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Composition of carotenoids and identification of aerial microalgae isolated from the surface of rocks in mountainous districts of Japan

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ABSTRACT

Four aerial microalgae were isolated from the surface of rocks in mountainous districts of Japan and identified as *Scenedesmus* sp. KGU-Y002, *Scenedesmus* sp. KGU-D002, and *Coelastrella* sp. KGU-H001 of the class Chlorophyceae and *Vischeria helvetica* KGU-Y001 of the class Eustigmatophyceae based on 18S rDNA analysis. Algal carotenogenesis was enhanced by increasing the light intensity from 40 to 214 μmol photons m^{-2} s^{-1}, with more or less decrease in the total chlorophyll content. Culture of these microalgae under high-light conditions resulted in production of astaxanthin (free and esters), adonixanthin (free and esters), lutein, canthaxanthin, and β-carotene as the major carotenoids. Under high-light culture conditions, strains KGU-Y002, KGU-D002, KGU-H001, and KGU-Y001 accumulated 5.75, 10.45, 2.60, and 11.50 mg g^{-1} dry weight cells (dwc) of total carotenoids when the total chlorophyll contents were 3.51, 5.88, 2.91, and 7.17 mg g^{-1} dwc, respectively. The astaxanthin produced might primarily be synthesized from β-carotene via accumulation of zeaxanthin and adonixanthin in strains KGU-Y002, KGU-D002, and KGU-H001. Strain KGU-Y001 was found to accumulate astaxanthin and violaxanthin with large amounts of β-carotene. These results revealed that the biosynthesis pathways for astaxanthin in these isolates were significantly different from those of astaxanthin in green microalgae.

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1. Introduction

In photosynthetic organisms, including algae and plants, carotenoids act as accessory pigments in light harvesting during the light phase of photosynthesis and are also able to photoprotect the photosynthetic machinery from excess light by scavenging reactive oxygen species with singlet oxygen and other free radicals [1]. Most carotenoids are involved in quenching singlet oxygen and trapping peroxyl radicals [2]. The bioactivities of astaxanthin (e.g., UV-light protection and anti-inflammatory activity) have been reported to impact human health conditions because of their strong antioxidant activity [3]. There are two classes of naturally occurring carotenoids, carotenes of lycopene, β-carotene, and α-carotene, which are hydrocarbons that are either linear or cyclized at one or both ends of the molecules, and xanthophylls such as astaxanthin, canthaxanthin, lutein, and zeaxanthin, which are the oxygenated derivatives of carotenes. Astaxanthin and lutein are synthesized from a common precursor (lycopene) through two divergent pathways with β-carotene and α-carotene as intermediates, respectively. Carotenoids are also widely used as colorants in natural foods including egg yolk, chicken, and fish. More than 750 carotenoids have been identified; however, only a few have been used commercially (lycopene, β-carotene, canthaxanthin, lutein, and astaxanthin, among the most common) [4].

Because many other antioxidant compounds are present in algal cells, one of the main advantages of the use of microalgae as a carrier of carotenoids is their positive impact on human health [4]. Astaxanthin is synthesized by *Chlorella*, *Chlamydomonas*, *Dunaliella*, and *Haematococcus* spp., which all belong to the Chlorophyceae family [5,6]. In almost all green microalgae, carotenoids are synthesized within plastids and only accumulate therein. However, a few green microalgae (e.g., *Haematococcus* sp.) can accumulate xanthophylls in oil bodies outside of plastids in the cytoplasm [5,7]. Formation of xanthophylls in algal cells can be influenced by nitrogen-limitation, oxidation, light intensity, temperature, metal ions, and salts [8,9]. Almost any factor that causes a cessation of growth can lead to efficient accumulation of carotenoids in algal cells. Therefore, the accumulation of carotenoids by microalgae can be up-regulated in response to oxidative stress in cells. For example, the green alga *Haematococcus pluvialis* and other Chlorophyceae accumulate astaxanthin under various culture conditions, such as high-light irradiance, high-salt, high-temperature, and nutrient-deficiency [5,9].

Although algae are generally known as freshwater and marine organisms, they can also grow on or within a variety of terrestrial habitats in which they have considerable ecological importance, such as rock, soil, snow, and ice. Many types of aerial microalgae have been isolated from terrestrial habitats and identified [10]. When conditions become

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too stressful for algae they enter dormant stages in order to survive, which is indicated by formation of a red–orange velvet-like layer. Since aerial microalgae are exposed to more severe conditions than aquatic algae (e.g., high light, rapid temperature changes, and drought), various carotenoids are produced to adapt to the environmental conditions. Therefore, aerial microalgae producing large amounts and many types of carotenoids can be isolated from parts of rocks and walls that have a red–orange color. Recently, the aerial microalgae *Trentepohlia aurea* and *Coelastrella striolata* var. *multistriata*, which were isolated from the surface of rocks, were shown to change from green to red under stress conditions such as nitrogen limitation, leading to production of large amounts of β-carotene, canthaxanthin, and astaxanthin [11–14]. However, no studies of the composition and accumulation of carotenoids in aerial microalgae other than those mentioned above have been conducted to date.

In this study, four aerial microalgae were isolated from the surface of rocks in mountainous districts of Japan. These isolates were able to accumulate a complex mixture of astaxanthin (free and esters), adonixanthin (free and esters), lutein, canthaxanthin, β-carotene, and other compounds under high-light culture conditions. The isolated aerial microalgae were characterized based on 18S rDNA and carotenoid analyses.

2. Materials and methods

2.1. Culture and irradiance conditions

Colonies of aerial microalgae were isolated from the surfaces of rocks in mountainous districts of the Tokyo (35° 72′ S Lat, 139° 16′ E Long) metropolitan area, as well as Tochigi (36° 97′ S Lat, 139° 60′ E Long) and Yamanashi (35° 74′S Lat, 138° 83′ E Long) Prefectures in Japan. The algal cells were cultured and maintained in Bold's basal (BB) medium at 25 °C under continuous illumination by cool-white fluorescent lamps (40 μmol photons m^{-2} s^{-1}) in 500 mL Erlenmeyer flask while bubbling with air. The BB medium contained 250 mg $NaNO_3$, 175 mg KH_2PO_4, 75 mg K_2HPO_4, 25 mg $MgSO_4 \cdot 7H_2O$, 25 mg NaCl, 50 mg EDTA, 30 mg KOH, 5 mg $FeSO_4 \cdot 7H_2O$, and 11 mg H_3BO_3 per liter of deionized water, and the pH was adjusted to 8.0 with NaOH prior to autoclaving [15]. The green vegetative cells were transferred to a 500 mL flat glass flask and further cultured for 2 weeks at 40 μmol photons m^{-2} s^{-1}. The suspended cells were then shaken reciprocally at 120 rpm under continuous illumination by cool-white fluorescent lamps (40 or 214 μmol photons m^{-2} s^{-1}) at 25 °C for 3 weeks in 100 mL Erlenmeyer flasks. Algal cell growth was determined by measurement of dry weight. Cells were collected using a 0.45 μm membrane filter, washed with distilled water, and then dried under reduced pressure before weighing. The max specific growth rate (μ_{max}) at the exponential phase was calculated according to $\mu_{max} = (\ln x_2 - \ln x_1) / (t_2 - t_1)$, where x_2 and x_1 are the dry weights of cells (g L^{-1}) at times t_2 and t_1, respectively. All experimental cultures were replicated in triplicate.

2.2. 18S rDNA for strain identification

Nucleic acid extraction of algal cells was performed by the bead-beating method, after which the 18S rDNA was amplified by PCR using the following universal primers: Primer A (5′-AACCTGGTTGAT CCTGCCAGT-3′) and Primer B (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) [16]. The 18S rDNA sequences were determined using an ABI PRISM™ BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM™ 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Sequencing was performed using the PCR primers and four internal primers previously used by Hamby et al.: 18G (5′-TGGCACCAGACTTGCCCT-3′), 18G′ (5′-AGGGCAAGTCTGGTGC CA-3′), 18H (5′-GCCCTTCCGTCAATTCCTTTAAGTTTCAGC-3′), and 18P′ (5′-CGAAAGCATTTGCCAAGG-3′) [17]. Sequences obtained for the 18S rDNA gene were aligned to published sequences obtained from GenBank using Clustal X ver. 2.0 [18]. A neighbor-joining tree [19] was constructed from these data by the bootstrap method (1000 replicates) using Clustal X ver. 2.0, and then visualized using FigTree ver. 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

2.3. Pigment extraction and saponification

After grinding the lyophilized samples in an agate mortar for 5 min, pigments were extracted from the powdered sample with dichloromethane/methanol (25:75, v/v). The extract was then dried in vacuo and dissolved in methanol solution. Saponification of carotenoid esters was performed as follows: NaOH dissolved in methanol was added to the pigment extract solution (final concentration: 20 mM) and the mixture was then kept at 5 °C in darkness for 12 h to allow complete hydrolysis of carotenoid esters [20].

2.4. Pigment analyses

Total chlorophyll amounts were estimated using a spectrometer (U-1800, Hitachi Tech Co., Ltd., Tokyo, Japan) according to the method described by Arnon [21]. The free carotenoids and carotenoid esters in the pigment extract were assayed by HPLC, which was performed using a reversed phase column (Inertsil ODS-2, 4.6 × 150 mm i.d., C18, 5 μm, GL Sciences Inc., Tokyo, Japan) and a pump (PU-1580, Jasco Co., Ltd., Tokyo, Japan) equipped with a UV/Vis detector (UV-1570, Jasco Co., Ltd., Tokyo, Japan). Aliquots of 20 μL were used for HPLC analysis. The mobile phase consisted of eluents A (dichloromethane:methanol: acetonitrile:water = 25:425:27.5:50, v/v) and B (dichloromethane: methanol:acetonitrile:water = 125:140:212.5:15, v/v). The following elution gradient was used: 0% B for 5 min, a linear gradient from 0 to 60% for 7 min, 60% B for 8 min, a linear gradient from 60 to 100% for 20 min, and then 100% B for 20 min. During analysis, the flow rate was maintained at 1.0 mL min^{-1}, the column temperature was 50 °C, and the absorbance was monitored at 480 nm. Commercial astaxanthin, zeaxanthin, lutein, canthaxanthin, and β-carotene (Funakoshi Co., Ltd., Tokyo, Japan) were used as standards. Adonixanthin and violaxanthin were identified based on HPLC data and adsorption spectra and quantified with calibration using pure astaxanthin and lutein standards [22,23]. Carotenoid analysis was conducted by liquid chromatography ion trap time of flight mass spectrometry (LCMS-IT-TOF, Shimadzu Co., Ltd., Kyoto, Japan). Liquid chromatography was performed under the same conditions as HPLC. The MS was conducted at a probe voltage of 4.50 kV, CDL temperature of 200 °C, block heater temperature of 200 °C, nebulizer gas flow of 1.5 L min^{-1}, ion accumulation time of 30 ms, MS range of *m/z* 200 to 2000, MS2 range of *m/z* 100 to 1000, and CID parameters of 50% energy and 100% collision gas.

2.5. Data analysis

Results presented are the means of at least three repeated experiments in a typical run to confirm reproducibility.

3. Results and discussion

3.1. 18S rDNA analysis and morphology of isolates

Four aerial microalgae were successfully isolated from the surface of rocks in mountainous districts of the Tokyo metropolitan area and Tochigi and Yamanashi Prefectures in Japan. Using PCR amplification and subsequent DNA sequencing, we determined almost full length of 18S rDNA of these isolates. The length of the 18S rDNA sequences of KGU-Y001, -Y002, -D002 and -H001determined in this study were 1723, 2271, 2637, and 2184 base pairs, respectively. The isolates were identified as *Scenedesmus* sp. KGU-Y002 (accession no.: AB742453), *Scenedesmus* sp. KGU-D002 (accession no.: AB743827), and *Coelastrella* sp. KGU-H001 (accession no.: AB742452) of the

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