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Accumulation and antioxidant activity of secondary carotenoids in the aerial microalga *Coelastrella striolata* var. *multistriata*

Katsuya Abe, Hiroaki Hattori, Morio Hirano *

Department of Applied Chemistry, Faculty of Engineering, Kogakuin University, 1-24-2 Nishi-Shinjuku, Shinjuku-ku, Tokyo 163-8677, Japan

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Abstract

The growth and nitrate uptake of the aerial microalga *Coelastrella striolata* var. *multistriata*, which was isolated from the surface of rocks, were characterized under a variety of conditions in this study. The maximum specific growth rate of the alga, having prominent inorganic nitrogen uptake in the fresh medium, was 0.30 d^{-1}, as calculated in the growth logarithmic phase. It was also shown that the alga had abilities to be reddish orange to green colour (depending on the nitrogen source concentration in the medium) and to synthesize very high amounts of a complex mixture of carotenoids, such as canthaxanthin, astaxanthin and β-carotene. The reddish orange cells of the alga could accumulate 56.0 mg of major secondary carotenoids per g biomass. In the content of carotenoids, canthaxanthin, astaxanthin and β-carotene in the cells were 47.5, 1.5, and 7.0 mg/g dwc, respectively. Additionally, it was shown that the algal extract containing those carotenoids had an antioxidant potential in lipid foods. In the near future, the aerial microalga *C. striolata* var. *multistriata* will be used as a functional material in a variety of commercial applications, such as feed supplements, natural antioxidants and food dyes.

Keywords: Aerial microalga; *Coelastrella striolata* var. *multistriata*; Canthaxanthin; Antioxidant

1. Introduction

Reactive oxygen species (single oxygen and peroxyl radicals) may react with biologically important components, such as DNA, proteins, or lipids, impairing their physiological functions (Halliwell, 1996; Sies, 1985). Such processes are considered as initial events in the pathogenesis of several diseases, including cancer, cardiovascular diseases, or age-related macular degeneration. Several classes of biological molecules are susceptible to attack by these free radicals, including certain amino acids, which react with radicals at quite rapid rates. In the lipids, polyunsaturated fatty acids also react at much slower rates, increasing with the number of double bonds in the molecule, to form lipid hydroperoxides (Kiritsakis & Dugan, 1985; Terao, Hirota, Kawakatsu, & Matsushita, 1981; Terao & Matsushita, 1977). Such lipid oxidation, not only results in an undesirable off-flavour, but also decreases the nutritional quality of lipid foods due to the loss of essential fatty acids. It was reported, 35 years ago, that singlet oxygen participates in the initiation step of oil oxidation, and the reaction rate of singlet oxygen with linoleic acid is about 1450 times greater than that of triplet oxygen (Rawls & Van Santen, 1970).

Carotenoids play major roles in oxygenic photosynthesis, where they function in light harvesting and protect the photosynthetic apparatus from excess light by energy dissipation. Most carotenoids are efficient antioxidants, quenching singlet oxygen, and trapping peroxyl radicals (Jyonouchi, Sun, & Gross, 1995; Krinsky, 1992, 1993; Miki, 1991; Surai et al., 2003). The effects of naturally occurring and food-approved carotenoids (for example, β-carotene and canthaxanthin) on the singlet oxygen oxidation of vegetables oils have previously been examined (Goulson & Warthesen, 1999; Jung & Min, 1991; Pánek,

* Corresponding author. Fax: +81 3340 0147.
E-mail address: bt10807@ns.kogakuin.ac.jp (M. Hirano).

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Fantová, Trojáková, Réblová, & Pokorný, 2001; Psomiadou & Tsimidou, 2002; Rahmani & Csallany, 1998). The singlet-oxygen quenching rates of the food-approved carotenoids increased as the number of conjugated double bonds of the carotenoids increased (Jung & Min, 1991). It has been shown in vitro that canthaxanthin also has antioxidant capabilities. The antioxidant activity of canthaxanthin has been recorded in membrane model systems, liposomes, and cell models (Clark, Faustman, Chan, Furr, & Riesen, 1999; Palozza et al., 1996; Rengel et al., 2000).

Among the ketocarotenoids found in higher plants, algae, fungi, or bacteria, astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) and canthaxanthin (β,β-carotene-4,4′-dione) are the most important from the biotechnological viewpoint. Thus, there is growing commercial interest in the biotechnological production of such secondary carotenoids (Baker, 2002). The secondary carotenoids are functionally defined as carotenoids that are not essential for photosynthesis and are not localized in the thylakoid membranes of the chloroplast. The ketocarotenoid astaxanthin is commonly used as a feed supplement in aquaculture for the production of salmon, trout and shrimp (Lorenz & Cysewski, 2000), while canthaxanthin is mainly used as a food dye for colouring egg yolks and chicken skin (Surai et al., 2003). In the past, astaxanthin has been found and identified in many species of algae, such as *Haematococcus pluvialis* (Grünewald, Hirschberg, & Hagen, 2001), *Chlorococcum* sp. (Zhang & Lee, 2001), *Chlorella zofingiensis*, *Neochloris wimmeri*, *Scenedesmus vacuolatus*, *Scotiellopsis oocystiformis* and *Protosiphon botryoides* (Orosa, Torres, Fidalgo, & Abalde, 2000). On the other hand, canthaxanthin production has been reported only in a few algal species {*C. zofingiensis* (Pelah, Sintov, & Cohen, 2004), *Chlorococcum* sp., and *Tetracystis intermedium* (Campo et al., 2000)}. The fresh water microalga *C. zofingiensis* could accumulate 8.79 mg/g biomass of total secondary carotenoids (containing about 97% canthaxanthin), when the alga was grown for 9 days under conditions of salt stress and low light.

We previously reported the growth and ammonium uptake of the aerial microalga, *C. striolata* var. *multistriata*, which was isolated from the surface of rocks at Shizuoka (Japan) (Abe, Takizawa, Kimura, & Hirano, 2004). It was shown that the alga had a unique ability to be a reddish-orange to green colour depending on the nitrogen source concentration in the medium. Using the chlorophyll formation of the alga, it was possible to estimate, spectrophotometrically, the total nitrogen content in water collected from the aquatic system. In addition, the reddish-orange cells of the alga were able to accumulate significant quantities of carotenoids (mainly as canthaxanthin, astaxanthin, and β-carotene). The aim of the present work is to study secondary carotenoid accumulation during culture of the aerial microalga, *C. striolata* var. *multistriata* under the stress condition of nitrogen deficiency and to examine activities of natural antioxidants in oil.

2. Materials and methods

2.1. Regents

All solvents and reagents were of analytical or laboratory grade. Deionized water was used throughout.

2.2. Algal material and culture conditions

Colonies of the microalga, *C. striolata* var. *multistriata*, were isolated from the surface of rocks at Shizuoka, Japan. The microalga was 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). Batch cultures were bubbled with air in flat glass bottles. The BB medium contained 250 mg of $NaNO_3$, 175 mg of KH_2PO_4, 75 mg of K_2HPO_4, 25 mg of $MgSO_4 \cdot 7H_2O$, 25 mg of NaCl, 50 mg of EDTA, 30 mg of KOH, 5 mg of $FeSO_4 \cdot 7H_2O$ and 11 mg of H_3BO_3 in 1 l of deionized water. The pH was adjusted to 8.0 with NaOH prior to autoclaving.

2.3. Measurement of algal growth

The growth of algal cells was determined by turbidity at 750 nm. For the dry weight measurement, cells were collected using a 10 μm membrane filter (Nihon Millipore, Tokyo), washed with distilled water, and then dried under reduced pressure before weighing.

2.4. Pigment analysis

Chlorophylls were extracted using methanol. Total amounts of chlorophyll were estimated using a spectrometer (UV-2450; Shimadzu, Kyoto) according to the method of Mackinney (1941). Monitoring of the free carotenoids and carotenoid esters in the pigment extract was carried out according to an HPLC method, as described previously (Abe et al., 2004). The HPLC analysis was performed with a reversed phase column (250 × 4.6 mm i.d., ODS-P Inertsil; GL Sciences, Tokyo), and a pump (PU-980; Jasco, Tokyo) equipped with a UV/Vis detector (UV-970; Jasco). Aliquots of 10 μl were used for HPLC analysis. The mobile phase consisted of eluents A (dichloromethane:methanol:acetonitrile:water, 5.0:85.0:5.5:4.5 v/v) and B (dichloromethane:methanol:acetonitrile:water, 25.0:28.0:42.5:4.5 v/v). For separation of carotenoids, the following gradient procedure was used (0% of B for 8 min; a linear gradient from 0% to 100% of B for 6 min; 100% of B for 40 min, at a flow rate of 1.0 ml/min and 25 °C). The absorbance detector was set at 480 nm. Pigments were extracted with 1 ml of dichloromethane and methanol (25:75, v/v). Saponification of carotenoid esters was performed as follows: NaOH (0.016 M) dissolved in methanol (0.25 ml), which was freshly prepared, was added to the pigment extract solution under a nitrogen atmosphere. The mixture was kept at 5 °C in darkness under nitrogen for 24 h for complete hydrolysis

of carotenoid esters. The saponified pigment extract solution was directly analyzed by HPLC. Pure standards of astaxanthin, lutein, and β-carotene were purchased from Sigma Chemical (St. Louis, MO, USA), and canthaxanthin and neoxanthine were from Wako.

2.5. Fatty acid analysis

After 1 ml of 5%HCl–methanol (Kokusan Chemical, Tokyo) was added to 10 mg of freezed-dried cells, the mixture was refluxed by heating at 100 °C for 3 h. After cooling to room temperature, fatty acid methyl esters were extracted into 1 ml of hexane. The obtained hexane layer was dehydrated, and then 1.5 μl of the hexane layer was withdrawn with a microsyringe and its fatty acid methyl ester content was determined by a gas chromatography system (GC-14B; Shimadzu, Kyoto) equipped with a capillary column (30 m × 0.25 mm internal diameter; J&W Scientific, CA, USA) and a hydrogen flame ionization detector. In this case, heptadecanoic acid was used as the internal standard for quantitative analysis.

2.6. Nitrate analysis

Ammonium was analyzed using an ammonium electrode (5002A-10C; Horiba, Kyoto). Nitrate ion was measured spectrophotometrically using the methods of the Japanese industrial standard for industrial wastewater (Japanese Industrial Standard, 1998). Nitrate was determined at 410 nm with 4-aminobenzenesulfonamide (Wako) and brucine (Tokyo Kasei Kogyo, Tokyo).

2.7. Photooxidation of methyl linoleate

Methyl linoleate (600 mg, Wako), containing oily pigment extract, was placed in test tube and subjected to oxidation under a fluorescent lamp for 4 h. The light intensity and temperature were 65 μE/m^2/s and 20 °C. The oxidation of methyl linoleate was determined by measuring peroxide values according to the JOCS method (Endo, Usuki, & Kaneda, 1984).

3. Results and discussion

3.1. Effects of nitrogen, light, and vessel on algal growth

In order to examine the effect of nitrogen concentration on growth, cells of the aerial microalga *C. striolata* var. *multistriata* were cultured using a flat glass bottle (700 ml) for 30 days in BB medium under various concentrations of nitrate (0, 250, 500 and 1000 mg/l $NaNO_3$ as the nitrogen source) at 40 μmol photons/m^2/s and 25 °C. The batch cultures were bubbled with air in flat glass bottles. Although the culture in the nitrogen-free medium inhibited cell division, higher biomass was obtained when the alga was cultured in BB medium containing more than 500 mg/l $NaNO_3$, as shown in Fig. 1(a). The alga entered the stationary phase of growth after 20 days with the higher rate of nitrate depletion. It was found that the maximum uptake rates of nitrate of the alga were almost the same under various concentrations of the nitrate-N (Fig. 1(b)). Because nitrogen deficiency will be a potential inducer, of secondary carotenoid formation in the microalga, the algal cells were grown in the medium containing 500 mg/l $NaNO_3$ (initial concentration), to promote production and accumulation of large amounts of secondary carotenoids in this study. To examine the effect of light intensity on growth, the algal cells were cultured for 30 days in BB medium containing 500 mg/l $NaNO_3$ at various light intensities (0, 7, 40, 65, 105, and 130 μmol photons/m^2/s) and 25 °C. As a result, the higher specific growth rate of 0.22 d^{-1} attained when the alga was cultured over 65 μmol photons/m^2/s (Fig. 2). Moreover, we tried to develop a culture method for the growth of *C. striolata* var. *multistriata* using two kinds of vessel. The alga was grown using a flat glass bottle (700 ml) and/or a slender test tube (400 mm × ∅ 30 mm) for 30 days in BB medium containing 500 mg/l $NaNO_3$ at 65 μmol photons/m^2/s and 25 °C. The maximum specific growth rate of 0.30 d^{-1} was obtained if the algal cells were cultured in the test tube. The alga entered the stationary phase of growth after 20 days with the highest rate of nitrate depletion in Fig. 3. The nitrate uptake rate reached 0.4 mg/

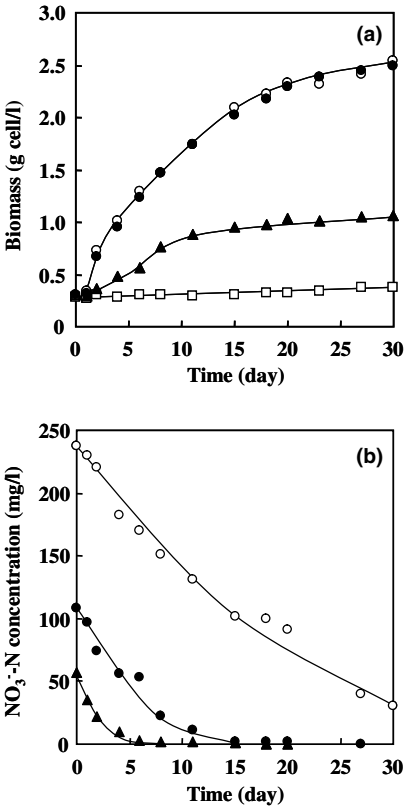

Fig. 1. Time course of the growth (a) and nitrate-N concentration of the medium (b) in a culture of *C. striolata* var. *multistriata*. The alga was cultured using a flat glass bottle for 30 days in BB medium under various concentrations of 0 (□), 250 (▲), 500 (●), and 1000 (○) mg/l $NaNO_3$ as the nitrogen source at 40 μmol photons/m^2/s and 25 °C.

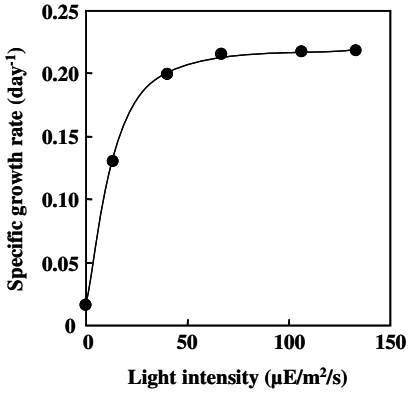

Fig. 2. Effect of light intensity on the specific growth rate in a culture of *C. striolata* var. *multistriata*. The alga was cultured using a flat glass bottle for 30 days in BB medium with 500 mg/l $NaNO_3$ added as the nitrogen source at 25 °C.

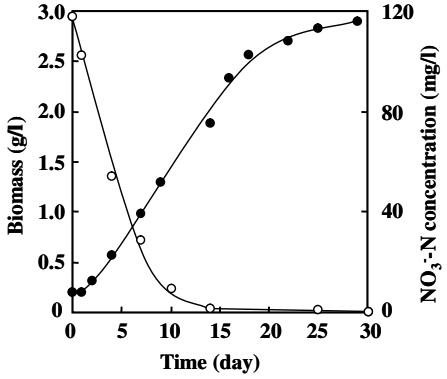

Fig. 3. Time course of growth (●) and nitrate-N concentration (○) of the medium in a culture of *C. striolata* var. *multistriata*. The alga was cultured using a slender test tube for 30 days in BB medium under various concentrations of 500 mg/l $NaNO_3$ as the nitrogen source at 65 μmol photons/m^2/s and 25 °C.

l/h, which was the same as that by *C. striolata* var. *multistriata* cultured in the medium with added NH_4Cl instead of $NaNO_3$ as the nitrogen source. This uptake rate was twice that obtained by other microalgae, such as *C. pyrenoidosa* and *Scenedesmus* sp. (Tam & Wong, 1989). The aerial microalga, *C. striolata* var. *multistriata* shows prominent ammonium and nitrate-N uptake in the fresh medium. When the alga was cultured in nitrogen-deficient medium under the same conditions, the cells were observed, in the slender test tube, to change from green to reddish-orange more rapidly than in the flat glass bottle (Fig. 4). Accordingly, the optimal growth of *C. striolata* var. *multistriata* was obtained at a combination of 500 mg/l $NaNO_3$ and a light irradiance of 65 μmol photons/m^2/s in the slender test tube to induce carotenoid synthesis.

3.2. Analyses of pigments and fatty acids of algal cells

The contents of carotenoids and chlorophylls in the extract solutions from the aerial microalga *C. striolata* var. *multistriata* cells were analyzed by HPLC and GC.

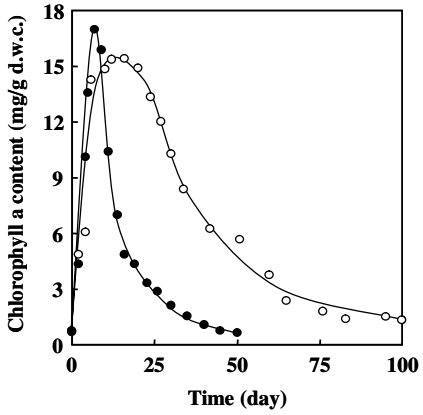

Fig. 4. Effect of culture vessel on chlorophyll content in a culture of *C. striolata* var. *multistriata*. The alga was cultured using a flat glass bottle (○) and a slender test tube (●) for 30 days in BB medium under various concentrations of 500 mg/l $NaNO_3$ as the nitrogen source at 65 μmol photons/m^2/s and 25 °C.

Analytical data of the pigment extracts from the green and reddish-orange cells of the alga are shown in Table 1. As a result, the reddish orange cells of the alga grown for 50 days under above-mentioned conditions could accumulate 56.0 mg of major secondary carotenoids per g dry weight cells (dwc) after the saponification treatment. This result is particularly surprising in view of the fact that the content of canthaxanthin in the cells was 47.5 mg/g dwc. While astaxanthin is a high-value carotenoid and identified in many kinds of microorganisms, canthaxanthin has also been characterized by comparatively high antioxidant activities in some in vitro systems. Natural canthaxanthin production has been investigated only in a few algal species. It was previously reported that the fresh water green microalga, *C. zofingiensis*, growing under conditions of salt stress and low light, accumulated high amounts of canthaxanthin (8.5 mg/g dwc) (Pelah et al., 2004). This study shows that the aerial microalga, *C. striolata* var. *multistriata*, has the ability to synthesize very high amounts of a complex mixture of carotenoids, such as canthaxanthin, astaxanthin, and β-carotene. These ketocarotenoids are commonly used as a feed supplement in aquaculture for the production of salmon, trout and shrimp. The biosynthesis of the ketocarotenoids in the algal cells will be

Table 1
Carotenoid content (mg/g dry weight cells) in the pigment extracts from the green and reddish orange cells of the aerial microalga, *C. striolata* var. *multistriata*, growing using a slender test tube in BB medium containing 500 mg/l $NaNO_3$ at 65 μmol photons/m^2/s and 25 °C

Pigment	Green cells	Reddish-orange cells[a]
Chlorophyll *a*	18.3	0.3
Chlorophyll *b*	6.0	0.3
β-Carotene	5.2	7.0
Canthaxanthin	3.2	47.5
Astaxanthin	n.d[b]	1.5

[a] After saponification treatment.
[b] n.d., not detected.

up-regulated under stress conditions, such as high light irradiance, salt addition and desiccative treatment.

In the fatty acid analysis, it is apparent that the alga contained fatty acids having 16 and 18 carbon atoms. Both saturated and unsaturated acids were present in the green and reddish orange cells of the alga (Table 2). The predominant saturated was palmitic acid (16:0) in both green and reddish-orange cells. On the other hand, it was found that the predominant unsaturated fatty acids in the green and reddish-orange cells were linoleic acid (18:2) and oleic acid (18:1), respectively. The reddish-orange cells of the alga could produce large amounts of fatty acids (total fatty acid content: 319 mg/g dwc), especially, 113.4 mg of oleic acid per g dwc.

3.3. Effect of algal extracts on photooxidation of oil

Effect of the algal extract (from *C. striolata* var. *multistriata* cells) on the peroxide value of methyl linoleate (ML) at 65 μmol photons/m^2/s and 20 °C is shown in Fig. 5. Using 20 mg of dried samples of the green and reddish-orange cells, the algal extract was prepared with dichloromethane and methanol, followed by saponification of carotenoid esters and fatty acids. As a result, the peroxide values of ML containing the algal extract from the orange cells were lower than that of the oil containing authentic carotenoid mixture, which was the same composition as carotenoids contained in the algal extract (0.95 mg canthaxanthin, 0.14 mg β-carotene and 0.03 mg astaxanthin). It was found that the algal extract sufficiently acted as antioxidants in ML, because it contained other natural antioxidants, such as tocopherols. On the other hand, the peroxide values of ML mixed with the algal extract from the green cells were higher, than that of the oil without the extract. This result was due to the chlorophyll-photosensitized singlet-oxygen oxidation of the oil. Consequently, the peroxide result suggests that canthaxanthin, with long-conjugated double bonds, has considerably greater antioxidant activity (same as astaxanthin). The algal extract from the cells of the aerial microalga *C. striolata* var. *multistriata* was demonstrated to have an antioxidant potential in lipid foods in this study. We will try to determine oxidation of oils by measuring peroxide formation and studying quenching mechanisms and quenching rate constants of the algal extract by using steady-state kinetic equations.

Table 2
Fatty acid content (mg/g dry weight cells) of the aerial microalga, *C. striolata* var. *multistriata*, growing using a slender test tube in BB medium containing 500 mg/l $NaNO_3$ at 65 μmol photons/m^2/s and 25 °C

Fatty acid	Green cells	Reddish-orange cells
C16:0	17.9	60.3
C16:1*cis*	1.0	19.9
C16:1*trans*	4.3	7.6
C18:	1.3	11.9
C18:1*cis*	13.1	113
C18:1*trans*	2.2	3.8
C18:2	22.7	54.1
C18:3α	28.3	48.3
Total fatty acid	90.8	319

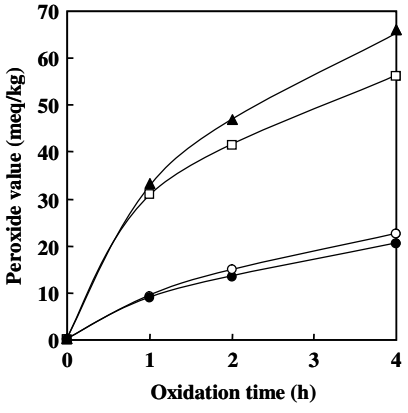

Fig. 5. Effect of the algal extracts of *C. striolata* var. *multistriata* on photooxidation of methyl linoleate. The photooxidation of methyl linoleate was carried out at 65 μmol photons/m^2/s and 20 °C. Symbols: □, control; ▲, the algal extract from the green cells; ○, authentic carotenoids mixture (containing 0.95 mg canthaxanthin, 0.14 mg β-carotene, and 0.03 mg astaxanthin); ●, the algal extract from the reddish orange cells (after the saponification).

In summary, the growth and nitrate uptake of the aerial microalga *C. striolata* var. *multistriata*, which was isolated from the surface of rocks, were characterized in the present study. The maximum specific growth rate of the alga, showing prominent inorganic nitrogen uptake in the fresh medium, was 0.30 d^{-1}, as calculated in the growth logarithmic phase. It was also shown that the alga had the ability to synthesize very high amounts of a complex mixture of carotenoids, such as canthaxanthin, astaxanthin and β-carotene. The algal extract containing these carotenoids had an antioxidant potential in lipid foods. In the near future, the aerial microalga, *C. striolata* var. *multistriata*, will be used as a functional material in a variety of commercial applications, such as feed supplements, natural antioxidants and food dyes.

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