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Identification and biological activities of carotenoids from the freshwater alga *Oedogonium intermedium*

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ABSTRACT

The chemical and biological properties of carotenoids in the freshwater alga *Oedogonium intermedium* were investigated in this study. Carotenoids were extracted from the alga by dichloromethane and purified by saponification. The carotenoid content was determined both spectrometrically and by HPLC, the carotenoids identified by HPLC-PDA-APCI-IT-TOF-MS and the extracts analysed for several health-related bioactivities. The crude and saponified extracts contained 3,411.2 ± 20.7 and 2,929.6 ± 5.9 μg carotenoids/g dry algal biomass, respectively. Seven major carotenoids were identified, namely neoxanthin, 9′-*cis*-neoxanthin, loroxanthin, violaxanthin, lutein, α-carotene and β-carotene, which were present in similar amounts in the alga. Both the crude and saponified carotenoid extracts exhibited significant antioxidant activities as well as potent inhibitory effects against several metabolically important enzymes including α-amylase, α-glucosidase, pancreatic lipase and hyaluronidase, but they were poor inhibitors of angiotensin converting enzyme (ACE). *Oedogonium* could be an important new source of carotenoids, specifically loroxanthin, which is lacking in terrestrial plants.

1. Introduction

Carotenoids are an important group of natural pigments that are widely distributed in nature. More than 700 different carotenoids have been identified, mostly in plants but also in animals, microorganisms and algae (Saini, Nile, & Park, 2015). Carotenoids are broadly divided into carotenes and xanthophylls with the former being tetra-terpenoid hydrocarbons that are made only of C and H, while the latter being oxidised carotenoids that contain some *O*-substituent groups such as hydroxyl, keto and epoxy groups (Saini et al., 2015). Carotenoids are excellent scavengers of singlet oxygen in plants and, thus, protect cellular components, such as chlorophylls, lipids, proteins and DNA, from oxidative damage (Raposo, De Morais, & De Morais, 2015). Carotenoids are also shown to exhibit protective effects against several human health disorders such as certain cancers and eye conditions (*e.g.*, cataracts/macular) and cardiovascular disease, and improve the function of the immune system, skin texture and gap-junction communication (Raposo et al., 2015). These properties have formed the basis for their wide application not only as food colorants, but increasingly also as functional ingredients in foods, nutraceuticals, cosmetics and other products. However, to date, the vast majority of research on carotenoids is concerned with those from fruit and vegetables, while other important sources of carotenoids, such as algae, have received much less attention.

Compared to terrestrial plants, algae offer a number of advantages as a source of carotenoids, including fast growth rates, year-round availability and low or potentially positive impact on the environment (Ahmed et al., 2014; Lawton, Cole, Roberts, Paul, & de Nys, 2017). Furthermore, many algal carotenoids are lacking in terrestrial plants (Sugawara, Ganesan, Li, Manabe, & Hirata, 2014). Commercial production and extraction of carotenoids from algae already exists, although it is limited to a few species of microalgae. For example, β-carotene from *Dunaliella salina* and astaxanthin from *Haematococcus pluvialis* are extracted for use as food colourants (Hu, Lin, Lu, Chou, & Yang, 2008), while *Muriellopsis* has been used commercially to produce lutein due to its high lutein content and high growth rate (Lin, Lee, & Chang, 2015). Recently, there has been a growing interest in sourcing carotenoids from marine macroalgae (seaweed), including siphonaxanthin from the green seaweed *Codium fragile* and fucoxanthin from the brown seaweed *Undaria pinnatifida* (Kotake-Nara, Asai, & Nagao, 2005; Sugawara et al., 2014). While these studies have focused on marine algae, freshwater algae, such as *Oedogonium*, represent another important source of carotenoids that is yet to be explored.

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Oedogonium is a genus that grows in freshwater and has recently been domesticated for intensive aquaculture (Lawton et al., 2017). Research on this group of freshwater macroalgae, done at commercial scales, has shown that *Oedogonium* can be used in wastewater treatment for aquaculture, as a bio-sorbent of metals and as a fertiliser and soil conditioner. It has also been analysed as a feedstock biomass for bioenergy applications since it has a high bio-crude yield compared with many other algal species (Cole et al., 2016). Proximate, biochemical and ultimate analyses of the dry biomass of *Oedogonium* has revealed a profile of carbohydrate content of ~40%, lipid 10%, protein 30% and mineral content 20% (Neveux et al., 2014). In addition to bioenergy applications, there is also potential for the development of this alternative protein as a higher value commodity for animal feeds. However, to date, there has been no research to investigate its bioactive components and related biological activities. The objective of this study was to investigate the carotenoid content, composition and health-related biological activities of the green alga *Oedogonium intermedium* cultured under intensive conditions. Carotenoid content of the alga was determined using both spectrophotometric and HPLC methods; carotenoids were identified by HPLC and LC-MS and the carotenoid extracts were analysed for their antioxidant capacities as well as *in vitro* inhibitory activities against several metabolically important enzymes including α-amylase α-glucosidase, pancreatic lipase, hyaluronidase and ACE.

2. Materials and methods

2.1. Chemicals and reagents

4-Methylumbelliferyl oleate (4-MUO), 4-nitrophenyl-a-D-glucopyranoside (*p*-NPG), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), acarbose, ACE from rabbit lung, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), DPPH (2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl), hyaluronic acid sodium salt from *Streptococcus equi*, hyaluronidase from bovine testes, pancreatic lipase from *Candida rugose, N*-Hippuryl-his-Leu (HHL), sodium citrate, sodium potassium tartrate tetrahydrate, α-amylase from *Bacillus subtilis* (380 U/mg), gallic acid, α-glucosidase from *Saccharomyces cerevisiae* (100 U/mg), TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) and trolox were purchased from Sigma-Aldrich (Sydney, Australia). Acetic acid, acetone, acetonitrile, 3,5-dinitrosalicylic acid, ammonium acetate, boric acid, citric acid, hexane, hydrochloride acid (32%), iron (III) chloride hexahydrate, methanol, potassium carbonate, potassium persulfate, sodium chloride, sodium phosphate diabasic, sodium phosphate monobasic and potato starch were purchased from Ajax Finechem Pty Ltd (Sydney, Australia). 4-Dimethylaminobenzaldehyde (DMAB) was purchased from BDH Chemicals Ltd (Poole, UK), dichloromethane from Merck (Sydney, Australia), methyl-tert-butyl ether (MTBE) from Fisher Scientific (Sydney, Australia), orlistat from Tokyo Chemical Industry (Tokyo, Japan) and potassium hydroxide and sodium hydroxide from Chem-Supply Pty Ltd (Sydney, Australia). All chemicals were of at least analytical grade unless stated otherwise. Solvents used in HPLC were of liquid chromatography grade and water used in all experiments was purified by reverse osmosis using the MilliQ RO system (referred to as MilliQ water).

2.2. Seaweed sample collection and storage

The freshwater alga *Oedogonium intermedium* (hereafter *Oedogonium*) was harvested in December 2012 from stock cultures grown in outdoor tanks at James Cook University (JCU), Townsville, Queensland, Australia. *Oedogonium,* a filamentous macroalga that naturally grows under eutrophic conditions in streams and drainage ditches, was cultured in two 10,000 L parabolic tanks, using dechlorinated tap water, enriched with Guillard's f/2 nutrient medium to 1 g/L, as described in Cole, Mata, Paul, and de Nys (2014). Monocultures of *Oedogonium* were maintained in open system throughout the sampling period and verified by visual inspection and microscopy. Biomass was harvested weekly, using large hand nets or pumped through nylon bags, to maintain the stocking density at 0.5 g fresh weight/L. Use of a coarse filter to harvest the *Oedogonium* ensured that any microalgae or microbes from the water column were not collected in the sample. The biomass was centrifuged and freeze-dried (Labogene ScanVac Coolsafe 110–4 Pro Freeze Dryer, Lynge, Denmark) for 48 h. The freeze-dried biomass was vacuum packed immediately in plastic bags and air freighted to the University of New South Wales, Sydney, Australia, where they were stored at −80 °C until use.

2.3. Extraction of carotenoids from seaweeds

The extraction procedure was a modified Bligh-Dyer method (Rao, Baskaran, Sarada, & Ravishankar, 2013). Briefly, 15 mg of freeze-dried samples was mixed with 1.2 mL of MilliQ water, 3 mL of methanol and 1.5 mL of dichloromethane, followed by vigorous mixing. After 10 min incubation at room temperature, the mixture was added with 1.5 mL each of distilled water and dichloromethane, and was mixed vigorously again. The organic layer was separated by centrifugation (10 min at 3,500*g*) and collected. The extraction step was repeated twice more using 3 mL of dichloromethane each time. The solvent was dried with nitrogen (N_2) gas, and the resultant extract was dissolved in 1 mL of methanol or DMSO for further analysis. The crude extract was then saponified with 10% methanolic KOH and kept overnight at room temperature in the dark; after which, the carotenoid extract was washed with water to remove the alkali, and dried over N_2 gas. Precautions were taken to minimise potential loss of carotenoids due to light, heat and oxidation. These precautions included conducting the extraction procedures under dim lighting, use of low temperatures, and evaporation of solvents with N_2 streams where applicable and performing each operation in the shortest possible times.

2.4. Determination of total carotenoids

Determination of total carotenoids was carried out spectrophotometrically according to Lichtenthaler (1987). The dried extract (15 mg dry algal biomass weight equivalent) was re-dissolved in 5 mL of methanol, and the absorbance of the extracts was measured at 470, 665.2 and 652.4 nm by a Spectramax Plus M2 spectrophotometer (Molecular Devices, Australia). The pigment contents (chlorophyll *a* and chlorophyll *b* and total carotenoids) were calculated using the Lichtenthaler equations (Lichtenthaler, 1987):

$$C_{chlorophyll\ a} = 16.72A_{665.2} - 9.16A_{652.4}$$

$$C_{chlorophyll\ b} = 34.09A_{652.4} - 15.28A_{665.2}$$

$$C_{carotenoid} = \frac{1000A_{470} - 1.63C_{chlorophyll\ a} - 104.96C_{chlorophyll\ b}}{221}$$

2.5. Identification and quantification of carotenoids

2.5.1. HPLC-PDA-APCI-IT-TOF-MS analysis of carotenoids

The analysis was performed using a Prominence LC system (Shimadzu, Kyoto, Japan) connected to a PDA detector (SPM-M20A, Shimadzu, Japan) followed by an ion trap-time of flight mass spectrometer (LCMS-IT-TOF, Shimadzu, Japan) equipped with an atmospheric pressure chemical ionisation (APCI) source. The extract was separated on a TSK gel ODS-80TsQA column (2.0 × 250 mm, 5 µm, Tosoh, Tokyo, Japan) at 40 °C. The injection volume was 5 µL and flow rate was 0.2 mL/min. The mobile phase consisted of methanol/water (90/10, v/v) containing 0.1% of ammonium acetate (A) and methanol/ethyl acetate (70/30, v/v) containing 0.1% of ammonium acetate (B). The gradient elution was performed as follows: 0–5 min 0% B; 5–20 min,