Biomedicine & Preventive Nutrition 2 (2012) 276–282

Available online at
SciVerse ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com/en

Original article

Purification and characterization of carotenoids from green algae *Chlorococcum humicola* by HPLC-NMR and LC-MS-APCI

Bhagavathy Sivathanu [a], Sumathi Palaniswamy [b,*]

[a] *Bharathiar University, Coimbatore, Tamilnadu, India 641 043*
[b] *Department of Biochemistry, Queen Mary's College, Mylapore, Chennai, India 600 004*

ARTICLE INFO

Article history:
Received 5 April 2012
Accepted 25 April 2012

Keywords:
Carotenoids
Green algae
Chlorococcum humicola
Carotenes
Xanthophils

ABSTRACT

The fresh water green algae *Chlorococcum humicola* (*C. humicola*), rich in bioactive components such as carotenoids, flavonoids, polyphnols and fatty acids, has a wide variety of health benefits used in the medical and food industry because of its possible role in the prevention of diseases like cancer. The present study focused on to elucidate the structural details of the existed carotenoids in the selected algae *C. humicola*. The total carotenoid extract quantitated and further fractionated in an open column chromatography (OCC) yields totally six major carotenoids. These fractions were confirmed by HPLC before subjecting to NMR and LC-MS-APCI analysis for their structural elucidation. The spectral data of these fractions revealed the six major fractions were violaxanthin, astaxanthin, lutein, zeaxanthin, α-carotene and β-carotene. The findings of the present study confirm that green algae *C. humicola* is a rich source of carotenoids and should be further focused well for its beneficial effect.

1. Introduction

Carotenoids are naturally occurring tetraterpenes found in various fruits, vegetables, plants, algae and bacteria. These are not only essential for human health but also effective as cancer preventing agents, life extenders, and inhibitors of ulcers, heart attacks and coronary artery diseases. Carotenoids act as antioxidants through a free radical mechanism by quenching singlet oxygen and oxidizing species in the prevention of cellular damages. The antioxidant activity of astaxanthin was reported to be ten times stronger than that of other carotenoids, viz., zeaxanthin, lutein, canthaxanthin and β-carotene. This particular carotenoid is of considerable academic and practical interest not only because of its abundance in nature but also high economic value as a pigment in fish feeds [1,2].

Carotenoids belong in the group of yellow or red pigments that occur widely in plants, animals and humans. They are synthesized in plants and in some microorganisms and are only introduced with diet into human and animal organisms, which are incapable of their de novo synthesis but sometimes capable of their structural modification. They are highly physiologically important and fulfil many tasks. Primarily, they always accompany chlorophyll and assist photosynthesis and photo taxis as auxiliary light absorbers or, on the other hand, protect plants and microorganisms against excessive irradiation. Further, they strongly interact with reactive oxygen species and thus act in plant and animal organisms as potent free radical quenchers, singlet oxygen scavengers and lipid antioxidants (some of them are vitamin A precursors). In addition to this physiological and medical importance, changes in the contents and structure of carotenoids can also act as markers of environmental damage [3,4].

Pigments like carotenoids, anthraquinone and chlorophyll have been produced from yeast, fungi, bacteria and algae. There is growing interest in microbial pigments due to their natural character, medicinal properties and nutritive value; production being independent of season, geographical conditions, controllable and predictable yield and safety to use. Among pigments of natural origin, carotenoids seem to play a fundamental role, their presence in the human diet being considered positively because of their action as pro-vitamin, antioxidant or possible tumor-inhibiting agents. Despite the availability of a variety of natural and synthetic carotenoids, there is currently a renewed interest in microbial sources of pigments because of the problems of seasonal and geographical variability in plant origin [5].

Consequently, carotenoids have been intensely studied by organic chemists, food chemists, biologists, physiologists, medical doctors and recently also by environmentalists and great demands have been placed on their identification and determination. Carotenoid research in the field of food chemistry is a very extensive area for the high reactivity of carotenoids follows from their structure and places great demands on the methods of their analysis. Carotenoids are long, aliphatic, conjugated double bond systems, i.e., polyenes. A part of them are hydrocarbons, are usually

* Corresponding author.
E-mail address: sumathisiva12@gmail.com (S. Palaniswamy).

2210-5239/$ – see front matter © 2012 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.bionut.2012.04.006

composed of eight isoprene units and have the molecular formula $C_{40}H_{56}$. The central portion of the molecule contains four isoprene units, the centre two of which are joined tail-to-tail and open chain or ring structures form the ends of this chain. These hydrocarbons are called carotenes. A great majority of natural carotenes have double bonds in the all-*trans* position, where R is an open-chain structure or a ring system. Only a few natural carotenes exhibit a *cis-trans* configuration [6].

Carotenoids have a strong lipophilic character and are usually analyzed in organic solvents. However, because of their biological activity, the characterization of these compounds is very important. Carotenoids have been classified into two groups, hydrocarbon carotenes and oxygenated xanthophylls. Because of their extended system of conjugated double bonds, carotenoids are usually unstable in the presence of light, heat, or oxygen. Therefore, the isolation, identification, and quantitation of these pigments can be challenging. Complex biological samples such as human serum, tissues, and plant material often contain compounds than can interfere with carotenoids or mixtures of carotenoids with similar structures. The identification and quantitation of specific carotenoids therefore requires highly sensitive and selective analytical methods. NMR spectroscopy is a useful tool for the investigation of molecular composition of plant tissues. Actually, the low concentration, the presence of a large number of metabolites, whose resonances make the spectrum overcrowded, and the loss of spectral resolution due to line broadening associated with the heterogeneity of the sample are the main drawbacks for the observation of carotenoids in vegetables. In this work ^{1}H NMR and LCMS analysis were carried out for the structural elucidation of the carotenoids available in the selected green algae *Chlorococcum humicola (C. humicola)*.

2. Materials and methods

2.1. Chemicals

High-performance liquid chromatography (HPLC) grade acetonitrile, methanol, and dichloromethane were purchased from Rankem Chemicals Ltd. (Mumbai, India). Analytical grade acetone, hexane, chloroform, methanol, and petroleum ether were purchased from Sisco Research Laboratories, Pvt, Ltd., (Mumbai, India). Standard carotenes β-carotene, xanthophylls (astaxanthin, lutein, zeaxanthin), and were obtained from Sigma Chemicals Co. (St. Louis, MO).

2.2. Algal culture and quantification of carotenoids

Fresh water, unicellular, nonmotile green algae *C. humicola* was obtained from the culture collected from the department of Plant Biology and Plant Biotechnology, RKM Vivekanantha College, Chennai, India. Algal culturing was carried out with autotrophic Bold's basal medium supplemented with sterile compressed air and kept under fluorescent light (20 μmol m^2 s) with 16 h light period and at 25 ± 2 °C temperature [7]. After growth, the culture was harvested by centrifugation [8], Algal samples were cleaned of epiphytes, necrotic parts were removed and the biomass was lyophilized, used for the extraction.

Twenty-five grams of fresh algal samples were used for carotenoid extraction with ethanol until all the pigments removed, filtered through a sintered glass filter (porosity 3; pore size 20–30 μ). An equal volume of diethyl ether was added to the combined ethanol extracts, followed by the addition of water droplets until two layers were formed. The ethereal epiphase, containing all the pigments, were washed free from ethanol with water, and the solvent removed. To avoid carotenoid oxidation/isomerisation during this process, BHT (2, 6-di-*tert*-butyl-*p*-cresol) and $CaCO_3$, were added. The combined extracts were concentrated in a rotary evaporator ($T < 35$ °C), yielding 12 g, and dried overnight at high vacuum (6 g). The crude extract was then saponified with equal volume of 10% methanolic KOH and kept overnight in the room temperature at dark, after which the carotenoid solution was washed with water to remove the alkali (pH: 7.0) dried over Na_2SO_4 yielding 4 g of saponified extract after evaporation of the solvent [9]. The total carotenoid content was analysed under HPLC and estimated spectrophotometrically at 450 nm in a Perkin-Elmer Spectrophotometer and calculated using the formula

$$\text{Total Carotenoids } (\mu g/g) = \frac{A_{Total} \times \text{Volume (ml)} \times 10^4}{A \times \text{Sample Weight (g)}}$$

2.3. HPLC analysis of carotenoids

The homogeneity and the purity of the different fractions were checked by HPLC. HPLC analysis of carotenoids were analyzed using an HPLC (Shimadzu 10AS, Kyoto, Japan) reverse phase 25 cm, 4.6 mm i.d., 5 μm, C18 column (Wakosil 11 5C 18RS) with an isocratic solvent system consisting of dichloromethane: acetonitrile: methanol (20:70:10, v/v/v) at a flow rate of 1.0 ml/min and the column temperature is maintained at 25 °C. The dried carotenoid samples were dissolved in 100 μl of mobile phase and used for HPLC analysis. Samples were handled on ice under dim yellow light to minimize isomerisation and oxidation of carotenoids by light irradiation. The identification of carotenoids were performed by comparing retention time, UV spectra and mass spectra of unknown peaks with reference to the standards and values reported in the literature. All the carotenoids were monitored at 450 nm with a UV-visible detector (Shimadzu, Kyoto, Japan) [10].

2.4. Fractionation of carotenoids

A part of total carotenoids dissolved in light petroleum was subjected to column chromatography on aluminium oxide grade III (100 × 10 mm column). For removal of neutral lipids, the column was washed twice with light petroleum. Top the column 1 cm of anhydrous sodium sulphate to ensure that no residual water gets into the absorbent. Pass about one bed volume of petroleum ether through the column and adjust the volume so that the solvent flow is about two to three drops per second. Once petroleum ether was added to the column, keep the top of the column covered with solvent at all times until chromatography is complete. With the dropper pipette add the carotenoid petroleum ether solution into the column and let the sample layer go down almost to the surface of the sodium sulphate layer. The column was developed by adjusting the mobile phase so as to isolate the available carotenoid fractions as quickly and efficiently as possible. Elute fraction 1 with 15–18% acetone in petroleum ether, fraction 2 with acetone, fraction 3 with 25–30% acetone in petroleum ether, fraction 4 with 40–45% acetone in petroleum ether, fraction 5 with petroleum ether, and fraction 6 with 2% acetone in petroleum ether. As acetone affects the absorption of carotenoids in petroleum ether, removed from all the fractions by washing with water in a separating funnel. The petroleum ether fractions were dried with anhydrous sodium sulphate. Aliquots from each isolate were dried under nitrogen, concentrated and stored at −20 °C was further used to verify the purity by HPLC [11]. The spectrum of each fraction was taken to find out the λ_{max} of the eluted fractions in UV-VIS spectrophotometer.

$$\text{Carotenoid Fractions } (\mu g/g) = \frac{F_{ractions} \times \text{Volume (ml)} \times 10^4}{A \times \text{Sample Weight (g)}}$$