTAGTCCTGAACAGTTT
MMP9	Forward	hMMP9-F TGTACCGCTATGGTTACACTCG
	Reverse	hMMP9-R GGCAGGGACAGTTGCTTCT
Filaggrin	Forward	hFilaggrin-F GGACAGGAACAATCATCGGGG
	Reverse	hFilaggrin-R CAACCTCTCGGAGTCGTCTG

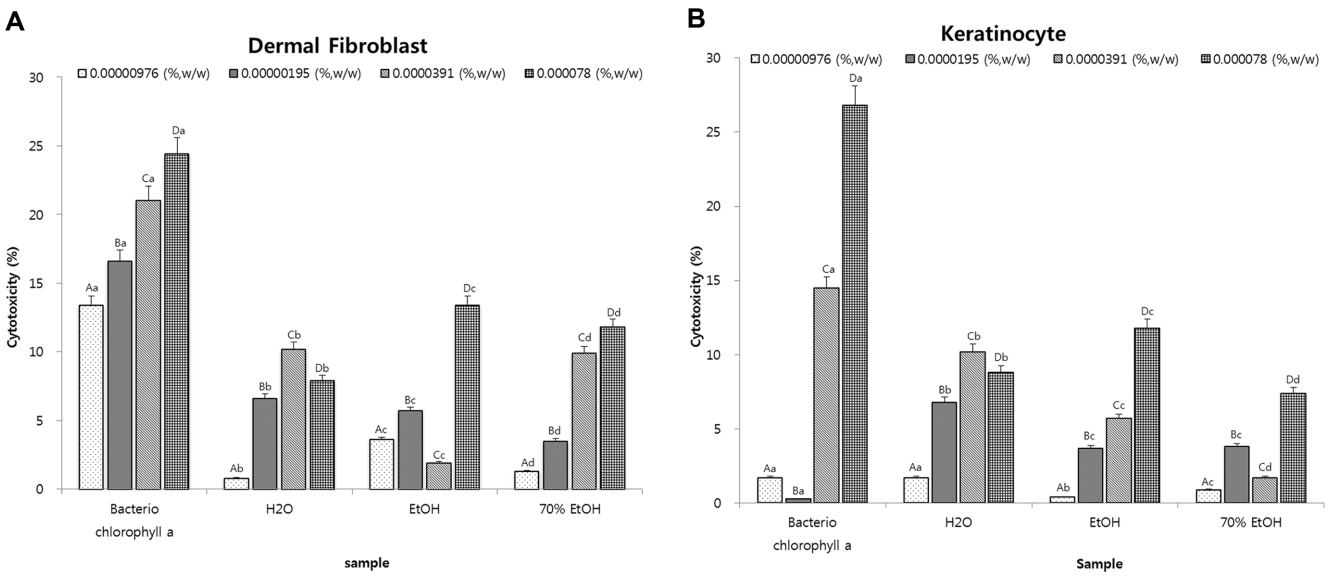

Fig. 1. Comparison of cell cytotoxicity against human skin cells at various concentrations of extracts of *Rhodobacter sphaeroides* and bacteriochlorophyll *a*.

Mean values ± SD from triplicate separate experiments are shown. Means with a different letter (A–D) within the same sample are significantly different at $p < 0.05$ and means with a different letter (a–d) within the same concentration are significantly different at $p < 0.05$.

cytotoxicity values ranged from 14% up to 27% indicating that bacteriochlorophyll *a* had somewhat high cytotoxicity in the range of 14% to 27% at 0.00078 (% (w/w)) of the highest concentration. The cytotoxicity of *Smilax china*, a natural herb that has been known to have antiwrinkle activities, was also reported as 5~30% [18], and thus the ca. 27% cytotoxicity of bacteriochlorophyll *a* at the highest concentration seemed to be acceptable for further experiments.

Observation of Skin Irritation of the Samples by Using Artificial Skins

After the evaluation of cell toxicity, to test possible hazards to human bodies, skin irritation was assessed using a 3D artificial skin model, EPI-300. As shown in Fig. 2, for the control, the cell viability of the no-treatment group treated with DPBS was assumed as 100%, and the cell viability after adding the samples dissolved by DMSO was estimated only as 36.4%. In considering the skin irritation of the sample when the cell viability was lower than 50%, for examining the skin irritation, the irritant was determined based on the viability of 50%. Except for the 70% EtOH extract, photosynthetic bacteria and bacteriochlorophyll *a* showed no skin irritation, which is close to water [12]. In addition, bacteriochlorophyll *a* and photosynthetic bacteria extracted using different solvents showed higher cell viabilities than that of DMSO, indicating that most samples are minimally irritating. Therefore, the hazards of photosynthetic bacterial samples to human skin were judged to be insignificant. Accordingly, the application of photosynthetic bacteria as a material for relieving skin aging is deemed not problematic.

Production of Procollagen and TNF-α from Human Skin Fibroblast Cells

The value of the photosynthetic bacteria extracts and bacteriochlorophyll *a* as materials for improvement of wrinkles due to aging was evaluated by investigating the generation of procollagen, a precursor of collagen and an important protein for maintenance of skin elasticity. The results are shown in Fig. 3. Compared with the no-treatment group treated with UV only, collagen generation of the experimental groups was generally increased. In the case of photosynthetic bacteria extracts, the quantity of generated collagen was in the range of 150–200 ng/ml. Collagen generation was not concentration dependent, and most extracts did not show significant differences from each other. In the case of bacteriochlorophyll *a*, larger values were identified, up to 220 ng/ml, indicating better wrinkle improvement effects than the other photosynthetic bacteria extracts. Because the relevant values are larger than those of aronia (which has been studied and reported relative to wrinkle improvement, because aronia showed collagen quantities in a range of 70–150 ng/ml at similar concentrations), the effects of bacteriochlorophyll *a* are considered to be higher [16].

To evaluate and compare the effects of photosynthetic *Rhodobacter sphaeroides* solvent extracts and bacteriochlorophyll *a* on TNF-α (which affects increases in collagenase generation) [2, 21], TNF-α secretion rates were investigated (Fig. 4). The TNF-α secretion rate of UV-irradiated but otherwise untreated cells was assumed as 100%. All samples were UV

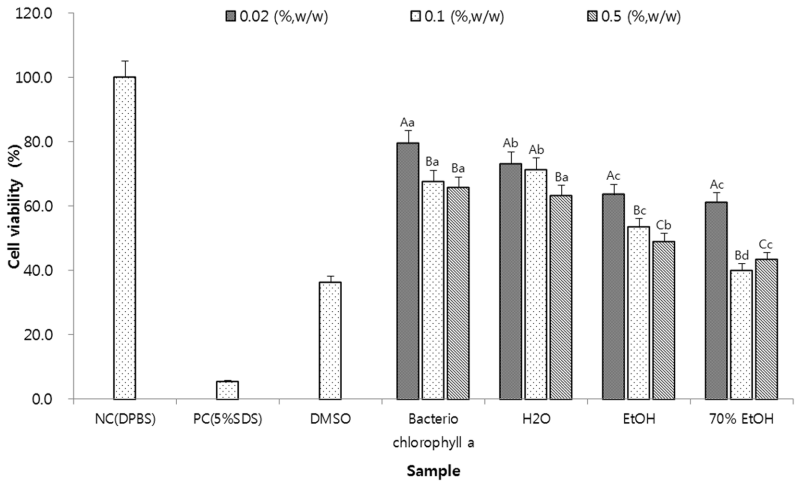

Fig. 2. Observation of skin cell irritation by the extracts of *Rhodobacter sphaeroides* and bacteriochlorophyll *a*.
Mean values ± SD from triplicate separate experiments are shown. Means with a different letter (A–C) within the same sample are significantly different at $p < 0.05$, and likewise means with a different letter (a–d) within the same concentration.

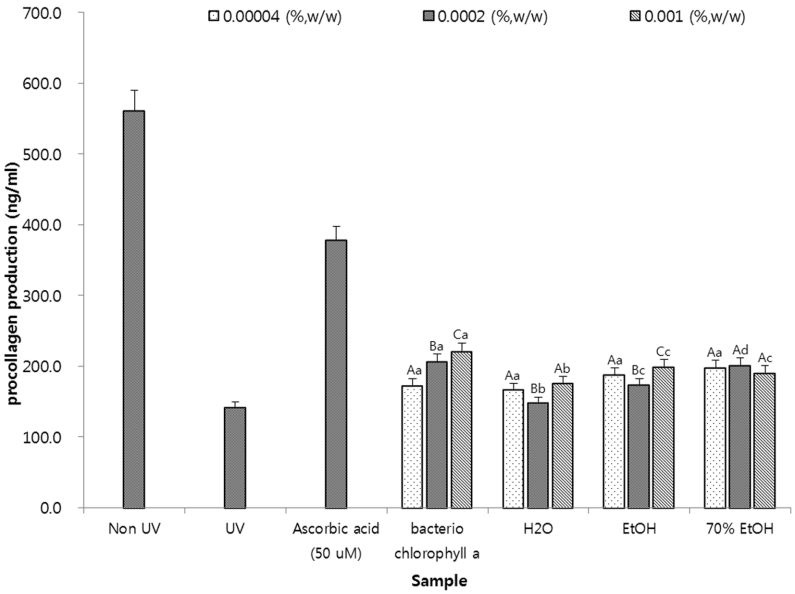

Fig. 3. Comparison of procollagen production from human fibroblast cell CCD-986sk after adding the extracts of *R. sphaeroides* and bacteriochlorophyll *a*.

Mean values ± SD from triplicate separate experiments are shown. Means with a different letter (A–C) within the same sample are significantly different at $p < 0.05$ and means with a different letter (a–d) within the same concentration are significantly different at $p < 0.05$.

irradiated before treatment with other substances. When the bacteria were treated with the positive control dexamethasone after UV irradiation, a TNF-α secretion rate of 59.2% was observed. Although the TNF-α secretion rates of photosynthetic bacteria samples from extraction solvent exceeded 100% in most cases, a TNF-α secretion rate of 67.6% was identified in the case of 100% ethanol extracts, and a TNF-α secretion rate of 82.3% was identified in the case of 70% ethanol extracts. However, the range of increase/decrease according to concentration was quite large. The TNF-α secretion rates of single-pigment bacteriochlorophyll *a* concentrations decreased in a concentration-dependent manner, and a secretion rate of 63.8% was identified at the highest concentration, indicating effects close to those of

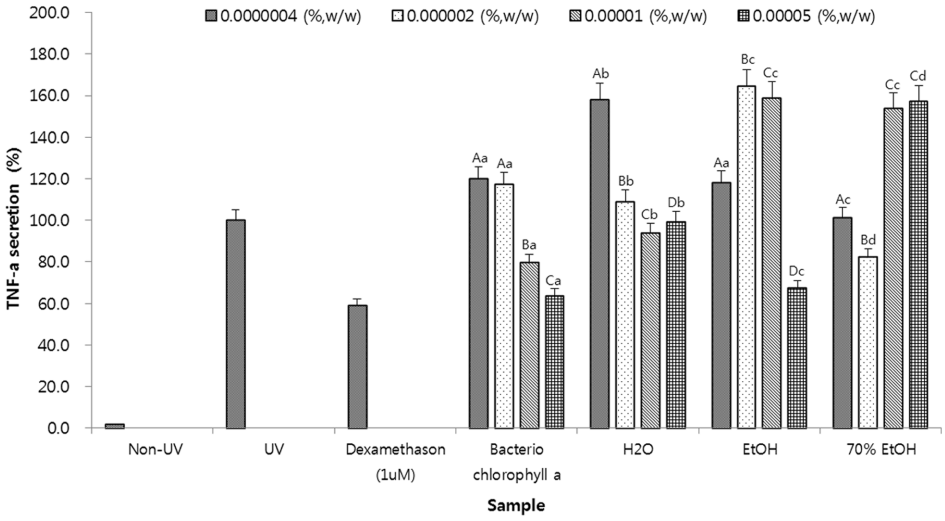

Fig. 4. The secretion of TNF-α from HaCaT cells after adding the extracts of *R. sphaeroides* and bacteriochlorophyll *a*.

Mean values ± SD from triplicate separate experiments are shown. Meana with a different letter (A–D) within the same sample are significantly different at $p < 0.05$ and means with a different letter (a–d) within the same concentration are significantly different at $p < 0.05$.

dexamethasone (positive control). When the above-mentioned results were compared with those of previous studies on *Angelica gigas* water extracts that reported TNF-α reduction effects, the superiority of the TNF-α reduction effects of photosynthetic bacteria and bacteriochlorophyll *a* was identified, because the lowest secretion rate was greater than 85% in the case of *Angelica gigas* water extracts [8]. Because TNF-α increases the generation of MMP-1, an enzyme that hinders collagen generation [2, 21], bacteriochlorophyll *a* that can reduce TNF-α is expected to be capable of reducing enzymes that hinder collagen generation, thereby showing efficacy for skin wrinkle improvement.

Up- and Down-Regulation of Skin-Aging-Related Gene Expression

To more precisely assess the anti-skin-aging activities of photosynthetic bacteria and bacteriochlorophyll *a*, the gene expression levels of keratinocyte-growth-related marker protein hKRT10-F keratin 10, cell-differentiation-related proteins hIVL-F involucrin, hTGM1-F transglutaminase-1 (GTase-1), and hLAMA5-F laminin 5 (a protein related to dermal-epidermal junctions important for skin elasticity) were measured from the growth of keratinocytes and skin fibroblasts [3, 7, 24, 27]. The gene expression of representative collagenase and MMPs was also observed. As shown in Table 2, in considering the no-treatment group as a control with an amount of expression of 1.0, the amounts of keratin 10 expression increased by the extract of *R. sphaeroides* was 0.1, which indicates that *R. sphaeroides* itself has no cell growth effects. However, bacteriochlorophyll *a* showed very high up-regulated gene expression up to 4.28 times higher than that of the control, indicating that it can greatly improve the fibroblast cell growth. In the case of involucrin, although the photosynthetic bacteria expression levels were less than those of the control, bacteriochlorophyll *a* showed notably high expression from the growth of human fibroblast cells, up to 15.5 times higher than that of the control. This result definitely proves that the pigment should positively affect the cell differentiation and eventually prevent the skin aging process. Moreover, for TGase-1 expression, excellent effects were also identified in treating with bacteriochlorophyll *a* by comparing with the case of photosynthetic bacteria in the same concentration. However, the expression levels of laminin 5 were found to be lower in both samples than in the control, indicating that there was no effect on dermal-epidermal junctions of the fibroblast cells.

In experiments conducted with the same genes for keratinocytes, in the case of keratin 10, the bacteriochlorophyll *a* group expression amount was identified with a value close to 15 times that of the control. The photosynthetic bacteria group showed values that were approximately 10% higher than that of the control. In the case of involucrin, the amounts of expression in both sample groups were quite close to those of the control, and thus no remarkable effect was shown. In the case of TGase-1, the bacteriochlorophyll *a* group showed 3.5 times higher gene expression than that of the control, and the photosynthetic

Table 2. Comparison of the gene expression levels after adding the extracts of *R. sphaeroides* and bacteriochlorophyll *a*.

Gene expression (fold)	Cell	Control	Bacteriochlorophyll *a*	*Rhodobacter sphaeroides*
Keratin 10	Fibroblast	1.00	4.28 ± 0.32^{A}	0.10 ± 0.01^{B}
	Keratinocyte	1.00	14.97 ± 1.13^{A}	1.19 ± 0.21
Involucrin	Fibroblast	1.00	15.50 ± 1.26^{A}	0.76 ± 0.13^{B}
	Keratinocyte	1.00	1.17 ± 0.32	0.81 ± 0.11^{A}
Transglutaminase (TGase)	Fibroblast	1.00	7.98 ± 0.65^{A}	0.35 ± 0.09^{B}
	Keratinocyte	1.00	3.49 ± 0.26^{A}	2.16 ± 0.15^{B}
Laminin5	Fibroblast	1.00	0.44 ± 0.04^{A}	0.29 ± 0.02^{B}
MMP-1		1.00	1.00 ± 0.12	3.04 ± 0.32^{A}
MMP-2		1.00	1.30 ± 0.08^{A}	1.61 ± 0.11^{B}
MMP-3		1.00	0.24 ± 0.02^{A}	1.96 ± 0.09^{B}
MMP-7		1.00	5.22 ± 0.39^{A}	2.09 ± 0.21^{B}
MMP-8		1.00	0.49 ± 0.07^{A}	0.02 ± 0.00^{B}
MMP-9		1.00	0.26 ± 0.06^{A}	2.58 ± 0.25^{B}
Filaggrin	Keratinocyte	1.00	1.64 ± 0.11^{A}	1.38 ± 0.17^{A}

Mean values ± SD from triplicate separate experiments are shown.

Means with a different letter (A-B) within the same gene expression are significantly different at $p < 0.05$.

bacteria group also showed 2.2 times up-regulated gene expression than that of the no-treatment control, such that the effects could be identified in both sample groups. These results show that although photosynthetic bacteria had almost no effect for skin cell growth or regeneration, bacteriochlorophyll *a* should have high skin anti-aging activities for both types of cells. It was interesting that some genes closely related to the differentiation of the cells located in the epidermis, such as keratin 10, involucrin, and TGase-1, were also up-regulated after treating the samples into the fibroblast cells [3, 10, 13], even though their expression levels were somewhat fluctuated and not much in consistency. However, in general, the expression levels were up-regulated and seemed to be correlated with the patterns of up-regulation of the genes in the keratinocytes. These results strongly imply that baceteriochlorophyll *a* should play an important role in increasing the cell differentiation of mainly keratinocytes and also possibly fibroblasts, and which results in greatly improving its anti-skin-aging activities. However, detail mechanisms of increasing those gene expression levels in the fibroblast cells by the bacteriochlorophyll *a* should be further investigated.

For the gene expression of the MMP series (collagenases) from the fibroblast cells, in general, the effects on skin wrinkle improvement could be observed. In the case of MMP-1, -2, and -7, the samples appear to have almost no effect on hindering collagenase because the gene expression levels of the samples were much higher than that of the control. However, in the case of MMP-3 and -9, although the gene expression levels by the photosynthetic bacteria were higher than the control, such that no effect could be expected, the bacteriochlorophyll *a* group showed notably low expressions close to 1/5 of those of the control group. In addition, in the case of MMP-8, both the photosynthetic bacteria group and the bacteriochlorophyll *a* showed values lower than those of the control, and in particular, the photosynthetic bacteria showed rather low expression amounting to 1/50 that of the control.

The amounts of expression of the protein filaggrin, which plays key roles in the maintenance of skin barriers, were also evaluated because filaggrin is a protein involved in important functions for keratinocyte differentiation as well as maintenance of skin moisture [29]. In general, the gene expression levels of filaggrin by both photosynthetic bacteria and the bacteriochlorophyll *a* were increased, compared with that of the control. The expression level was increased by ca. 1.4 times after treating with photosynthetic bacteria extract and at least 1.6 times high with bacteriochlorophyll *a*, indicating that bacteriocholorophyll *a* should have an effect for maintaining the skin barriers. These results imply that the photosynthetic bacteria and bacteriochlorophyll *a* are able to prevent optical aging of the skin and also to provide skin aging improvement by preventing moisture loss [11]. Generally speaking, although skin cell regeneration and differentiation and wrinkle improvement cannot be expected from the photosynthetic bacteria *per se*, excellent effects on gene expression related to skin cell regeneration and differentiation and wrinkle improvement can be expected from bacteriochlorophyll *a*. Based on these results, we could conclude that bacteriochlorophyll *a* can be considered effective for skin aging improvement among cosmetic materials.

Measurement of Antioxidant Activities of the Samples

Fig. 5 demonstrates the correlation between skin anti-aging activities with the antioxidant activity of both samples, *R. sphaeroides* extract and bacteriochlorophyll *a*. Ascorbic acid, a strong positive control, was used, and its activity was assumed as 100% for calculation of the activity of each sample as a relative percentage. Although photosynthetic bacteria water extracts and 70% ethanol extracts showed rather low scavenging activity, not exceeding 15%, 100% ethanol extracts showed high activity of up to 57.2%. Although pigment bacteriochlorophyll *a* contained in photosynthetic bacteria did not show concentration-dependent activity values (unlike photosynthetic bacteria extracts), a value of 63.8% (the highest) was identified such that its antioxidant activity could be identified. Black chokeberry (aronia) and blueberry showed DPPH free radical scavenging activity in a range of 40−60% at the same concentration, indicating that the antioxidant activity of photosynthetic bacteria extracts and the pigment bacteriochlorophyll *a* contained in photosynthetic bacteria is effective to the extent that is comparable to that of other natural products [4]. Referring to the fact that photosynthetic bacteria serve as electron donors, the antioxidant activity was assessed using the DPPH free radical scavenging activity assay. In the case of photosynthetic bacteria extracts from extraction solvents, large deviations were identified according to the solvents. Therefore, the selection of solvent appears to be highly important for extraction.

Discussion

In this work, we first showed the skin anti-aging activities of bacteriochlorophyll *a* isolated from photosynthetic bacteria, *Rhodobacter sphaeroides*, even though this bioactive pigment

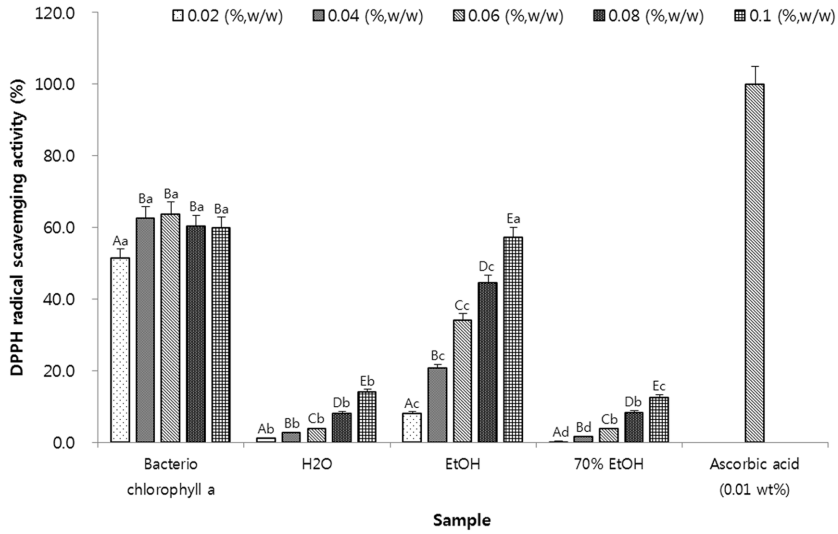

Fig. 5. DPPH free radical scavenging activities of *R. sphaeroides* and bacteriochlorophyll *a*.
Mean values ± SD from triplicate separate experiments are shown. Means with a different letter (A–E) within the same sample are significantly different at $p < 0.05$ and means with a different letter (a–d) within the same concentration are significantly different at $p < 0.05$.

existed in relatively large quantities and has also been known to have antioxidant activities. It was also found that the bacteriochlorophyll *a* and the extracts of photosynthetic bacteria were safe in terms of cell cytotoxicity and skin irritation. The bacteriochlorophyll *a* also proved its skin anti-aging activity by maintaining larger induction of procollagen production than those by photosynthetic bacteria extracts. In addition, when the secretion rates of cytokine TNF-α (which promotes increases in the generation of collagenase MMP-1) were measured [2, 21], bacteriochlorophyll *a* showed secretion rates close to those of the positive control dexamethasone such that its effects could be identified. Although low TNF-α secretion rates were shown by the extracts as well, the secretion rates were not concentration dependent and showed large deviations, indicating problems in utilization to a certain extent. Its skin anti-aging efficacy was also confirmed by measuring the gene expression level of the proteins involved in cell differentiation, cell growth, and wrinkle improvement, and the genes for skin cell differentiation and growth were greatly up-regulated in treating cells with bacteriochlorophyll *a*, compared with the extracts from photosynthetic bacteria itself. The expression of genes for wrinkle improvement was not significantly observed in photosynthetic bacteria, but the effects of bacteriochlorophyll *a* could be well identified for certain genes (keratin 10, involucrin, transglutaminase, MMPs, and filaggrin). In addition, in observing the genes related to skin barriers for helping to reduce the skin aging process, bacteriochlorophyll *a* also showed better skin protection activities. The antioxidant effect of bacteriochlorophyll *a* was also found to be better than those of photosynthetic bacteria extracts, and these results strongly indicate that higher antioxidant activity of the bioactive pigment could positively affect both skin wrinkle and anti-aging activities, even though anti-inflammation activity of the pigment was shown and could be partially involved in its skin anti-aging process. These results could also be supported by other reported data on the association between the removal of active oxygen by antioxidant effects and the wrinkle improvement and skin aging improvement [25]. Therefore, it can be concluded that bacteriochlorophyll *a* could be used as a new biomaterial for reducing the skin aging process, and possibly photosynthetic bacteria *Rhodobacter sphaeroides* itself, too. However, further studies on the secretion of the proteins related MMP and/or involucrin and TGase, *etc*. should be carried out to more clearly explain the anti-skin-aging mechanisms of this pigment [3, 24].

References

1. Ahn KJ. 2001. The studies on the factors of photosynthesis in photosynthetic bacteria. *Bullet. Inst. Basic Sci.* **15:** 79-92.
2. Baugh MD, Hollander AP, Evans GS. 1998. The regulation of matrix metalloproteinase production in human colonic fibroblasts. *Ann. N. Y. Acad. Sci.* **859:** 175-179.
3. Choo JH, Lee SH. 2013. The effect of chrysin on the transcriptional activity of vitamin D receptor in human

keratinocytes. *J. Soc. Cosmet. Sci. Kor.* **39:** 75-81.
4. Chung HJ. 2014. Comparison of total polyphenols, total flavonoids, and biological activities of black chokeberry and blueberry cultivated in Korea. *J. Kor. Soc. Food Sci. Nutr*. **43:** 1349-1356.
5. Dietz BM, Kang YH, Liu G, Eggler AL, Yao P, Chadwick LR, *et al*. 2005. Xanthohumol isolated from *Humulus lupulus* inhibits menadione-induced DNA damage through induction of quinone reductase. *Chem. Res. Toxicol*. **18:** 1296-1305.
6. Eimhjellen KE, Aasmundrud O, Jensen A. 1963. A new bacterial chlorophyll. *Biochem. Biophysic. Res. Commun*. **10:** 232-236.
7. Giannelli G, Falk-Marzillier J, Schiraldi O, Stetler-Stevenson WG, Quaranta V. 1997. Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. *Science* **277:** 225-228.
8. Han HS. 2013. Anti-inflammatory effect of *Angelicae gigantis* Radix water extract on LPS-stimulated mouse macrophages. *Kor. J. Herbol*. **28:** 113-119.
9. Holzapfel W, Finkele U, Kaiser W, Oesterhelt D, Scheer H, Stilz HU, *et al*. 1989. Observation of a bacteriochlorophyll anion radical during the primary charge separation in a reaction center. *Chem. Phys. Lett*. **160:** 1-7.
10. Hong IK, Kim EJ, Seok JH, Kim BH, Jang JD, Joe GJ, *et al*. 2014. Effects of *Eucommia ulmoides* Oliver extract on inhibition of β-hexosaminidase and keratinocyte differentiation. *J. Soc. Cosmet. Sci. Kor.* **40:** 21-28.
11. Hyung SH, Min KJ, Kim YC. 2010. Alleviating effect of *Gardeniae fructus* water extract on inflammation and skin-barrier damage. *J. Cosmetol. Sci*. **6:** 63-72.
12. Jung KM, Moon JY, Lee SH, Kim CW, Park CB, Kim BH. 2008. The validation of alternative methods of reconstructed human skin equivalents for the assessment of skin irritation. *J. Alternat. Anim. Exp*. **2:** 31-38.
13. Kim BR, Lee SM, Hwang TY, Kim HS. 2013. Anti-oxidative and skin barrier effects of natural plants with a supercritical extract. *Kor. J. Food Preserv.* **20:** 597-601.
14. Kim DS, Lee HJ. 2004. Characterization of enzymes against oxygen derivatives produced by *Rhodobacter sphaeroides* D-230. *Kor. J. Microbiol*. **40:** 94-99.
15. Kim JS, Seo YC, Choi WY, Kim HS, Kim BH, Shin DH, *et al*. 2011. Enhancement of antioxidant activities and whitening effect of *Acer mono* Sap through nano encapsulation processes. *Kor. J. Med. Crop Sci.* **19:** 191-197.
16. Kim NY, Kim JH, Choi GP, Lee HY. 2014. Comparison of anti-skin wrinkle activities of *Aronia melanocarpa* extracts by extraction methods. *Kor. J. Med. Crop Sci*. **22:** 217-222.
17. Lee ES, Lee JW. 2000. Isolation of ubiquinone formation photosynthetic bacteria *Rhodobacter* sp. N2. *Kor. J. Food Nutr*. **13:** 558-562.
18. Lee SY, Lee JY. 2013. Inhibitory efficacy of *Smilax china* L. on pro-collagen type-1 activity and *mmp-1* gene expression in fibroblasts (CCD-986sk). *J. Life Sci*. **23:** 1239-1245.
19. Lee YS, Kim HS, Kim KS, Kim SD. 2000. IL-6 mRNA Expression in Mouse Peritoneal Macrophage and NIH3T3 Fibroblasts in Response to *Candida albicans*. *J. Microbiol. Biotechnol*. **10:** 8-15.
20. Li JJ, Dong Z, Dawson MI, Colburn NH. 1996. Inhibition of tumor promoter-induced transformation by retinoids that transperase AP-1 without transactivating retinoic acid response element. *Cancer Res*. **56:** 483-489.
21. Macnaul K, Chartrain N, Lark M, Tocci M, Hutchinson N. 1990. Discoordinate expression of stromelysin, collagenase, and tissue inhibitor of metalloproteinases-1 in rheumatoid human synovial fibroblasts: synergistic effects of interleukin-1 and tumor necrosis factor-alpha on stromelysin expression. *J. Biol. Chem*. **265:** 17238-17245.
22. Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65:** 55-63.
23. Oelze J, Drews G. 1970. Variations of NADH oxidase activity and bacteriochlorophyll contents during membrane differentiation in *Rhodospirillum rubrum*. *Biochim. Biophys. Acta Biomembranes* **219:** 131-140.
24. Park JS. 2008. Epidermal homeostasis and dry skin management. *J. Soc. Cosmet. Sci. Kor*. **34:** 1-8.
25. Park SN. 1997. Skin aging and antioxidant. *J. Soc. Cosmet. Sci. Kor*. **50:** 329-341.
26. Pentland AP, Shapiro SD, Welgus HG. 1995. Agonist-Induced expression of tissue inhibitor of metalloproteinases and metalloproteinases by human macrophages is regulated by endogenous prostaglandin E_2 Synthesis. *Soc. Invest. Dermatol*. **104:** 52-57.
27. Suh SS, Seo HH, Lee HJ, Hwang JI, Park MR, Moh SH, *et al*. 2014. Anti-corrugation activity of micosporine-like amino acid mixtures from *Chlamydomonas* sp. *J. Kor. Acad. Ind. Coop. Soc*. **15:** 5393-5399.
28. Tai SP, Kaplan S. 1985. Intracellular localization of phospholipid transfer activity in *Rhodopseudomonas sphaeroides* and a possible role in membrane biogenesis. *J. Bacteriol.* **164:** 181-186.
29. Youn DH, Shin HT. 2012. The anti-bacterial effects and epidermal permeability barrier function of red onion juice produced in Jeon-Nam province in Korea. *Kor. J. Orient. Prev. Med. Soc*. **16:** 43-56.