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Research Article

Chlorella growth factor extraction and its effect on gene expression of types I and III collagen in skin fibroblast cells

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Abstract

Skin protects the body from outer factors as a barrier, and contains an important cosmetic role. Skin aging is related to collagen degradation and increasing multiple enzymes, including matrix metalloproteinase (MMP_S), which degrade collagen. *Chlorella vulgaris* is a marine alga that exhibits anti-aging activity. The beneficial effects of *Chlorella* on skin make it a proper ingredient to be used in anti-aging products. In this study, the effect of *Chlorella* growth factor (CGF) on types I and III collagen in human fibroblast cell line Hu02 was investigated. To find an effective extraction method in the present study, CGF was extracted using hot water extraction, enzymatic hydrolysis, and ultrasonication plus enzymatic hydrolysis, and the yields were compared. The yield of ultrasonication plus enzymatic hydrolysis method (CGF-3) had the strongest absorbance at 260 nm and highest solid recovery, compared to the other two methods. Using quantitative PCR, it was confirmed that CGF increased collagens I and III expression in skin fibroblast cells. This finding indicated that CGF induced collagen synthesis in Hu02 cells. Gene expression of types I and III collagen were elevated to 3.144-fold and 1.14-fold in the CGF-3 group, respectively, comparing to the control group.

Keywords: Chlorella growth factor, Collagen synthesis, Anti-aging, Human skin

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Introduction

Skin, as the largest organ, has multiple functions of which one of the most important is protection against various harmful agents. In recent years, people have begun to pay attention to skin beauty and health. Skin aging is a biological process that is related to reduction of collagen production, which decreases skin elasticity and several enzymatic activities (Kim *et al.*, 2017). Therefore, maintaining collagen levels in the dermis is important for maintaining healthy skin. Collagen in the extracellular matrix (ECM) is made by dermal fibroblasts. There are several types of collagen, which constitutes 80-90% of the dermis (Yoon *et al*., 2012). Type I collagen, which accounts for 85% of total collagen, is the most essential protein in skin connective tissue and provides tension, elasticity, and flexibility to the skin (Chen *et al.*, 2011). Type III collagen is another important collagen found in the skin (Park *et al*., 2009). During the aging process some enzymes such as matrix metalloproteinases (MMP_S) increase and levels of extracellular matrix components including collagen decrease, which reduce skin elasticity (Kim *et al.*, 2017). Recent studies found that marine resources including marine algae are good sources of biological substances with a variety of bioactive functions (Berthon *et al.*, 2017). For example, *Chlorella vulgaris*, a green alga, is a microalga that grows in fresh water and is popular as food supplement or additive (Li *et al*., 2002; Saberi *et al.*, 2017), or colorant and food emulsion in the market (Fernandes *et al*., 2012). Chlorella growth factor (CGF) is known to be a unique group of substances available in the nucleus of *Chlorella*. It is rich in nucleic acids (RNA and DNA), amino acids, peptides, vitamins, minerals, and polysaccharides. CGF has numerous biological functions, including antitumor, anti-inflammatory and antioxidant activities (Yasukawa *et al.*, 1996).

Although a number of studies are in progress to examine biological effects of *C. vulgaris*, effect of CGF of *Chlorella vulgaris* on collagen production in human fibroblast cells have not yet examined.

Materials and methods

Preparation of CGF

Dried *C. vulgaris* powder (Parsian Microalgae Co. Tehran, Iran) was suspended in 100 ml of distilled water at a concentration of 4% (w/w). For hot water extract, the *C. vulgaris* suspension was maintained at 60°C for 3h. After separation at 10000 rpm centrifuging for 30 min, the supernatant was freeze-dried to yield CGF-1. For the enzymatic hydrolysis, the *C. vulgaris* suspension was hydrolyzed using 2 ml protease (Sigma-Aldrich, USA) and 2 mL β-(1→3)-D-Glucanase (Sigma-Aldrich, USA) at 50°C for 3h. After centrifugation at 10000 rpm for 30 min, the supernatant was freeze-dried to obtain CGF-2. For the ultrasonic assay and enzymatic hydrolysis, the *C. vulgaris* suspension treated with ultrasonic disintegration for

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30 min was hydrolyzed using 2 ml protease and 2 mL β-(1→3)-D-Glucanase at 50°C for 3h. After centrifugation at 10000 rpm for 30 min, the supernatant was freeze-dried to yield CGF-3. The freeze-dried supernatants containing CGF were reported (Josephine *et al*., 2015).

Characterization of the extract

The extraction yield was expressed as solid recovery and calculated as ratio weight of the lyophilized extract to the weight of the *Chlorella* powder. Absorbance at 260 nm was used as a simple index for quality control of *Chlorella* growth factor content. In this work triplicate samples were dissolved in water separately and UV absorbance was measured at 260 nm using a spectrophotometer. Absorbance of samples was then compared with control CGF, which was a commercial product. Among the three CGF extraction methods, CGF-3 with the highest absorption, compared to the other two methods, was selected for the next steps and its effect on collagen production in skin fibroblast cells was investigated (Josephine *et al.*, 2015).

Cell culture

The human skin fibroblast cells were maintained in complete Dulbecco' s modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Sigma-Aldrich, USA), 2 mM L-Glutamine, 100 U/mL penicillin, and 100 mg/ml streptomycin in a humidified 5% CO_2 incubator at 37°C. The Hu02 cells were cultured to 70-80% confluence and were used between passages 5 and 15 (Chen *et al.*, 2011).

MTT assay

The cells were plated in 96-well plates at a density of 10^4 cells/well, and subsequently treated with CGF-3 (3200, 1600, 800, 400, 200, 100, 50, 25, 12.5 and 6.25 µg/ml) for 24 h. Each concentration was tested three times to confirm the results. The cells were then incubated with 10 µL of MTT solution for 4 h at 37°C, and the absorbance was quantified at 590 nm using a microplate reader. Cell viability was calculated as the ratio of absorbance of treated cells to that of untreated cells (Wachesk *et al.*, 2013).

Treatment with CGF-3

The cells were incubated for 24 h with 400 µg/mL CGF-3.

Quantitative PCR (qPCR)

Using TRIzol reagent (Sigma-Aldrich, USA), Total RNA was isolated from the cells and the extracted RNA was used as a template for cDNA synthesis using oligo (dT). The PCR amplification mixtures (total volume, 20 µL) contained 10 µL of TOPreal qPCR 2X PreMIX SYBR-Green (Enzynomics Inc., Daejeon, Korea), 2 µL cDNA template, 0.8 µL forward primer, 0.8 µL reverse primer and 6.4 µL of RNase-free water. Using StepOne Real-Time PCR system (Applied Biosystems Inc., Foster City, CA, USA), qPCR was performed as follows: pre-incubation at 95°C for 10 min, 40 cycles of 95°C for 10 sec,

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annealing at 60°C for 15 sec, and elongation at 72°C for 15 sec. Gene expression levels were normalized to those of GAPDH and calculated using the comparative $\Delta\Delta C_T$ method (Kim *et al.*, 2017). Three technical replicates were performed for each sample. The oligonucleotide primers used for PCR are listed in Table 1.

Table 1: The oligonucleotide primer sequences used in the real-time PCR (5'→3').

Gene	Forward primer	Reverse primer
Type I collagen	CTCCCCAGCCACAAAGAGTC	CCGTTCTGTACGCAGGCAGGTGAT
Type III collagen	AGCTGGCTACTTCTCGCTCT	TCCGCATAGGACTGACCAAGA
GAPDH	CATGAGAAGTATGACAACAGCCT	AGTCCTTCCACGATACCAAAGT

Statistical analysis

The results are expressed as mean±standard deviation (SD). To determine statistical significance, analysis of variance (ANOVA) with Tukey's range test was conducted using SPSS software, version 22. A value of $p<0.05$ was considered to indicate statistically significant difference.

Results

Yield of CGF

Extraction yields of CGF-2 (38%) and CGF-3 (36%) were significantly higher than the 16% extraction yield of CGF-1 (Fig. 1, $p<0.05$). However, no significant difference was found between extraction yields of CGF-2 and CGF-3.

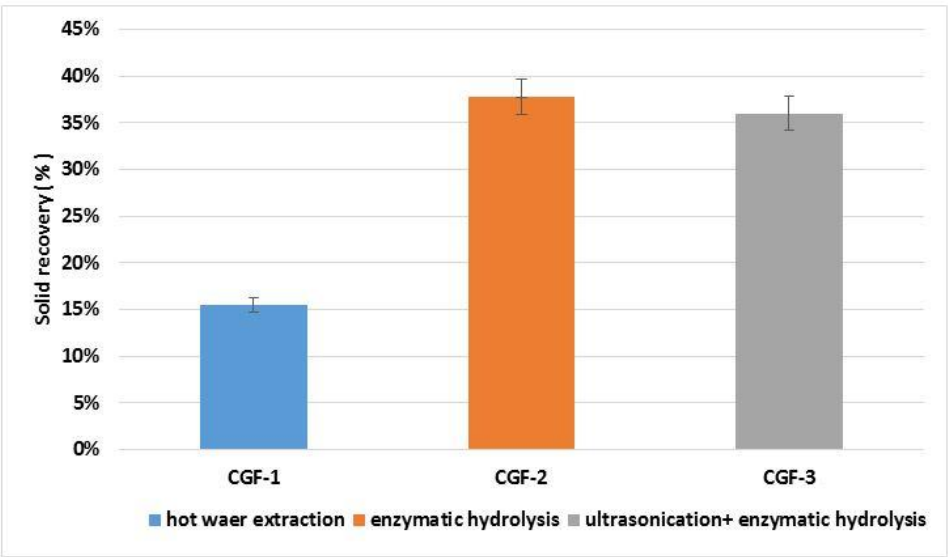

Figure 1: The yield of CGF derived from different methods expressed as solid recovery and calculated as ratio weight of the lyophilized extract to the weight of the *Chlorella* powder. Error bars show standard deviation.

UV absorbance of CGF-1, CGF-2 and CGF-3 was measured at 260 nm. The samples were dissolved in water and then diluted to 400 $\mu g mL^{-1}$. There were

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significant differences among UV absorption of CGF-1, CGF-2 and CGF-3 (Fig. 2, $p<0.05$). The UV absorbance of CGF-3 was not significantly different from that of the control CGF ($p>0.05$), whereas absorbance of CGF-1 and CGF-2 were lower than that of CGF-3 and the control CGF. Among the three extraction methods, the maximum absorption at 260 nm was related to CGF-3.

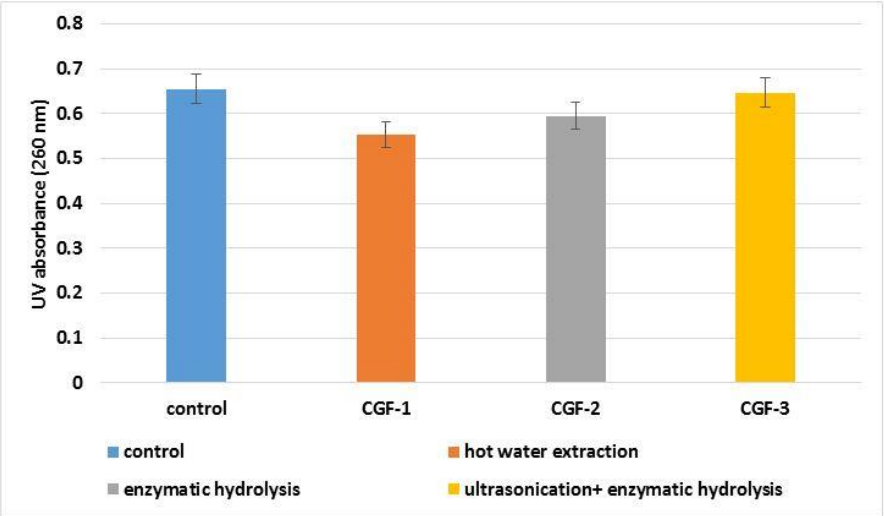

Figure 2: UV absorbance of CGF at 260 nm detected using a UV spectrophotometer. Error bars show standard deviation.

Type I and III collagen determination

CGF-3 increased the gene expression of types I and III collagen. Collagen is an important component of connective tissue. With age, collagen decomposes, resulting in increased skin aging. In this study, qPCR was performed to examine the effect of CGF on collagen expression in Hu02 cells and it was found that the mRNA expression levels increased following treatment with CGF-3 (Fig. 3).

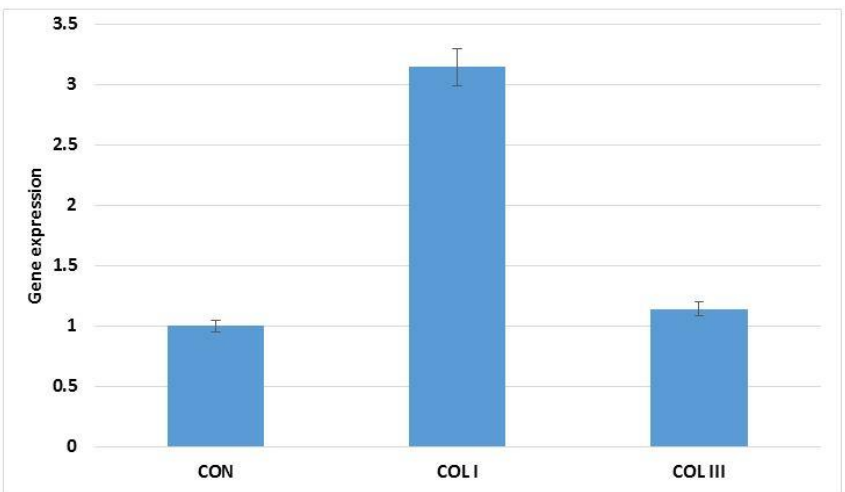

Figure 3: Effect of CGF-3 on gene expression of types I and III collagen. Collagen expression was quantified by qPCR. Error bars show standard deviation.

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Discussion

Efficient extraction method is an important factor to obtain high-value products for different purposes. Different methods have been identified to extract various bioactive ingredients from marine algae. Bead milling is a proper method to release intracellular components from the microalga *C. vulgaris* comparable to the combined PEF-temperature treatment (Postma *et al*., 2016). PEF, Pulsed Electric Fields, is a suitable cell disruption method for selective recovery of small-sized cytoplasmic compounds (Carullo *et al*., 2018). CGF extraction using hot water from *C. vulgaris*, as a best growth stimulatory, increases the biomass and lipid production (Josephine *et al*., 2015). In this study, three disruption methods were tested. CGF-3 obtained by ultrasonication and enzymatic hydrolysis was the most effective cell disruption method. The yield of CGF-1 was obviously lower than that of the other two products, which indicated poor efficiency of hot water extraction. The high yield of CGF-3 observed in the present study might be due to the rupture of the cell wall by enzymes and ultrasonic waves. Ultrasonic method can be used as a simple method to disrupt small amounts of biomass. Enzymatic hydrolysis has the advantage of being specific and gentle, and enzymes have a specific effect on hemicellulose and saccharides of the cell wall (Zheng *et al*., 2011). Our study found out that two enzymes with an appropriate dosage disrupted the cell wall of *C. vulgaris* effectively.

Skin is the largest organ in the human body that has an important role in many physical functions. Skin aging is the complex process that induces many changes such as thinning, dryness, fragility, fine lines and wrinkles (Wang *et al*., 2015). Since collagen, the remarkable component of the dermis, must be maintained to fight the skin aging, it is necessary to prevent its decomposition. Molecular mechanisms of skin aging can stimulate AP-1 (activator protein 1). It, therefore, increases metalloproteinases (MMP_S: MMP-1, MMP-3, and MMP-9) expression and collagen degradation (Chen *et al*., 2011). There is an increasing demand for bioactive substances from natural products such as plants, mushrooms, microbial metabolites and marine algae.

Extract of microalgae has a remarkable value for cosmetic product developments due to its high content of nutrients such as polyunsaturated fatty acids, essential amino acids, vitamins A, B, C, and E, antioxidants and immunologically effective compounds (Berthon *et al*., 2017; Khani *et al*., 2017). There are different bioactive compounds from marine algae that can affect collagen and MMP_S levels and are considered as anti-aging compounds. It has been reported that PYP1-5, *Pyropia yezoensis* peptide, promotes collagen synthesis by activating the TGF-β/SMAD signaling pathway and suppress the MMP-1 protein (Kim *et al*., 2017). Porphyra-334 from *P. yezoensis*, is a natural compound found in a wide variety of

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organisms, which increases levels of procollagen and type I collagen and suppresses the expression of MMP_S following UVA irradiation (Ryu *et al*., 2014). The treatment of fucosterol on HaCaT, a natural sterol compound from brown algae, increases type-1 procollagen production and decreases MMP_S production (Kim *et al*., 2013a). It has been demonstrated that sargachromanol E, the phenolic compound from *Sargassum horneri*, suppresses the expression of collagenases such as MMP-1, MMP-2, and MMP-9 (Kim *et al*., 2013b).

One of the most important algae that has an important role in collagen production and also contains anti-aging characteristics is different species of *Chlorella*. It has been reported that microalgae such as *Chlorella* promotes collagen synthesis and prevents wrinkle formation by acting on the epidermis to erase vascular imperfection (Berthon *et al*., 2017). Moreover, *Chlorella* contains β-1, 3-glucan which is a free-radical scavenger (Iwamoto, 2004). A study conducted by Chen *et al*. (2011) demonstrated that a *Chlorella*-derived peptide exerts a protective effect against UVB irradiation on human skin fibroblasts. However, effects of *C. vulgaris* on collagen synthesis in human dermal fibroblasts remained unclear. In this study, we examined *Chlorella* growth factor, CGF, for its anti-aging function by promoting collagen synthesis in human fibroblasts. First, we extracted CGF, CGF-3, then we investigated whether CGF-3 increased collagen in Hu02 cells. Collagen decomposes due to various factors, which promotes wrinkle formation and skin aging (Varani *et al*., 2000). CGF-3 increased types I and III collagen mRNA expression. These results indicated that CGF-3 promotes collagen synthesis (Fig. 3). The positive effect of CGF-3 on type I collagen production, considering that type I collagen is the major structural component of the extracellular matrix (Chen *et al*., 1999), could be due to its anti-aging effects. In this study, CGF extract from *C. vulgaris* acted as an anti-aging factor by modulating expression of the genes associated with aging in skin such as collagen. The findings of this study demonstrated that ultrasonication with enzymatic hydrolysis is an effective method for producing CGF, CGF-3, from *C. vulgaris*. Moreover, CGF-3 promoted types I and III collagen synthesis in Hu02 cells. Our results suggested that CGF-3 may have beneficial effects on skin aging.

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