Crude protein from spirulina increases the viability of CCD-986sk cells via the EGFR/MAPK signaling pathway

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Abstract. Spirulina, an edible blue-green alga, has great potential for various applications in human health, possibly including reduced skin aging. The mechanisms by which spirulina crude protein (SPCP) may influence human skin fibroblast viability are not yet understood; therefore, a human dermal fibroblast cell line (CCD-986sk) was used as a cell model system to study the influence of SPCP on human skin fibroblast viability. An enzyme-linked immunosorbent assay showed that collagen formation improved in SPCP-treated cells in a dose-dependent manner, while elastase activity was decreased. In addition, western blot analysis showed a dose-dependent decrease in the expression of the aging-associated gene matrix metalloproteinase-8, a collagen-degradative enzyme. It was also shown that SPCP upregulated epidermal growth factor receptor (EGFR) activity, leading to activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway. Together, these results demonstrated that SPCP increases human fibroblast viability by activation of the EGFR/MAPK signaling pathway. This contribution sheds light on the molecular mechanism for SPCP increasing the viability of human skin cell and provides a potential efficient cosmeceutical for protecting human skin.

Introduction

The blue-green microalga, spirulina (*Arthrospira platensis*), has recently been used as a food source due to its high protein content and nutritional value (1,2). Spirulina has been extensively studied owing to its nutrient composition, which has been demonstrated to effectively treat a number of medical conditions (such as anti-cancer, inhibiting senescence and enhancing the non-specific cellular immune function) (3). Spirulina has also been reported to have an inhibitory effect on ultraviolet B-induced skin inflammation (4,5). It has an antioxidant defense system that removes reactive oxygen species that may damage cells (6-8). Previous studies have reported that early skin aging can be repaired using an extract from microalgae, which prevents wrinkle formation and has a tightening effect (9); thus, such extracts have been used in many skin care products (10). Despite these benefits, the mechanisms underlying the protective effects of spirulina crude protein (SPCP) on skin cells are largely unknown.

The prevention of skin aging has attracted considerable attention, both scientifically and cosmetically. Owing to the disruption of its barrier function over time, the aged skin has a dry appearance and has an increased risk of skin disorders (11). Deterioration of skin elasticity and the skin extracellular matrix (ECM) are characteristics of the aging skin. Reduction of the elastic properties in dermal layers can lead to the formation of wrinkles in humans and animals (12-14). Furthermore, three-dimensional alteration of elastic fibers indicates the marked and continuous upregulation of an elastin-degrading enzyme in the aging skin. The ECM of the dermis consists of collagen and is produced by fibroblasts (15). In particular, type I collagen is the most prevalent of the fibril-forming collagens (16,17). Matrix metalloproteinase-8 (MMP-8), a zinc-dependent endopeptidase, is present at sites of acute inflammation and potently degrades type I collagen (18,19). Anti-wrinkle strategies therefore include enhancing skin elasticity and collagen content. Additionally, the promotion of proliferation and differentiation of human dermal fibroblasts can also help delay the aging process of the skin (20-22).

Fibroblast proliferation is regulated by many growth factors. Epidermal growth factor receptor (EGFR) is one of the most well-known cell proliferation proteins in the body (23), and it has been reported that EGFR levels in dermal fibroblasts decline with age (21). A previous study has demonstrated that EGFR inhibitors may promote human skin aging (24). Green *et al* (25), reported that EGFR expression levels are lower in the dorsal skin of old rats (day 23 or day 51) compared with younger ones (neonatal, day 1). Furthermore, EGFR

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expression levels were lower in old human dermal fibroblasts (Passage 16 or greater) compared with young human fibroblasts (Passage 9 or less) (26). The mitogen-activated protein kinase (MAPK) signaling pathway is one of the EGFR-activated downstream signaling pathways and is an important regulator of cell proliferation (27,28). Therefore, in the present study, the effects of SPCP on the EGFR/MAPK signaling pathway in CCD-986sk cells were investigated. These results shed light on the molecular mechanism for SPCP increasing the viability of human skin cell and provide a potential efficient cosmeceutical for protecting human skin.

Materials and methods

Preparation of SPCP. Spirulina powder [40 g, New Zealand Nutritionals (2004) Ltd., Burnside Christchurch, New Zealand] was soaked in distilled water (1l) and mixed for 4 h at room temperature. Following centrifugation at 2,399 x g at 4°C for 10 min, it was incubated for 4 h with three volumes of ethanol at 4°C. The solution was centrifuged at 2,399 x g at 4°C for 10 min. The supernatant was filtered and concentrated using rotary evaporation at 40°C (29), and the concentrated solution was precipitated overnight with 80% saturated $(NH_4)_2SO_4$ solution at 4°C. After standing at 4°C for 10 min, the precipitate was dissolved in and dialyzed against distilled water. The dialysate was concentrated (Rotary evaporator, 40°C) and freeze-dried (1000 μg/ml), and subsequently used as the SPCP preparation in subsequent experiments.

Assessment of the SPCP protein profile. The protein profile of the SPCP was analyzed by subjecting the extract to SDS-PAGE and Coomassie Brilliant Blue staining. In brief, the SPCP were mixed with 5 x sample loading buffer [50 mM Tris-HCl, 2% SDS, 10% Glycerol, 0.02% Bromophenol blue (BPB), 5% 2-mercaptoethanol], and SPCP containing 75 μg of protein were separated by 15% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue for 1 h at room temperature and washed with destaining solution until the bands appeared (3). SPCP of 20 kDa and ~16 kDa were performed a quadrupole time of flight mass spectrometry (Q-TOF MS/MS) analysis by Peptron, Inc. (Daejeon, Korea).

Cell culture. The CCD-986sk human dermal fibroblast cell line (CRL-1947; American Type Culture Collection, Manassas, VA, USA), derived from normal female skin tissue, was cultured in Dulbecco's Modified Eagle's Medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin at 37°C with 5% CO_2 in a saturated humidified incubator (30). The CCD-986sk cells were cultured to 60-80% confluency in 100-mm diameter plates, and the medium was replaced every 2 days.

Cell viability assay. Cell viability was determined using the CellTiter 96 AQueous One Solution Reagent (Promega Corporation, Madison, WI, USA). CCD-986sk cells were seeded in a 96-well plate at a density of $0.5x10^4$ cells/well. After 24 h of incubation, the cells were incubated with serum-free medium (SFM) for 4 h at 37°C. Serum contains several hormones, which are stimulatory for cell growth

and mask the effects of SPCP. To avoid the complication, SFM was used in all of the groups tested (control and SPCP-treated groups) (31). Subsequently, various concentrations of SPCP (6.25, 12.5 or 25 μg/ml in SFM) or SFM alone (control) were used to treat the cells. The cells were further cultured for 24 h. Subsequently, the cells were exposed to the 3-(4,5-dimethylthiazol-2-yl)-5-(3-caboxymethoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium (MTS) assay solution at 37°C for 30 min, and the optical density at 490 nm was measured using a Synergy HTX microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Data are expressed as the percentage of viable SPCP-treated cells compared with viable cells in the SFM-treated control.

Elastase activity. Elastase activity was measured using N-succinyl-Ala-Ala-Ala-p-nitroanilide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Cells were seeded into 6-well plates at a density of $5x10^4$ cells/well. After 24 h of culture, the cells were incubated with SFM for an additional 4 h at 37°C. Various concentrations of SPCP (6.25, 12.5 or 25 μg/ml in SFM) or SFM alone (control) were used to treat the cells, and they were further cultured for 24 h. The cells were collected in radioimmunoprecipitation assay buffer (iNtRON Biotechnology, Seongnam, Korea) with 1% protease inhibitor using a cell scraper. The cell supernatant was collected by centrifugation (1,8341 x g; 4°C; 10 min). Subsequently, the supernatant (98 μl) and 25 mg/ml N-succinyl-Ala-Ala-Ala-p-nitroanilide (2 μl) were added to each well (32). After incubation at 37°C for 30 min, the optical density at 410 nm was measured using a Synergy HTX microplate reader (BioTek Instruments, Inc.). Data are expressed as a percentage of elastase activity in treated cells compared with the SFM-treated control.

Procollagen type I C-peptide (PIP) solid phase enzyme immunoassay. The level of procollagen was measured with the Takara MK101 kit (Takara Bio, Inc., Otsu, Japan). Cells were seeded into 6-well plates at a density of $5x10^4$ cells/well. After 24 h of incubation, the cells were incubated with SFM for 4 h at 37°C, followed by treatment with various concentrations of SPCP (6.25, 12.5, or 25 μg/ml in SFM) or SFM (control) for an additional 24 h. The cell medium was collected and centrifuged (13,475 x g; 4°C; 10 min). Subsequently, 100 μl of antibody-peroxidase conjugate solution (included in the Takara MK101 kit) and 20 μl of supernatant were added to each well. After incubation for 3 h at 37°C, each well was washed four times using 400 μl of PBS each time. A total of 100 μl of 3,3',5,5'-tetramethylbenzidine substrate solution was then added to each well and incubated for 15 min at room temperature (32). The reaction was terminated with 100 μl of stop solution (1 N H_2SO_4). The absorbance at 450 nm was measured using a Synergy HTX microplate reader (BioTek Instruments, Inc.).

Western blotting. CCD-986sk cells were cultured to 50-60% confluency and then incubated with SFM for 4 h at 37°C. The medium was replaced with three concentrations of SPCP (6.25, 12.5 or 25 μg/ml in SFM) or SFM (control). The cells were cultured for another 24 h and proteins were extracted using radioimmunoprecipitation lysis buffer

(iNtRON Biotechnology) with 1% protease inhibitor, followed by centrifugation at 18,341 x g at 4°C for 10 min. Protein concentrations were determined using the Bicinchoninic assay. The protein samples (30 μg) were resolved by 7.5-12.5% SDS-PAGE and subsequently transferred electrophoretically to polyvinylidene difluoride membranes. After washing with methanol, the membranes were blocked with 1% bovine serum albumin (MP Biomedicals LLC, USA) in TBS + Tween-20 [10 mM Tris-HCl, 150 mM NaCl (pH 7.5) and 0.1% Tween-20] and incubated overnight at 4°C with specific primary antibodies (all 1:1,000) (33). After washing twice, the membranes were incubated for 2 h at room temperature with the secondary antibodies (all 1:10,000). The second antibodies were horseradish peroxidase (HRP)-conjugated anti-rabbit lg G (cat. no. 7074S; Cell Signaling Technology, Inc., Beverly, MA, USA), donkey anti-goat lgG (cat. no. A50-101p; Bethyl Laboratories, Inc., MA, USA) and anti-mouse lgG (cat. no. 7076S; Cell Signaling Technology, Inc.). The following primary antibodies obtained from Santa Cruz Biotechnology, Inc., were used: Goat anti-phosphorylated (p)-EGFR antibody (cat. no. sc-12351), goat anti-EGFR antibody (cat. no. sc-03), mouse anti-Shc antibody (cat. no. sc-967), rabbit anti-growth factor receptor bound protein (GRB)2 antibody (cat. no. sc-255), rabbit anti-Son of sevenless homolog (Sos) antibody (cat. no. sc-259), rabbit anti-H-Ras antibody (cat. no. sc-520), mouse anti-p-Mitogen-activated protein kinase kinase (MEK)-1/2 antibody (cat. no. sc-81503), mouse anti-MEK-1/2 antibody (cat. no. sc-81504), mouse anti-p-extracellular signal-regulated kinase (ERK) antibody (cat. no. sc-7383), mouse anti-ERK 1 antibody (cat. no. sc-271269), rabbit anti-ERK-2 antibody (cat. no. sc-154), and rabbit anti-GAPDH antibody (cat. no. sc-25778). The signals were detected using an Enhanced Chemiluminescence western blot kit (Thermo Fisher Scientific, Inc.) using a bioanalytical imaging system (Azure Biosystems, Dublin, CA, USA). The densities of the bands which normalized to GAPDH were analyzed using Multi-Gauge software, v.3.0 (Fujifilm Life Science, Tokyo, Japan).

Statistical analysis. For all assays, at least three independent experiments were performed. The mean ± standard deviation of the expression values was calculated using Excel software v.2007 (Microsoft Corporation, Redmond, WA, USA). The differences among multiple groups were evaluated using one-way analysis of variance followed by Bonferroni post hoc test using SPSS statistical software for Windows, v.20.0 (IBM Corp., Armonk, NY, USA).

Results

Positive effects of SPCP on human skin cell growth. SPCP is a complex mixture of proteins, which were separated by SDS-PAGE and stained with Coomassie Brilliant Blue revealing ~20 and ~16 kDa bands (Fig. 1). The two bands were analyzed by Q-TOF MS/MS. The results revealed that the 20 kDa band didn't correspond to any known protein while the ~16 kDa protein band was C-phycocyanin α chain, which has many pharmacological benefits (34). To investigate the effects of SPCP on the viability of CCD-986sk cells, an MTS assay on cells treated with various doses of SPCP was performed. The viability of cells treated with 6.25, 12.5 or
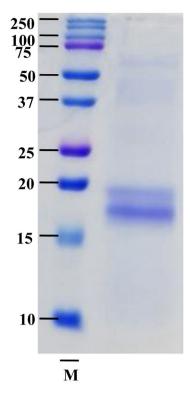

Figure 1. Protein profile of SPCP. SPCP was loaded on a 15% polyacrylamide gel and stained with Coomassie Brilliant Blue. M, marker.

25 μg/ml SPCP increased by 18±4.41%, 33±1.62% (P<0.01) and 42±2.82% (P<0.001), respectively, compared with the control (Fig. 2). Together, the results indicated that SPCP effectively promoted the growth of CCD-986sk cells at in a dose-dependent manner.

Inhibitory effect of SPCP on elastase activity. Elastase activity in CCD-986sk cells following various treatments with SPCP was measured. Compared with the control, SPCP treatment significantly decreased the elastase activity in a dose-dependent manner (P<0.05 or P<0.001; Fig. 3), with a maximum decrease of 42±6.15% observed in cells treated with 25 μg/ml SPCP.

Effect of SPCP on PIP levels. To determine whether SPCP affected the secretion of PIP, secreted PIP levels were measured using ELISA. SPCP treatments significantly increased PIP levels in a dose-dependent manner (P<0.05 or P<0.001; Fig. 4A). Compared with the basal level of 77±2.78 ng/ml, PIP concentrations of 100±2.36, 121±9.45 and 187±7.92 ng/ml were induced by 6.25, 12.5, and 25 μg/ml SPCP, respectively. This result indicated that SPCP promoted the secretion of PIP.

Western blotting was used to determine the effect of SPCP on MMP-8 expression in CCD-986sk cells. MMP-8 protein expression levels were significantly decreased by SPCP treatment in a dose-dependent manner (P<0.001; Fig. 4B). These results suggested that SPCP promoted the synthesis of collagen and inhibited the expression of MMP-8.

Activation of the EGFR signaling pathway by SPCP in CCD-986sk cells. As SPCP treatment was revealed to enhance the viability of CCD-986sk cells (Fig. 2), the potential regula-
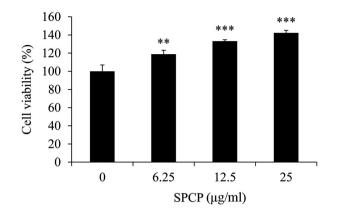
Figure 2. SPCP treatment increases the viability of CCD-986sk cells. Cell viability was determined by the MTS assay following treatment with various concentrations of SPCP for 24 h. Data are presented as the mean ± standard deviation of three independent experiments; **P<0.01 and ***P<0.001 vs. Control (0 µg/ml). SPCP, spirulina crude protein.

Figure 3. SPCP treatment reduces elastase activity in CCD-986sk cells. Elastase activity was determined by ELISA following treatment with various concentrations of SPCP for 24 h. Data are presented as the mean ± standard deviation of three independent experiments; *P<0.05 and ***P<0.001 vs. Control (0 µg/ml). SPCP, spirulina crude protein.

tion of the EGFR signaling pathway by SPCP was investigated. The EGFR signaling pathway was assessed by analyzing the total and p-EGFR protein expression levels. The expression levels of p-EGFR significantly increased following treatment of CCD-986sk cells with SPCP (P<0.001; Fig. 5A). SPCP treatment induced the expression of essential linkers of epidermal growth factor receptors to MAP kinase, including the adaptor proteins SHC and GRB2, and the guanine nucleotide exchange protein, SOS (P<0.01 or P<0.001; Fig. 5B). These results indicated that SPCP stimulation may activate the EGFR pathway in a dose-dependent manner.
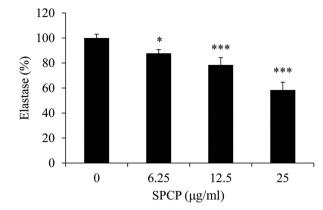
Activation of the MAPK pathway by SPCP in CCD-986sk cells. To determine whether EGFR activation induced activation of the MAPK signaling pathway, the expression levels of Ras were investigated following treatment with various doses of SPCP for 24 h. The expression levels of Ras significantly increased after SPCP treatment compared with untreated control cells (P<0.001; Fig. 6A). Meanwhile, SPCP also enhanced the phosphorylation and activation of MEK and ERK in a dose-dependent manner (P<0.001; Fig. 6B). Taken together, these results indicated that SPCP treatment stimulated CCD-986sk cell viability by activating the EGFR/MAPK/ERK signaling pathway.

Discussion

Microalgae proteins currently represent one of the most promising protein sources from food due to their abundant and balanced amino acid composition (35,36). *A. platensis* is an edible, photosynthetic, spiral-shaped, multicellular blue-green alga that possesses anti-inflammation and antioxidant properties. In the present study, spirulina was extracted with ethanol and $(NH_4)_2SO_4$. This extract (SPCP) contained proteins as determined by Coomassie Brilliant Blue staining. Q-TOF MS/MS analysis showed that the ~16 kDa protein band was C-phycocyanin α chain, which has been reported to have many pharmacological benefits, including anti-inflammatory, antioxidant and anticancer (34,37). The MTS assay showed that SPCP promoted the viability of human fibroblasts in a

dose-dependent manner. Cell viability increased by 18±4.41%, 33±1.62%, and 42±2.82% associated with the control after treatment with 6.25, 12.5 and 25 µg/ml SPCP, respectively. The results were confirmed by experiments performed in Hs27 cells (data not shown). The present study results suggested that SPCP treatment may lead to skin cell growth by enhancing the activation of growth factors in normal human fibroblasts.

Dermal fibroblasts are the primary cell type responsible for the production, maintenance and remodeling of the ECM in human skin (38). Skin fibrosis and aging are caused by an imbalance between the generation and degradation of ECM proteins, which results in severe alterations in the skin connective tissue (39). Skin aging is associated with a loss of ECM components from the dermis, including collagen, elastin, fibrillin, and proteoglycan (40). The proportion of type I collagen in dermis diminishes with intrinsic and extrinsic skin aging. In the present study, cell collagen production induced by SPCP treatment was 30-142% higher compared with the control group. The expression levels of the collagen-degrading protein MMP-8, which serves as a key enzyme in the degradation of collagen and stimulates the degradation of other major dermal components (which subsequently leads to aging) (41), was investigated by western blot analysis. These were decreased significantly in a dose-dependent manner in cells treated with SPCP. The increase in collagen might be due to a decrease in the expression of MMP-8, but this needs to be investigated further. Treatment of CCD-986sk cells with SPCP inhibited elastase activity and its activity was reduced in a dose-dependent manner.

Recent advance in understanding the role of endogenous growth factors in the aging process provides opportunities to develop novel anti-aging cosmeceutical products (24). Growth factors can have a prominent role in reversing the outcomes of skin aging (22). Topical application of human growth factors has been shown to reduce the signs and symptoms of skin aging, as well as increase dermal collagen synthesis, in several clinical studies (26). The MTS assay results obtained from the present study, showed that SPCP increased the viability of CCD-986sk cells. In order to explore the mechanism, growth factor protein expression in SPCP-treated CCD-986sk
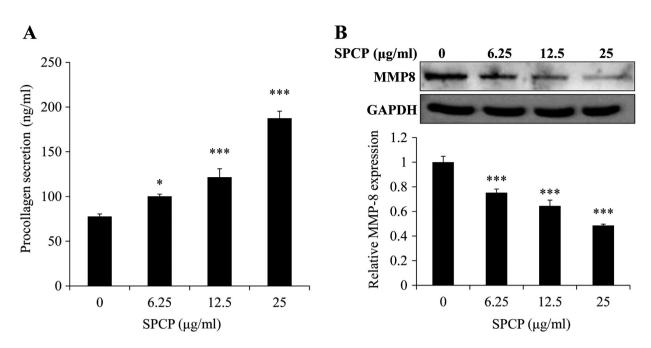
Figure 4. SPCP treatment induces procollagen secretion in CCD-986sk cells. (A) Procollagen secretion was determined using ELISA following treatment with various concentrations of SPCP for 24 h. (B) Changes in MMP-8 protein expression levels were measured by western blotting. Data are presented as the mean ± standard deviation of three independent experiments; $^*P<0.05$ and $^{***}P<0.001$ vs. Control (0 μg/ml). MMP, matrix metalloprotease; SPCP, spirulina crude protein.
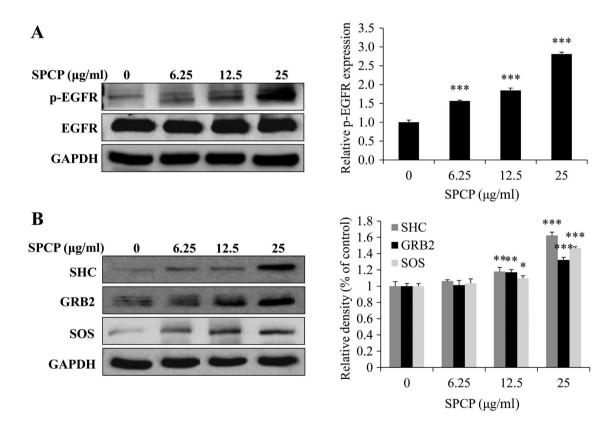

Figure 5. Treatment with SPCP activates the EGFR pathway in CCD-986sk cells. (A and B) CCD-986sk cells were treated with SPCP after incubation with serum-free medium for 4 h and expression levels of (A) total EGFR and pEGFR, and of (B) SHC, GRB2 and SOS were measured by western blotting. Data are presented as the mean ± standard deviation of three independent experiments; $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. Control (0 μg/ml). EGFR, epidermal growth factor receptor; GRB2, growth factor receptor bound protein 2; p, phosphorylated; SOS, son of sevenless homolog; SPCP, spirulina crude protein.

cells were investigated. It is reported that the activation of EGFR and insulin growth factor (IGF)-1R serve an important role in cell proliferation (23). Thus, the activation of EGFR and IGF-1R in CCD-986sk cells was measured. The results showed that EGFR was activated by SPCP rather than IGF-1R (data not shown). Furthermore, EGFR associated with skin
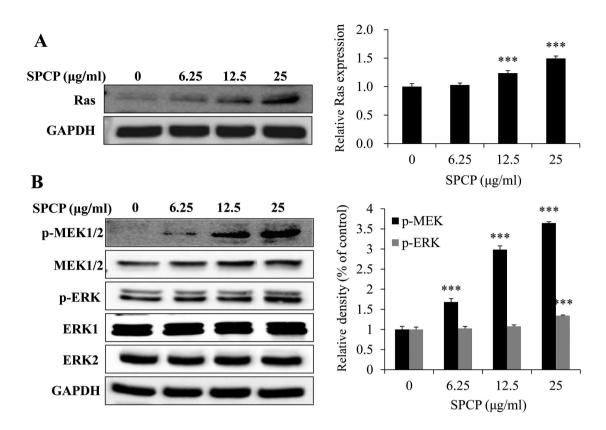
Figure 6. Treatment with SPCP activates the mitogen-activated protein kinase pathway in CCD-986sk cells. (A and B) CCD-986sk cells were treated with various concentrations of SPCP after incubation with serum-free medium for 4 h. Expression levels of (A) Ras and of (B) total and phosphorylated MEK and ERK were measured by western blotting. Data are presented as the mean ± standard deviation of three independent experiments. ***$P<0.001$ vs. Control (0 μg ml). ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase; p, phosphorylated; SPCP, spirulina crude protein.

aging (21). Based on the present data, it was found that SPCP enhanced the secretion of PIP and inhibited the activity of elastase. These results are beneficial for delaying skin aging. In the present study, p-EGFR was increased in SPCP-treated cells compared with the control cells. Additionally, the essential linkers from epidermal growth factor receptors to MAP kinase, including the adaptor proteins GRB2, SHC and SOS, were induced by SPCP treatment in a dose-dependent manner. These data indicated that SPCP stimulation induced EGFR pathway activation in a dose-dependent manner. A previous study reported that one of the main activated downstream signaling pathways is the MEK-ERK1/2 signaling pathway, which controls cell proliferation and differentiation (42). There are three proteins [ERK, c-Jun N terminal kinase (JNK) and p38] in MAPK signaling pathway (43). Only ERK1 and ERK2 are activated in response to growth factors, whereas JNK and p38 are more responsive to environmental stresses and inflammatory cytokines (44). The activation of this downstream signaling pathway was investigated and Ras levels were increased in SPCP treated cells, followed by phosphorylation and activation of MEK and ERK in a dose-dependent manner. The phosphorylation of ERK serves an important role in regulation of cell proliferation (43).

In summary, the expression levels of both MMP-8 protein and elastase were decreased by SPCP, leading to increased collagen levels. Additionally, SPCP treatment activated the EGFR and MAPK/ERK signaling pathways. To demonstrate the reproducibility of these results, other human dermal fibroblasts derived from different donors, such as different age and

gender will be used for further experiments. Based on these findings, it seems that the viability of human dermal fibroblasts was activated by SPCP. Overall, these results suggest that SPCP may be used as a cosmeceutical for potential applications in protecting human skin; however, further investigation is required. To further determine the effects of SPCP, hairless mice should be used to explore the change of epidermal thickness and collagen fibers with the application of SPCP.

Availability of data and materials

The analyzed data sets of the present study are available from the corresponding author on reasonable request.

Authors' contributions

PL, YHC and TJN made substantial contributions to the conception and design of this study; PL, MKL and JWC performed the

experiments; PL, MKL and YHC performed the data analysis; PL and YHC interpreted the experimental results and wrote the manuscript. All authors read and approved the final manuscript and agreed to the publication of the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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