3 Production of Carotenoids from Cultivated Seaweed

Masahiko Iha and Ritsuko Fujii

Abstract

Cladosiphon (C.) okamuranus, a brown alga endemic to the Nansei Islands, Japan, has been conventionally ingested as food. Nowadays, it is a major aquatic product of the Okinawa Prefecture with an annual production of around 20,000 tons. The life cycle of *C. okamuranus* comprises the macroscopic sporophyte (algal body) generation and the microscopic gametophyte generation. The germlings in the latter generation can proliferate when floating in seawater. This floating form has been exploited in techniques involved in the commercial production of *C. okamuranus* seedlings.

Brown algae contain fucoxanthin, a carbonyl carotenoid known to have anticancer, anti-obesity, and antidiabetic effect in addition to the anti-oxidation effect. We found that the fucoxanthin content of cultivated floating form of *C. okamuranus* discoid germlings becomes up to 50 times that of the mature alga. Since the discoid germlings repeatedly grow like microorganisms, although they are large algae, they are utilized to produce fucoxanthin. We optimize the culture conditions by changing the temperature, light intensity, photoperiod, light wavelength, and nutrient salt conditions for optimal fucoxanthin productivity. The cultivation has been successful to industrial plant scale, culminating in the use of 1 ton of cultivating medium.

In brown algal cells, fucoxanthin is primarily found bound to the photosynthetic pigment–protein complexes known as fucoxanthin–chlorophyll protein (FCP). Consequently cultivated floating form of *C. okamuranus* also shows high content of FCP. Isolation and characterization of pigments bound to the FCP were determined precisely, and ultrafast spectroscopies were applied to elucidate the photosynthetic function of fucoxanthin bound to the pigment–protein complexes. This cultivation method has also been applied to the other edible brown algae. We found that the optimal cultivation conditions as well as the yields of fucoxanthin and FCP highly depend on the species.

The floating form cultivation was also applied to a large-sized edible green alga, *Codium intricatum*, which is uniquely producing a carbonyl carotenoid, siphonaxanthin. This has several anti-disease effects and is also a primal photosynthetic pigment which is found bound to photosynthetic antenna complex usually called siphonaxanthin–chlorophyll protein (SCP). We are working on the improvement of productivity, scale-up of

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N. Misawa (ed.), *Carotenoids: Biosynthetic and Biofunctional Approaches*, Advances in Experimental Medicine and Biology 1261, https://doi.org/10.1007/978-981-15-7360-6_3

production, and development of cultivation technology of new macro algae.

Keywords

Fucoxanthin · FCP · Cultivation · Brown algae · Siphonaxanthin

3.1 Introduction

Okinawa, Japan's southernmost island group, has a subtropical climate and unique biological resources. Approximately 500 seaweed species have been confirmed to inhabit the seas surrounding the Okinawa Islands and Nansei Island, including numerous indigenous species. Especially several of them, such as *Cladosiphon okamuranus*, *Acetabularia ryukyuensis*, and *Pseudodichotomosiphon*, are precious species only found in very limited areas. Owing to this unique and diverse biota, various useful organisms have been identified. Indeed, various seaweed species can be readily collected from marine waters surrounding our affiliated company in Uruma City, Okinawa Prefecture.

Peoples living in these places have a culture of ordinarily eating some of those seaweeds, such as *Gracilaria blodgettii* and *Chondrus ocellatus*. A few species among them, including *Cladosiphon okamuranus*, *Monostroma nitidum*, and *Caulerpa lentillifera*, are actively commercially cultivated and are important aquatic products in Okinawa Prefecture.

Seaweed contains various functional compounds that are not found in terrestrial plants, and we have been conducting research and development to utilize seaweed resources unique to Okinawa. In particular, we have been working on the production of functional ingredients by taking advantage of the life history stages of large algae; culturing microscopic floating form results in the consolidation of the ingredients found in the later stage of sporophyte algae.

3.2 Cultivation of *C. okamuranus* Discoid Germlings in Floating Form

C. okamuranus is an edible seaweed indigenous to the Nansei Islands, in which the Yaeyama Islands and Amami Oshima Island represent the southern limit (latitude 24°N) and the northern limit (latitude 29°N), respectively (Fig. 3.1). *C. okamuranus* grows well in wide pools in coral reefs surrounding the inner and outer parts of bays, inhabiting the areas with a low water level of 0–13 m in spring tides, but preferring the 0–8 m tidal range. The preferred habitats are defined as relatively quiet but having good communication with the open sea and "clean sea" that realizes both high underwater visibility and low nutrient concentration (Toma 1991).

C. okamuranus algal body is a major aquatic product in Okinawa Prefecture where cultivation techniques were established in the 1970s, and the annual production now reaches 20,000 tons. The

Fig. 3.1 Photo of *Cladosiphon okamuranus* algal body

cultivation of algal body is performed on a horizontal cultivation net putting under a few meters depth at coastal waters. The cultivation net can accept seedlings of *C. okamuranus* spontaneously from the environment. But occasionally the net is dipped into the condensed pool of *C. okamuranus* seedlings prior to settling in seawater.

The life cycle of *C. okamuranus* was well studied, and it comprises the generation of a macroscopic sporophyte (alga body) and a microscopic gametophyte. Unilocular sporangia, formed in sporophytes during March–June, release zoospores, which are joined to form germlings that adhere to rock or other substrates, grow into a discoidal shape, and eventually form an upright algal body. However, if a zoospore fails to settle and remains floating on seawater, it grows into the lint-like floating form and undergoes vegetative propagation (Fig. 3.2). This phenomenon has been exploited in techniques for the *C. okamuranus* seedling production.

C. okamuranus contains fucoidan, which has been shown to exert various physiological effects (e.g., anticancer, immune activation, and anti-ulcer effects) and is used as a raw material for functional foods. In addition, it also contains fucoxanthin as a major carotenoid pigment (Fig. 3.3), which has been reported to have radical scavenging/singlet oxygen elimination properties and anticancer, anti-obesity, and antidiabetic effects, among others (Maeda et al. 2005). The chemical compounds present in the floating form of *C. okamuranus* have been investigated: fucoxanthin has been found at a concentration ~50 times that found in the mature alga (See Fig. 3.4). Thus, we applied the floating form, which repeatedly grows like microorganisms, albeit it is a large alga, to the production of fucoxanthin. Specifically, we identified optimal culture conditions by changing the temperature, light intensity, photoperiod, light wavelength, and nutrient conditions and using fucoxanthin productivity as an index (Iinuma et al. 2009).

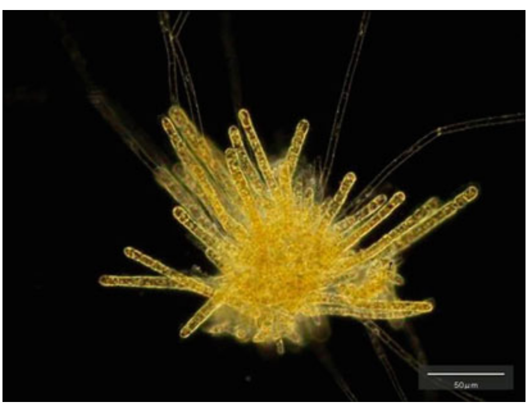

Fig. 3.2 Micrograph of *C. okamuranus* discoid germlings floating in seawater

Cultivation in an open pond has cost advantages with regard to the scale-up of cultures. Scaling-up is facilitated in the case of *C. okamuranus* as it exhibits an allelopathic effect, inhibiting the growth of other algae (Kakisawa et al. 1988). *C. okamuranus* is cultured stepwise in culture tanks in increasing size, from flask scale to industry plant level, culminating in the use of 1-ton tanks (open ponds), in which about 1-kg wet weight of *C. okamuranus* discoid germling per 1 ton of seawater can be produced.

3.3 Production of Fucoxanthin and Fucoxanthin Chlorophyll *a/c* Protein

Fucoxanthin is widely found in heterokontophyta, such as brown algae and oceanic microalgae, and is believed be the second-most abundant carotenoid on Earth after β-carotene. Recently, studies on the functionality of fucoxanthin have been increasingly conducted, and fucoxanthin has been shown to possess various beneficial physiological activities (e.g., anti-obesity effects). We put cultured *C. okamuranus* into practical use for fucoxanthin production. High-purity fucoxanthin is used as a reagent in research and as a standard for quantitative analysis. In addition, it is extracted, separated/purified, stabilized, and commercialized as a raw material for functional foods.

Fucoxanthin is a primary pigment binding to photosynthetic antenna proteins, which play a role in light harvesting during photosynthesis.

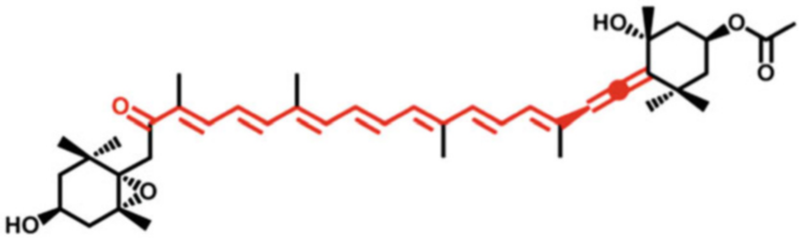

Fig. 3.3 Chemical structure of fucoxanthin

We hypothesized that the involvement of antenna proteins played a role in the changes in fucoxanthin content achieved under culture conditions used to culture. Brown algae, such as *C. okamuranus*, contain fucoxanthin chlorophyll *a/c* protein (FCP), a unique antenna protein that is not found in higher plants. FCP has been previously isolated from several diatoms and a few brown algae (Büchel 2018). *C. okamuranus* discoid germling is characterized by high fucoxanthin content and is suitable for the separation, purification, and production of FCP under controlled culture conditions. Using the cultivated discoid germling as a starting material, we extracted thylakoid membranes and successfully isolated and purified FCP by successive solubilization using a surfactant, sucrose density gradient centrifugation, and various chromatographic techniques (Fujii et al. 2012a, b). The isolated and purified FCP had a molecular weight of about 56 kDa and was found to be a homo- or hetero-trimer of 18.2- and 17.5-kDa subunits based on the results of two-dimensional polyacrylamide gel electrophoresis. (Recently, X-ray crystallography reveals that the similar FCP from a diatom consists of two dimers having molecular weight of 37 kDa apoproteins (Wang et al. 2019). The subunit composition of brown algal FCP, however, was not resolved yet.) It was also found that trimeric FCP binds five fucoxanthin

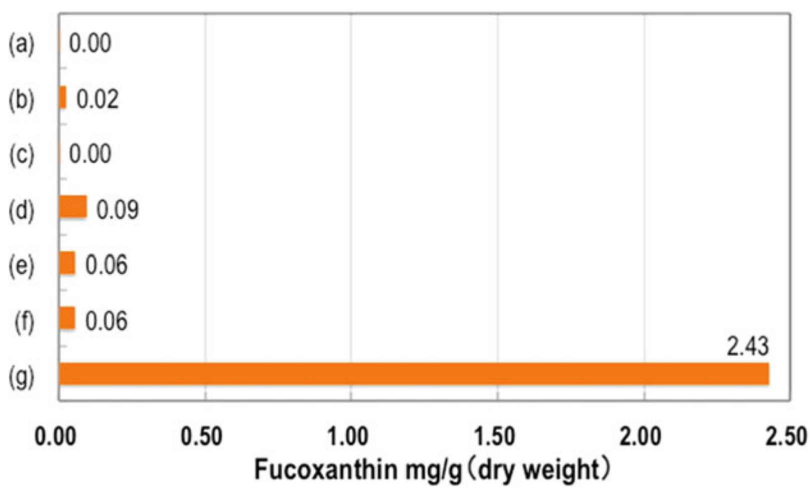

Fig. 3.4 Comparison of fucoxanthin contents (mg/g dry weight) in *Eisenia bicyclis* (**a**), Laminariaceae (**b**), *Sargassum fusiforme* (***c***), *Sargassum fulvellum* (***d***), *Undaria pinnatifida* (***e***), *C. okamuranus* alga body (**f**), and *C. okamuranus* discoid germlings (**g**)

molecules per one subunit (Fujii et al. 2012a, b). A series of ultrafast spectroscopies were applied to the trimeric FCP, and optical properties of fucoxanthin bound to the FCP have been extensively investigated (Kosumi et al. 2012).

3.4 Cultivation of Various Brown Algae in Microalgal Forms

Using the same cultivation technique as that used for *C. okamuranus*, we attempted to cultivate various other edible large algae with the aim of producing both fucoxanthin and FCP. Thus far, we have succeeded in culturing the brown algae *Nemacystus decipiens* and *Petalonia binghamiae* and have isolated and purified fucoxanthin and FCP from both algae. Interestingly, we found that these large algae exhibited differences in fucoxanthin content, pigment proportion, and optimal culture conditions during preliminary cultivations. We expect that physical and chemical studies on FCP preparations from different origins will advance and that comparisons between them will provide new knowledge.

3.5 Cultivation of *Codium intricatum* Trichomes in Floating Form

Yokohama and coworkers first identified a carbonyl carotenoid siphonaxanthin (Fig. 3.5) from a large-sized green alga, *Umbraulva japonica*, that grows under 20-m depths around Shimoda, Kanagawa Prefecture (Kageyama and Yokohama 1978). They found that siphonaxanthin absorbs green region of light (~540 nm) in vivo, although it absorbs blue light (~450 nm) in vitro when isolated and dissolved into organic solvents. Siphonaxanthin has been found in a group of green algae called siphonous green algae (Arimoto et al. 2019), which habited at relatively deeper side of intertidal zone. Siphonaxanthin was found bound to the photosynthetic antenna complexes known as siphonaxanthin–chlorophyll protein (SCP) (Yokohama 1981; Anderson 1983; Anderson 1985; Chu and Anderson 1985). It is very similar to the well-known photosynthetic antenna, known as light-harvesting II in higher plants and other green algae, but lutein in LHCII is substituted to siphonaxanthin. The green part of sunlight is mainly penetrated through the water column of a few tens of meters at coastal zones. The chemical structure of siphonaxanthin is similar to that of lutein, except for the addition of oxygen in two parts: a carbonyl and a hydroxyl groups. Thus the siphonous green algae were considered as the ancestor of other green algae and/or higher plants in a carotenoid biosynthesis point of view (Kageyama and Yokohama 1978). They also pointed out the similarity of the optical property of siphonaxanthin to that of fucoxanthin. Both of them absorb around 450 nm in methanol, but absorb around 540 nm when bound to the photosynthetic pigment–protein complexes. The large bathochromic shift may be caused by the dipole–dipole interaction from pigments surrounding the carbonyl carotenoids in the protein moiety, although detailed structures of both fucoxanthin and siphonaxanthin bound to the pigment–protein complexes have not yet been elucidated.

Moreover, siphonaxanthin has been reported to have physiological effects, such as angiogenesis-inhibiting action (Sugawara et al. 2014). Therefore, we aimed to apply our cultivation technique to siphonous green algae for the mass production of siphonaxanthin (and SCP) for further investigations.

Samples of several species of siphonous green algae were collected off the coast of Uruma City, Okinawa Prefecture, and their siphonaxanthin content was determined. Among them, *Codium intricatum* was selected by considering three criteria, the suitability for culture, sufficient high siphonaxanthin content in natural growth, and eating experience (Fig. 3.6). After unialgal culture, growth conditions were optimized on the basis of the growth rate and siphonaxanthin production in order to establish a culture technique for zygote in floating form (Fig. 3.7). The culture system was identical to that used for *C. okamuranus* discoid germlings, except that the scale of production was increased. Optimized culture conditions resulted in an increase of

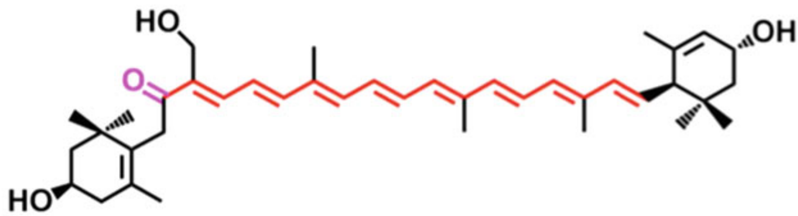

Fig. 3.5 Chemical structure of siphonaxanthin

approximately tenfold in siphonaxanthin content compared with the siphonaxanthin content of naturally grown *C. intricatum* (Oka et al. 2012; Uragami et al. 2014). We have also established a method for the separation and purification of siphonaxanthin and siphonaxanthin chlorophyll *a/b* protein from cultured *C. intricatum* (a Japanese patent).

Investigations of the physiological function using siphonaxanthin provided from the cultivated *C. intricatum* are in progress. Albeit anti-obesity and antidiabetic effects of siphonaxanthin have been shown thus far, as was the case for fucoxanthin, siphonaxanthin has been found to have a blood lipid-lowering effect that is not found in fucoxanthin. Further experiments will elucidate the new nutraceutical feature(s) of siphonaxanthin.

3.6 Prospects for the Future

We are currently working on improving productivity, scaling up production, and developing new cultivation techniques for large algae, with the aim of producing photosynthetic antenna proteins and carotenoids using proprietary culture techniques.

Because large algal cultured successfully thus far show compositions different from those of mature algal bodies, they have potential for use in the production of new functional ingredients.

Recently, the whole genome of *C. okamuranus* has been analyzed (Nishitsuji et al. 2016). We expect that biosynthetic pathways for various functional ingredients of *C. okamuranus* will be identified. In addition, *C. okamuranus* may be useful as a model organism in the genetic research of brown algae because its genome size (214 Mbp) is about the same as that of *Arabidopsis thaliana*.

Antenna proteins play the most important light-harvesting role in the initial stage of photosynthesis. Marine algae have antenna proteins that enable the highly efficient use of the weak solar energy received in deep waters of the sea. Fucoxanthin and siphonaxanthin are conjugated with antenna proteins in brown and green algae growing, respectively. An increasing number of

Fig. 3.6 Photo of *Codium intricatum* algal body

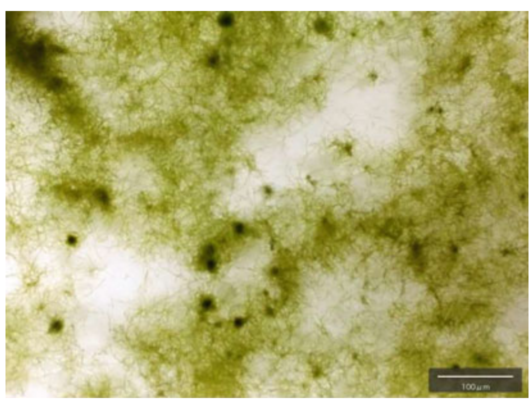

Fig. 3.7 Micrograph of *Codium intricatum* trichomes (filamentous form)

functions have been identified for fucoxanthin and siphonaxanthin, as has been the case for other carotenoids.

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