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Carotenoid composition in the green microalga *Chlorococcum*

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Abstract

The carotenoid composition in cells of a ketocarotenoid-producing alga, *Chlorococcum*, was investigated by high-performance liquid chromatgraphy (HPLC). Astaxanthin (free and esters), adonixanthin (free and esters), canthaxanthin, β-carotene, lutein, and some *cis*-isomers of ketocarotenoids were found. The alga *Chlorococcum* cells could produce a large amount of ketocarotenoid esters, like the alga *Haematococcus pluvialis*. The esters of astaxanthin and adonixanthin, and free canthaxanthin were the major carotenoids in the alga *Chlorococcum* cells, while astaxanthin alone was the major carotenoid in the alga *Haematococcus pluvialis*. The alga *Chlorococcum* cells might synthesize astaxanthin from β-carotene via canthaxanthin and adonixanthin in parallel, respectively, which was controlled by the culture conditions.

Keywords: Carotenoid; Astaxanthin; Alga; *Chlorococcum*; HPLC

1. Introduction

Astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) is a ketocarotenoid oxidized from β-carotene. Due to its attractive red colour and higher antioxidative activity than α-carotene, β-carotene, lutein, lycopene, canthaxanthin and vitamin E (Naguib, 2000; Rengel, Diez-Navajas, Serna-Rico, Veiga, Muga, & Milicua, 2000), astaxanthin can be used as a food colourant and in medicine (Johnson & An, 1991; Lorenz & Cysewski, 2000). Recent studies have demonstrated the preventive effects of astaxanthin against the invasion of cancer cells (Kozuki, Miura, & Yagasaki, 2000). Astaxanthin is a high-value carotenoid and has been found and identified in several microorganisms including the algae *Haematococcus pluvialis* (Donkin, 1976; Droop, 1955; Johnson & An, 1991), *Chlorella zofingiensis* (Bar, Rise, Vishkautsan, & Arad, 1995; Rise, Cohen, Vishkautsan, Cojocarum, Gottlieb, & Arad, 1994), and *Chlorococcum* sp. (Liu & Lee, 1999; Zhang, Lee, Ng, & Phang, 1997), the yeast *Phaffia rhodozyma* (Andrewes, Phaff, & Starr, 1976; Johnson & Lewis, 1979), and the marine bacterium *Agrobacterium aurantiacum* (Fraser, Miura, & Misawa, 1997; Yokoyama & Miki, 1995). The biosynthetic pathways for the formation of the ketocarotenoid astaxanthin are different in various microorganisms.

The alga *Chlorococcum* is another promising commercial source of ketocarotenoids due to its relative fast growth rate, ease of cultivation in outdoor systems, and high tolerance to extreme pH and high temperature (Zhang & Lee, 1999). In the alga *Chlorococcum*, astaxanthin is synthesized from β-carotene through various pathways which are different from other astaxanthin-producing microorganisms (Liu & Lee, 1999). The investigations for the carotenoid composition and biosynthetic pathway of astaxanthin in the alga *Chlorococcum* are important for the optimization of astaxanthin production. The objective of the present work is to analyse the carotenoid (free and esters) composition in cells of a ketocarotenoid-producing alga *Chlorococcum*, by high performance liquid chromatography (HPLC).

2. Materials and methods

2.1. Organism and growth medium

The green microalga *Chlorococcum* cells were isolated from flora collected from the rocky wall of Taiping Mountain, Hong Kong. The orange-red flora pellets

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were cultured aerobically on the basal medium at room temperature for 30 days to enrich the target microorganism. Serial dilutions for flora in the enrichment culture, above, were plated on the basal medium plates and incubated at 20 °C for 10 days. Pigmented colonies were subcultured on the same plates with the streak method to obtain pure isolates. One green clone was purified and selected for further experiments.

The basal medium (pH 6.1) consisted of (mg/l): KNO_3, 1250; KH_2PO_4, 1250; $MgSO_47H_2O$, 1000; EDTA, 500; H_3BO_3, 114.2; $CaCl_22H_2O$, 111; $ZnSO_47H_2O$, 88.2; $FeSO_47H_2O$, 49.8; $CuSO_45H_2O$, 15.7; $MnCl_24H_2O$, 14.2; MoO_3, 7.1; $Co(NO_3)_26H_2O$, 4.9. Unless otherwise stated, the alga *Chlorococcum* was grown autotrophically in 250-ml Erlenmeyer flasks containing 60 ml basal medium at 120 rpm and at 25 °C under illumination at a light intensity of approximately 90 μmol/m^2s (fluorescent lamps). The inoculum of the batch culture always came from a 7-day-old culture suspension. If heterotrophically grown, glucose (0.3%, w/v) was added to the basal medium.

2.2. Chemicals

Astaxanthin, β-carotene, lutein, chlorophyll *a*, and chlorophyll *b* were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Canthaxanthin was a kind gift from Professor Sammy Boussiba (Ben-Gurion University of the Negev, Israel). Sodium hydroxide, HPLC grade methanol, acetonitrile, and dichloromethane were purchased from BDH Laboratory Supplies (Poole, UK).

2.3. Extraction, saponification and analysis of carotenoids

Algal cells were collected by centrifuging the culture fluid at 3000 ***g*** for 10 min; the supernatant was discarded, the cell pellet was rinsed with distilled water and then freeze-dried using a Heto FD3 freeze-dryer (Hetoholten, Allerod, Denmark) to a constant weight.

Raw extracts of pigments were obtained by grinding an algal cell pellet in a mortar with a pestle for 1 min, followed by adding 2 ml of extraction solvent, the mixture of dichloromethane (25%) and methanol (75%), and re-grinding the algal cells, together with the extraction solvent, for 1 min. The extract solution, mixed with the algal cells, was then separated by centrifugation at 10 000 *g* for 5 min, and the supernatant, containing pigments, was collected. After the extraction procedure was repeated three times, the pigments were almost completely extracted. The combined pigment extracts were re-centrifuged at 10 000 *g* for 5 min and kept in the dark at −20 °C for analysis.

Saponification of carotenoid esters was carried out according to Yuan and Chen (2000). Six millilitres of 0.107 M NaOH dissolved in methanol, which was freshly prepared, was added to 30 ml of the pigment extract solution under a nitrogen atmosphere. The mixture (36 ml) was evaporated and concentrated to 30 ml under nitrogen during the process of adding NaOH, and then kept at 5 °C in darkness under nitrogen for 12 h in the water bath of a 9105 Refrig/Heat Circulating Bath (Polyscience, Niles, IL, USA) for complete hydrolysis of carotenoid esters. The saponified pigment extract solution was directly separated and determined by HPLC.

Monitoring and quantification of the free carotenoids and carotenoid esters in the pigment extract and saponified extract solution were carried out using a reversed phase-high performance liquid chromatography (RP-HPLC) method previously developed by Yuan and Chen (1998). A Waters liquid chromatograph, with a 996 photodiode array detector, was used. The pigments were separated on a Beckman Ultrasphere C_{18} reversed phase column (5 μm; 250×4.6 mm) at room temperature. Aliquots of 20 μL were used for HPLC analysis. The mobile phase consisted of eluent A (dichloromethane: methanol: acetonitrile: water, 5.0:85.0:5.5:4.5, v/v) and eluent B (dichloromethane: methanol: acetonitrile: water, 25.0:28.0:42.5:4.5, v/v). Separation of carotenoids was achieved by the following gradient procedure: 0% of B for 8 min; a linear gradient from 0 to 100% of B within 6 min; 100% of B for 40 min, at a flow rate of 1.0 ml/min. The absorption spectra of carotenoids were displayed between 250 and 700 nm. Peaks were measured at a wavelength of 480 nm to facilitate the detection of ketocarotenoids.

3. Results and discussion

3.1. Analysis of carotenoids and carotenoid esters in the algal cells

The contents of carotenoids and chlorophylls in the pigment extracts and the saponified extract solutions from the alga *Chlorococcum* cells were analysed using the HPLC method. The typical chromatograms of the pigment extracts from the alga *Chlorococcum* cells cultured with and without adding glucose are shown in Figs. 1 and 2, respectively. The retention time, absorption maxima, and identification of carotenoids and chlorophylls are shown in Table 1, which shows that the carotenoid composition in *Chlorococcum* cells was complex. Like the alga *Haematococcus pluvialis*, in which ketocarotenoids (mainly astaxanthin) were the major carotenoids and existed mainly as ketocarotenoid esters of various fatty acids, a great amount of ketocarotenoid esters were produced in *Chlorococcum* cells. But the composition of ketocarotenoid esters in *Chlorococcum* cells was more complicated than that in *Haematococcus pluvialis*. Therefore, the pigment extracts

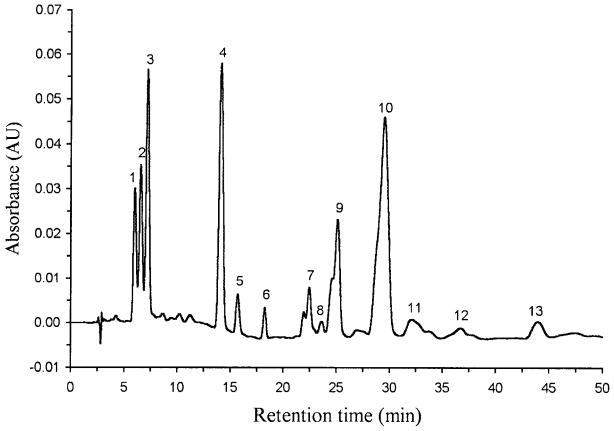

Fig. 1. High-performance liquid chromatgraphy (HPLC) chromatogram of the pigment extract from the alga *Chlorococcum* cells, cultured with added glucose. For the identification of peaks, see Table 1.

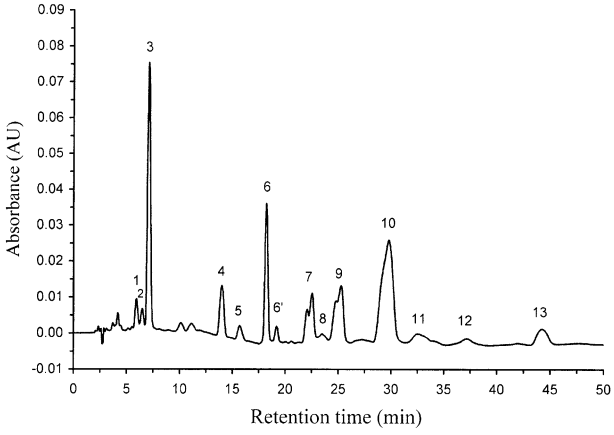

Fig. 2. High-performance liquid chromatgraphy (HPLC) chromatogram of the pigment extract from the alga *Chlorococcum* cells, cultured without adding glucose. For the identification of peaks, see Table 1.

were saponified for further investigation of the composition of ketocarotenoid esters by HPLC analysis.

Typical chromatograms of the saponified extract solutions from *Chlorococcum* cells, cultured with and without adding glucose, are shown in Figs. 3 and 4, respectively. The identification of carotenoids in the saponified extract solutions is also shown in Table 1. As shown in Figs. 3 and 4, after the carotenoid esters were hydrolyzed, the contents of two free-carotenoids (peaks 1 and 2) markedly increased, indicating that this alga differed obviously from *Haematococcus pluvialis* which produced mainly astaxanthin esters (Yuan & Chen, 1998). *Chlorococcum* cells could produce ketocarotenoid esters composed of both astaxanthin and another ketocarotenoid, which was identified as adonixanthin.

Since no adonixanthin standard was available in the present work, the identification of peak 2 (Figs. 1–4) was tentative. By photodiode array detection, the spectra of two ketocarotenoids and their ester were obtained and are shown in Figs. 5 and 6. The differences in the

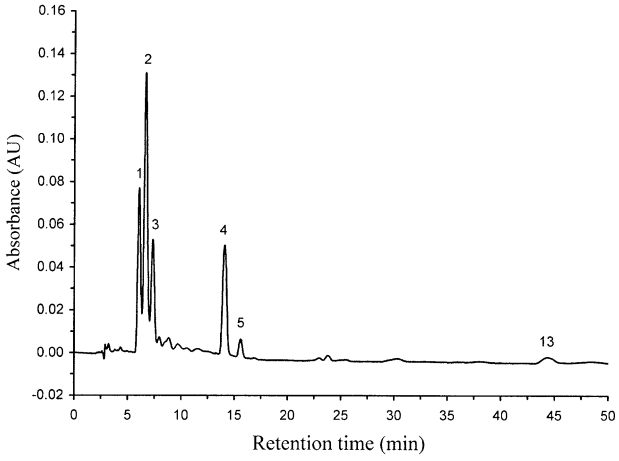

Fig. 3. High-performance liquid chromatgraphy (HPLC) chromatogram of the saponified extract solution from the alga *Chlorococcum* cells, cultured with added glucose. For the identification of peaks, see Table 1.

Table 1
The identification of carotenoids and chlorophylls in the alga *Chlorococcum* cells

Peak no.	Retention time (min)	Absorption maxima (nm)	Pigment
1	6.0	480.0	*Trans*-astaxanthin
2	6.6	(459.2) 469.2	Adonixanthin[a]
3	7.2	445.1 474.0	Lutein
4	14.1	479.4	Canthaxanthin
5	15.7	377.8 469.2	*Cis*-canthaxanthin
6	18.1	464.4 652.3	Chlorophyll *b*
6′	19.1	464.4 652.3	Chlorophyll *b*′
7	22.0	480.0	Astaxanthin esters
	22.5	(464.3) 474.0	Adonixanthin esters
8	23.6	435.4 663.6	Chlorophyll *a*
9	24.6	482.5	Astaxanthin esters
	25.1	(464.3) 474.0	Adonixanthin esters
10	28.8	482.5	Astaxanthin esters
	29.5	(464.3) 474.0	Adonixanthin esters
11	32.2	(464.3) 474.0	Adonixanthin esters
	33.5	355.4 464.4	*Cis*-adonixanthin esters
12	36.7	(464.3) 474.0	Adonixanthin esters
13	44.0	454.7 483.6	β-Carotene

[a] Tentatively identified.

spectra and absorption maxima of free and esterified ketocarotenoids (Figs. 5 and 6) were perhaps due to the change of elution solvent, in which they were detected (Britton, Liaaen-Jensen, & Pfander, 1995; Zang, Sommerburg, & Van-Kunk, 1997). In comparison with the spectrum of astaxanthin, which contained two keto group (Fig. 5), Fig. 6 shows an asymmetrical absorption spectrum with the wavelengths of maximum absorption at 469.2 nm for the free form and 474.0 nm for the ester form, indicating that the carotenoid contained only one keto group (Britton et al., 1995). The retention time of peak 2 was between those of astaxanthin and lutein, indicating that the compound might contain two

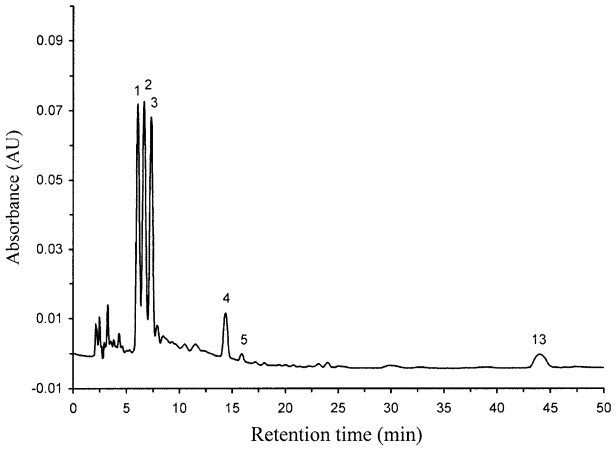

Fig. 4. High-performance liquid chromatgraphy (HPLC) chromatogram of the saponified extract solution from the alga *Chlorococcum* cells, cultured without adding glucose. For the identification of peaks, see Table 1.

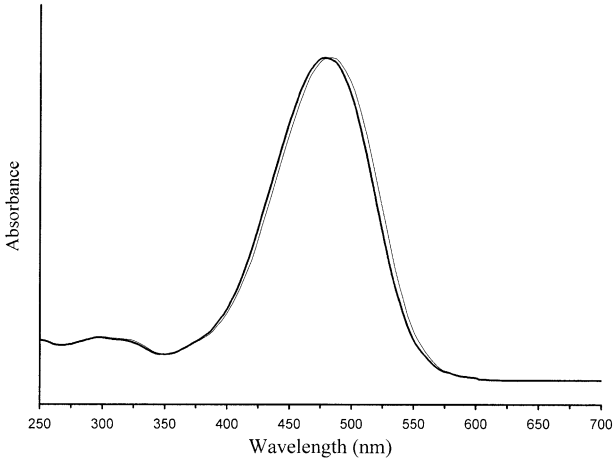

Fig. 5. The absorption spectra of astaxanthin (dark line) and astaxanthin esters (dotted line).

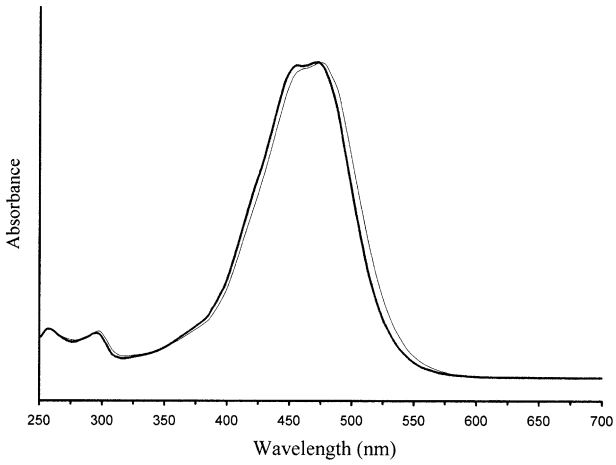

Fig. 6. The absorption spectra of adonixanthin (dark line) and adonixanthin esters (dotted line).

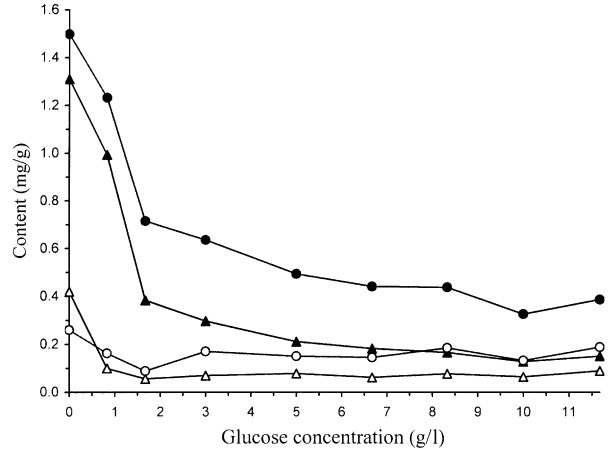

Fig. 7. The effect of glucose concentration on the production of free astaxanthin (△), free adonixanthin (○), total astaxanthins (▲), and total adonixanthins (●).

hydroxyl groups. In addition, by comparing its spectrum (by photodiode array detection) with published data (Harker & Hirschberg, 1997), peak 2 was tentatively identified as adonixanthin.

3.2. Effect of glucose on the contents of carotenoids and chlorophylls

Table 2 shows the composition of carotenoids in *Chlorococcum* cells under different culture conditions. It was obvious that the composition of carotenoids was related to the addition of carbon sources and irradiance. This result shows that the content of carotenoids in the *Chlorococcum* cells incubated in the carbon-free basal medium was higher than that in the basal medium supplemented with 0.3% glucose. When incubated in the carbon-free basal medium, the difference in contents of astaxanthin and adonixanthin in *Chlorococcum* cells were small. But the content of adonixanthin was higher than that of astaxanthin when glucose was supplemented in the basal medium. In comparison with the production of pigments under irradiance, when no irradiance was provided, lutein and β-carotene were the main pigments and only a very small amount of ketocarotenoids was produced in *Chlorococcum* cells. A large amount of ketocarotenoids was accumulated only under irradiance.

The results, for carotenoids and chlorophylls in the pigment extracts and saponified extract solutions from the *Chlorococcum* cells, indicated that the concentration of glucose in the culture medium could affect the formation of carotenoids and chlorophylls. Fig. 7 shows the content change of free astaxanthin, free adonixanthin, total astaxanthins, and total adonixanthins along with the change of glucose concentration. Evidently the contents of astaxanthins and adonixanthins (on cell dry weight) decreased along with the increase in the concentration of glucose in culture medium, especially

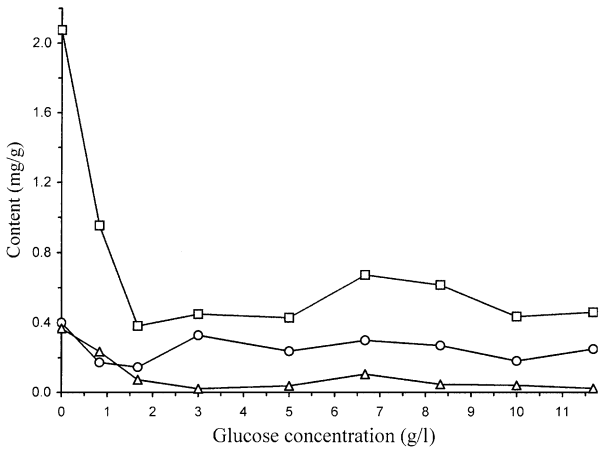

Fig. 8. The effect of glucose concentration on the production of lutein (□), canthaxanthin (○), and β-carotene (△).

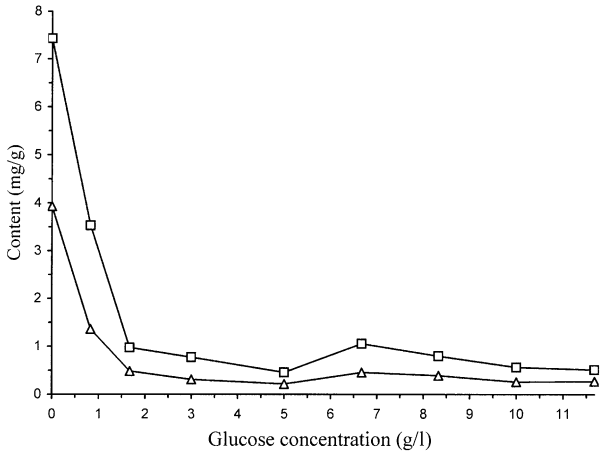

Fig. 9. The effect of glucose concentration on the production of chlorophyll *a* (□) and chlorophyll *b* (△).

Table 2
Carotenoid composition (% of total carotenoids) of the alga *Chlorococcum* cells under different culture conditions ($n = 3$)

Pigment	Without glucose under irradiance	With 0.3% glucose under irradiance	With 0.3% glucose without irradiance
Astaxanthin	23.2±1.1	17.2±0.9	3.8±0.2
Adonixanthin	26.5±1.4	36.7±1.6	4.5±0.3
Canthaxanthin	7.1±0.3	18.9±1.0	3.6±0.2
Lutein	36.7±1.5	25.9±1.2	75.5±3.2
β-Carotene	6.5±0.4	1.3±0.1	12.6±0.7

for astaxanthins (Fig. 7). Figs. 8 and 9 show the changes in the contents of lutein, canthaxanthin, β-carotene, and chlorophylls, respectively, along with the increase of glucose concentration. These results indicate that, although the cell dry weight concentration would increase when the concentration of glucose increases, the contents of lutein, β-carotene, chlorophylls *a* and *b* would decrease when glucose is added to the culture medium. When the concentration of glucose exceeded 0.2%, the contents of these carotenoids and chlorophylls changed slightly (Figs. 8 and 9). The effect of glucose on the production of canthaxanthin was not obvious (Fig. 8). The results for lutein, β-carotene, and canthaxanthin in the pigment extract and the saponified extract solution were consistent, indicating that no obvious loss or isomerization of these carotenoids occurred during the saponification. In the saponified extract, neither chlorophyll *a* or *b* were detected (Figs. 3 and 4), indicating that chlorophylls were completely degraded during saponification.

3.3. Biosynthesis pathway of astaxanthin

It is generally agreed that β-carotene serves as a precursor of ketocarotenoids. Astaxanthin was synthesized from β-carotene by two hydroxylation reactions, at C-3 and 3′, and two steps of direct oxidation to ketone groups at C-4 and 4′ (Rise et al., 1994; Yokoyama & Miki, 1995). The order of the reactions and the intermediate products in astaxanthin biosynthesis are different for various astaxanthin-producing microorganisms. The possible intermediates in the astaxanthin biosynthesis pathway contained β-carotene, echinenone, β-cryptoxanthin, canthaxanthin, zeaxanthin, 3-hydroxy-echinenone, 3′-hydroxy-echinenone, adonirubin (phoenicoxanthin), and adonixanthin (4-ketozeaxanthin) (Fraser et al., 1997; Harker & Hirschberg, 1997; Yokoyama & Miki, 1995). In the alga *Haematococcus pluvialis*, astaxanthin is synthesized from β-carotene through echinenone, canthaxanthin, and adonirubin (Donkin, 1976; Fan, Vonshak, Gabbay, Hirshberg, Cohen, & Boussiba, 1995; Grung, D'Souza, Borowitzka, & Liaaen-Jensen, 1992). In the red yeast *Phaffia rhodozyma*, astaxanthin is produced from β-carotene via echinenone, 3-hydroxy-echinenone, and adonirubin (Andrewes et al., 1976; Johnson & An, 1991). In the alga *Chlorella zofingiensis*, the hydroxylation step in astaxanthin biosynthesis might take place before oxygenation and astaxanthin (about 70%) is produced via the oxygenation of zeaxanthin while canthaxanthin (about 30%) might be the last stage of the oxygenation of β-carotene (Rise et al., 1994). In the astaxanthin-producing bacterium *Agrobacterium aurantiacum*, the biosynthesis pathway of astaxanthin is controlled by the culture conditions, especially dissolved oxygen in the medium (Yokoyama & Miki, 1995). Gene transfer

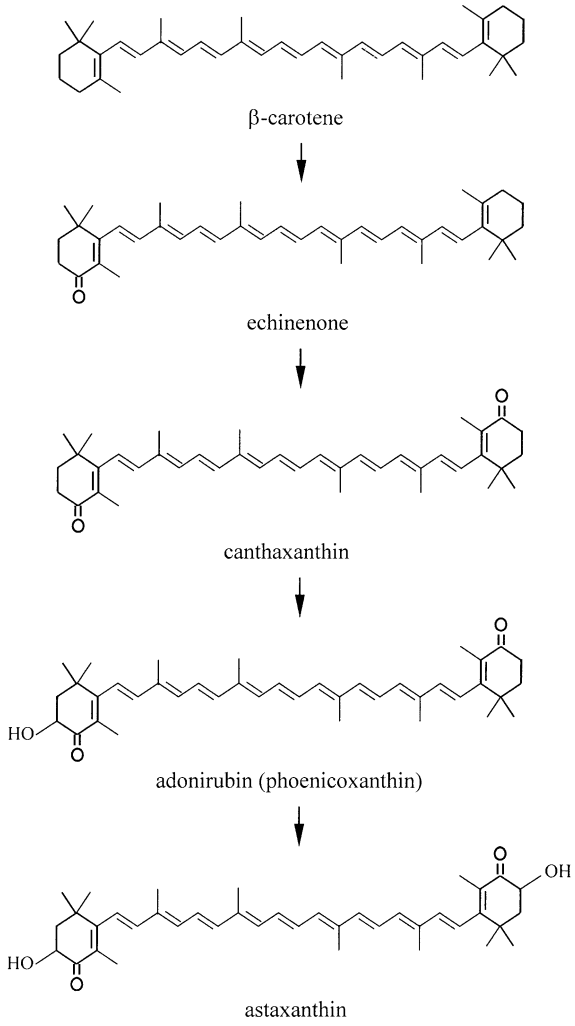

Fig. 10. Postulated pathway for the biosynthesis of astaxanthin in the algae *Haematococcuss pluvialis* and *Chlorococcum*.

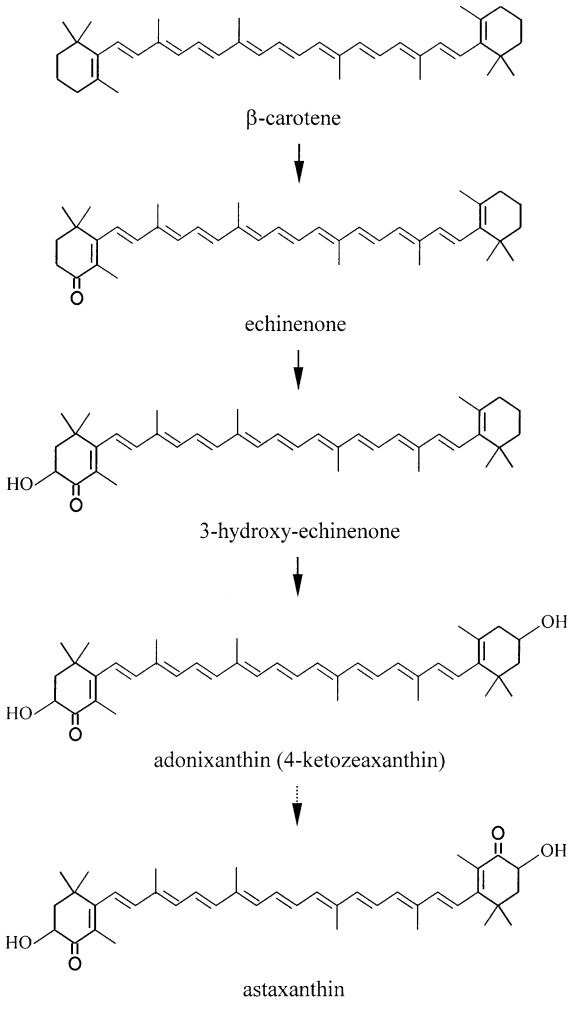

Fig. 11. Postulated pathway for the biosynthesis of adonixanthin and astaxanthin in the alga *Chlorococcum*.

technology has also been developed to aid alteration of the carotenoid biosynthesis pathway so as to produce astaxanthin and related ketocarotenoids in the blue-green alga *Synechococcus* sp. PCC7942, where other carotenoids, such as β-carotene and zeaxanthin, were already present at high concentrations in the cells (Harker & Hirshberg, 1997). In the green alga *Chlorococcum* (Liu & Lee, 1999), astaxanthin is synthesized from β-carotene by various pathways which are different from other astaxanthin-producing microorganisms. Liu & Lee (1999) postulated five possible pathways leading to the biosynthesis of astaxanthin but could not confirm which was the main biosynthetic pathway. All possible pathways started with the introduction of a keto group at C-4 or a hydroxyl group at C-3, followed by random addition of other keto and hydroxyl groups at both sides of the β-end groups.

Unlike *Haematococcus pluvialis*, in which astaxanthin esters were the main ketocarotenoid, the alga *Chlorococcum* cells could accumulate a large amount of adonixanthin and canthaxanthin as well as astaxanthin. In the present analysis, no obvious amounts of other intermediate carotenoids were detected, suggesting that the conversion of β-carotene to adonixanthin or canthaxanthin was a very rapid reaction. As shown in Figs. 1–4, astaxanthins (free and esters), adonixanthins (free and esters), and canthaxanthin were the main ketocarotenoids in the *Chlorococcum* cells, indicating that the pathways for astaxanthin biosynthesis in the *Chlorococcum* cells were significantly different from those in the alga *Haematococcus pluvialis*, and even more complicated. On the one hand, canthaxanthin was present in the *Chlorococcum* cells, indicating that astaxanthin was synthesized from β-carotene via canthaxanthin (Fig. 10). On the other hand, the presence of adonixanthin indicated that astaxanthin might be synthesized from β-carotene via adonixanthin (Fig. 11). Nevertheless, a large amount of adonixanthin was present in the *Chlorococcum* cells, indicating that the biosynthesis of astaxanthin through adonixanthin was

difficult. Thus, it seems possible that adonixanthin is the last stage of the hydroxylation of echinenone and astaxanthin is synthesized mainly via hydroxylation of canthaxanthin in the alga *Chlorococcum* cells, as in the alga *Haematococcus pluvialis* (Fig. 10).

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