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Characterization of Light-harvesting Pigments in Spores of *Ulva fasciata* Delile

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Results of *in vivo* absorbance spectra, high-performance liquid chromatography (HPLC) and *in vivo* fluorescence spectra were compared to characterize pigments in spores and vegetative tissues of *Ulva fasciata*. *In vivo* absorbance spectra of these spores revealed a broad absorbance capacity from 470 to 550 nm not observed in vegetative tissue of the same thalli. Calculation of the 4th derivative of the absorbance spectrum corresponds with wavelength to an absorbance peak of one of the pigments present in the sample. The chlorophyll and carotenoid composition, determined by standard HPLC methods with a three solvent gradient system and C_{18} reversed-phase column chromatography revealed that lutein and β-carotene were the main novel contributors in the blue-green region (475 to 495 nm). The ratio of β-carotene/ Chlorophyll *a* in spores was 3- to 4-fold higher than in vegetative tissues; the ratio of lutein/Chl *a* in spores was 1.5- to 2-fold higher than in vegetative tissues. *In vivo* fluorescence spectra for room temperature PSII emission in these unicells revealed a broad spectral range for light-harvesting capacity, with enhanced harvesting from 470 nm to 495 nm, a 5 nm shift to longer wavelengths for spores when compared with vegetative tissues of the same thalli. The enhanced in *in vivo* absorbance and *in vivo* fluorescence by spores suggests that distinct carotenoids function in light-harvesting.

Key Words: high-performance liquid chromatography, *in vivo* absorbance spectra, *in vivo* fluorescence spectra, light-harvesting pigments, *Ulva fasciata*

INTRODUCTION

The diversity of algal pigments, chlorophylls, carotenoids and phycobilins, can be seen as evidence of the dynamic spectral environment found in coastal and deeper water regions. Within this heterogeneous classification, carotenoids are not only the most widespread of the naturally occurring groups of accessory pigments, but probably also have the most varied functions: fluorescence quenching (Demming-Adams and Adams 1990; Laczko and Maroti 1992; Olaizola *et al.* 1994), photoprotection (Hans 1984; Young 1991; Green and Koslova 1992; Cunningham *et al.* 1993; Hagen *et al.* 1993; Ladygin and Shirshikova 1993; Maegawa *et al.* 1993; Sandmann *et al.* 1993), light-induced xanthophyll cycles (Senger *et al.* 1993), and light-harvesting photosynthesis (Goodwin 1980; Hans *et al.* 1983; Granovskaya *et al.* 1992). The varied presence of pigments has been documented through the analysis of *in vivo* absorbance (Goodwin 1980; D'Agnolo *et al.* 1994; Smith and Alberte 1994; Beach *et al.* 1995), and *in vivo* fluorescence spectra (Kageyama *et al.* 1977; Owen *et al.* 1987; Mimuro *et al.* 1992; D'Agnolo *et al.* 1994), for a range of unicellular algae including dinoflagellates (Ricketts 1966, 1970; Jeffrey *et al.* 1975; Prezelin *et al.* 1976; Vesk and Jeffrey 1977; Jeffrey 1980; Chisholm *et al.* 1988), diatoms (Barrett and Jeffrey 1971; Larkum and Barett 1983), eustigmatophytes (Owens *et al.* 1987), chloromonads (Haxo *et al.* 1984; Fawley 1993), prymnesiophytes and cryptomonads (Vesk and Jeffrey 1977; Jeffrey 1980). Light-harvesting appears to be a common role for many carotenoids (Goodwin 1965, 1980; Goodwin and Britton 1988) (i.e. fucoxanthin in the Bacillariophyta and Phaeophyta; peridinin in the Dinophyta: Larkum and Barrett 1983; Anderson 1985). In general, the specific roles of minor carotenoids, that are invariably present in algae are poorly understood.

Among the chlorophytes, chloroplast pigments are similar to those of land plants; chlorophylls *a* and *b* are present. In addition, varied carotenoids are present in green algae (Lee and Soh 1991; Shin and Smith 1995) depending on the differences in habitats. For members of

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the Ulvales, Cladophorales and Siphonocladales, lutein was detected only in shallow-water species, whereas large amounts of loroxantin and lutein (Sasa *et al.* 1992; Yokohama *et al.* 1992) or siphonaxanthin were detected in deep-water species. For species of *Ulva*, a spectral component attributable to siphonaxanthin-like carotenoids was observed in *U. japonica* (Yokohama 1981) and *U. rigida* (Pollesello *et al.* 1992). The siphonaxanthin-like carotenoid(s) efficiently transferred excitation energy to photosystem II (PS II) (Anderson 1985) in *U. japonica* (Kageyama *et al.* 1977). However, the extent to which carotenoids enhance overall light-harvesting for reproductive unicells of a macroalgae has not been investigated. Following Beach *et al.* (1995), *U. fasciata* appeared to have produced an increased relative amount of carotenoids in spore producing portions of the sporophyte. Mathematical deconvolution of blue-green region of those spectra demonstrate a 490 nm peak in both gametophyte and sporophyte thalli of *U. fasciata*. For the other algae, maxima in this spectral region have been attributed to carotenoids (Smith and Alberte 1994). Beach *et al.* (1995) suggested that the pools of carotenoids change resulting in a broadening of the absorbed photosynthetically active radiation (PAR) into the blue-green region for *U. fasciata* spores and gametes.

The objectives of this study are: 1) to characterize pigments in vegetative and spores of the green alga, *U. fasciata* using analytical tools: *in vivo* absorbance spectra and standard HPLC method, and 2) to elucidate functional roles of accessory pigments in different phases of the life cycle via *in vivo* fluorescence spectra.

MATERIALS AND METHODS

Field sampling and spore release

The materials for this investigation were collected at Kaëalawai Beach (tide height of site approximately +0.2 feet) from the south coast of O'ahu Island in Hawaii. Whole thalli of *U. fasciata* was collected haphazardly and transported to a laboratory in a plastic bag with seawater. All plant surfaces were cleaned several times with a soft brush in 0.2 μm filtered seawater. The material was blotted dry, separated, and individual plants were placed in dry Petri dishes under 50 μmol $m^{-2}s^{-1}$ fluorescent lights at room temperature (19.5°C). Twelve hours later the thalli were barely covered with filtered seawater (approximately 30 ml). This treatment led to release of motile cells typically as a dense suspension of cells. Identification of motile cells was accomplished at 1000X magnification with phase contrast microscopy (Olympus model BH-2). *In vivo* absorbance spectra, HPLC analyses and *in vivo* fluorescence spectra were performed within 2 h of collection.

In vivo absorbance spectra

In vivo absorbance spectra for vegetative tissues and suspensions of spores were measured using a Shimadzu UV Vis-2101 spectrophotometer with a 150 mm external integrating sphere attachment on algal tissues of dimensions 1.3 by 3.0 cm following Beach *et al.* (1995). Absorbance spectra of vegetative tissues were obtained by examining non-overlapping layers of vegetative algal tissue. Absorbance spectra of spores were measured by scanning suspensions of spores in seawater in a cuvette (spores density: 4.5 to 6.5 x 10^7/ml). Before beginning the investigation of spores, the cuvette was gently inverted several times to mix the spores to minimize self shading (i.e. "package effects") or settlement on cuvette walls. Fourth derivative spectra were generated with the algorithms provided by the manufacturerís software PC revision 2.2 with a lambda of 16 nm.

High performance liquid chromatography

An HPLC system (Shimadzu) consisting of a SPD-10AV UV-VIS detector, SCL-10A system controller, SIL-10A auto injector and LC-10AS pump were employed. Reversed phase columns, Axxion 25 cm x 4.6 mm ID, 5 μm particle sizes (90000 to 1000000 plates m^{-1}) were used. The gradient systems used are shown in Table 1. In the analytical system, an initial 2 min gradient from 100 % A to 100 % B provided improved separation of polar compounds (Wright and Shearer 1984; Wright *et al.* 1991). The program returned to initial conditions and re-equilibrated for 5 min before the next sample injection. Methanol, acetonitrile, ethyl acetate and water were HPLC grade regents. Flow-rate was maintained at 1 ml/min. Standards for Chl *a*, Chl *b*, β-carotene, and lutein were purchased from Sigma.

Pigment extraction from vegetative tissues and motile cells for HPLC

Vegetative algal tissue was measured and sized to 1.0 x 1.3 cm then ground for 30 seconds in a mortar with a pre-chilled pestle in 4.7 ml 100% acetone. HPLC grade water (0.3 ml) was added to make up 90 % acetone and the sample was repeatedly ground. Debris were separated by centrifugation (Bring Kmann Inc. Model 545C) at 1.4 x 10^4 rpm for 2 min. After centrifugation, an aliquot

Table 1. HPLC solvent system program.

Time (min)	Flow rate (ml min^{-1})	% A	% B	% C	Conditions
0	1.0	100	0	0	Injection
2	1.0	0	100	0	Linear gradient
2.6	1.0	0	90	10	Linear gradient
13.6	1.0	0	65	35	Linear gradient
20	1.0	0	31	69	Linear gradient
22	1.0	0	100	0	Linear gradient
25	1.0	100	0	0	Linear gradient
30	1.0	100	0	0	Equilibration

Solvent A: 80:20 methanol : 0.5 M ammonium acetate (pH 7.2 v/v)
Solvent B: 90:10 acetonitrile (210 nm UV cut-off grade): water (v/v)
Solvent C: 100 ethylacetate (HPLC grade)

of the extract (2 ml) was placed in sample vials. Pigment extraction from spores followed standard methods (Wright et al. 1991). The collected spores were filtered (Whatman 0.2 μm) and then were ground in 5 ml of 90 % acetone. Two ml of the supernatant from the unicells were collected for HPLC injection. Two hundred μl of extraction sample was injected for HPLC.

In vivo fluorescence emission spectra

Vegetative algal tissue was placed in quartz cuvette and placed in line fluorescence spectra (S/N QM-1058, Photon Technology International) to examine for standard room temperature emission. The vegetative thalli was placed onto a support of nytex mesh (1.3 x 4 cm) and then put into a four-sided fluorescence cuvette. The fluorescence scanning range was 350 to 650 nm. Self-shading of spores was minimized by a moderate stirring speed in the cuvette. The spectrum sampling interval and slit width were set at 0.5 and 0.2 nm. The fluorescence spectra were generated and corrected for variations in the baseline with the algorithms provided by the manufacturer (OSCARTM software, version 2.060a).

RESULTS

In vivo absorbance and 4th derivative spectra

Absorbance spectra of spores for *Ulva fasciata* confirmed distinct enhancement in absorbance over the vegetative thalli in from 470 to 550 nm (Fig. 1). A 5 nm shift to longer wavelength for spores was observed from 490 nm. This increased *in vivo* absorbance by spores is consistent with the presence of carotenoids (Owen *et al.* 1986) . Calculation of the 4th derivative spectra for the *in*

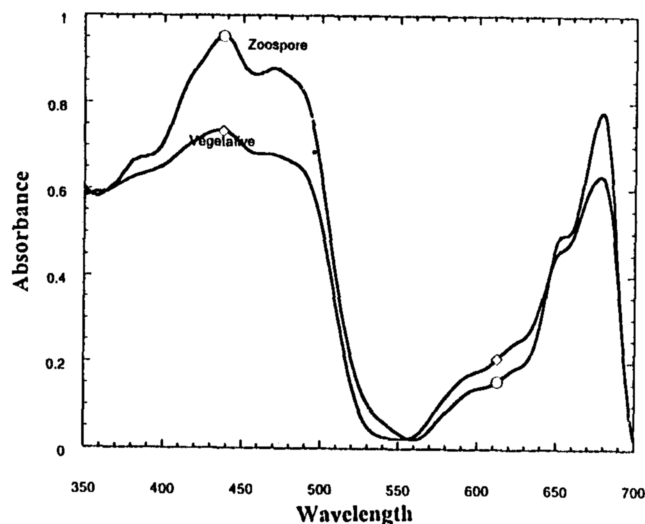

Fig. 1. *In vivo* absorbance spectra of vegetative thallus and spore of *U. fasciata.*

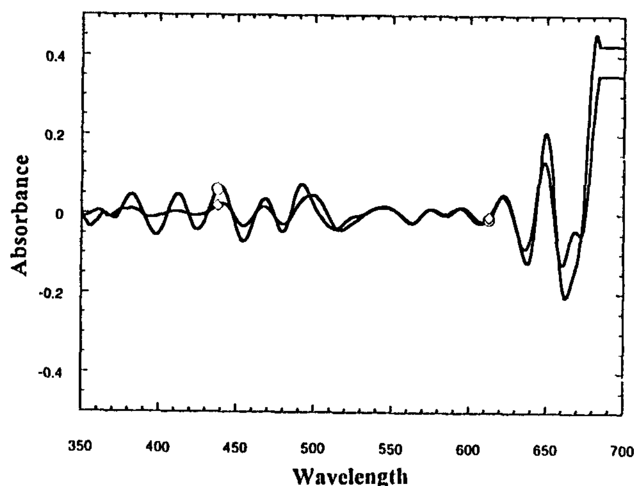

Fig. 2. Fourth derivative spectra of vegetative thallus and spore of *U. fasciata.*

vivo absorbance spectra for vegetative thalli and spores resolved Chl a maxima at 441, 622 and 682 nm, and b absorbance maxima at 468 and 650 nm. Fourth derivative deconvolution of this spectral region demonstrated that the 490 nm peak of spores of *U. fasciata,* attributed to carotenoid absorbance in other algae (Smith and Alberte 1994), had shifted 12 nm to a longer wavelength (Fig. 2).

HPLC spectra

The pigments of vegetative tissues and spores that were characterized by HPLC with a three solvent gradient system analysis, are summarized in Table 2. HPLC analysis of vegetative tissues and spores of *U. fasciata* exhibited consistent pigment composition. The retention

Table 2. Major carotenoids and chlorophyll from *Ulva fasciata*.

Carotenoids	R_t (min)	Wavelength (nm)		
Unknown	13:11		-	
Lutein	15:28	425	446	474.5
Chlorophyll *b*	18:33	456	599	643
Chlorophyll *a*	19:58	433	619	664.5
Unknown	21:68		-	
Unknown	22:04		-	
β-carotene	22:74	456	495	-

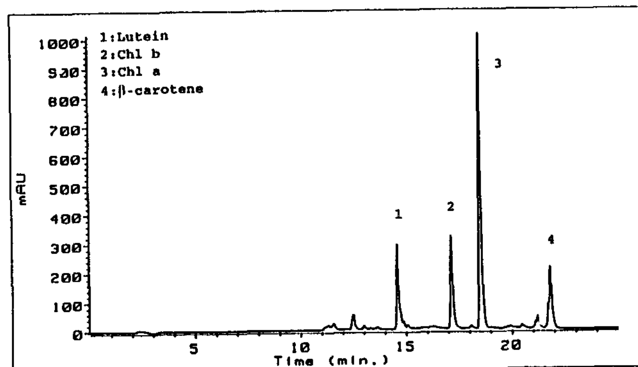

Fig. 3. HPLC traces of pigment of spore of *U. fasciata*.

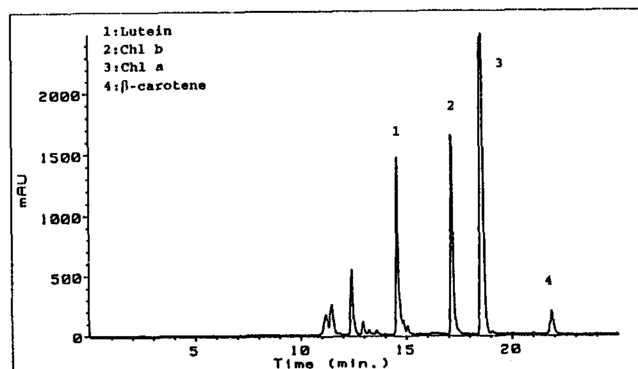

Fig. 4. HPLC traces of pigment of vegetative thallus of *U. fasciata*.

times (R_t) of Chl *a*, *b*, lutein and β-carotene from HPLC were $\approx$ 15.28, 19.58, 18.33 and 22.74 min. The major carotenes lutein (absorbance maxima in 90 % acetone: 425, 446, 474.5 nm), β-carotene (absorbance maxima in 90 % acetone: 456, 495 nm), and an unidentified minor carotene were identified as the main contributors to absorbance in the 470 to 550 nm range (Fig. 3 and 4 and Table 2). Further analysis by HPLC indicated the presence of at least two additional minor carotenoids ($R_t \approx$ 21.68 and 22.04 min.) were extracted from spores.

Pigment analysis by HPLC confirmed that spores had a 3.5-fold-higher Chl *a* : β-carotene ratio than vegetative

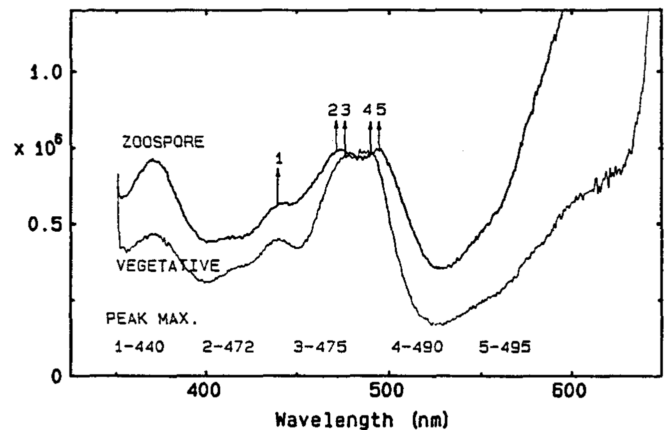

Fig. 5. *In vivo* fluorescence spectra at room temperature of thallus and spore of *U. fasciata*.

tissues. The Chl *a* : *b* ratio of spores and vegetative tissues also varied. However, the ratio of Chl *a*:lutein and β-carotene in spores was fairly uniform at 1.5 to 2- fold higher than vegetative tissues.

In vivo fluorescence spectra

Fluorescence excitation spectrum for room temperature PS II emission in *U. fasciata* demonstrated a broad spectral range for light-harvesting capacity, with as much as a 5 nm shift to longer wavelengths for spores when compared with vegetative tissues: maximum emission ranged from 472 to 495 nm in spores and 475 to 490 nm in vegetative tissues (Fig. 5).

DISCUSSION

Pigmentation of reproductive cells was generally held to be consistent with that of vegetative thalli. Here we report the occurrence of pigments that vary substantially from spore to vegetative thallus for a green alga. In this study, similar enhancements of *in vivo* absorbance and fluorescence excitation spectra in the 490 nm region demonstrate that carotenoids that absorb in this wavelength region function in light-harvesting for spores. Based on the pigment content and estimated contribution to absorbance in the 490 nm region , β-carotene and possibly lutein are the major carotenoids functioning in light-harvesting pigments in spores. The *in vivo* absorbance spectra of spores increased and broadened the absorbance from 470 to 490 nm consistent with the presence of increased Chl *b* and carotenoids. Absorbance maxima consistent with lutein (absorbance maxima: 425, 446, 474.5 nm) and β-carotene (456, 495 nm) were detected. The lutein and β-carotene absorbance maxima

and R_t (R_t $\approx$ 15.28 and 22.74 min) of HPLC for Chl *a*, *b* and novel carotenoids correspond to their absorbance and R_t reported by Wright *et al.* (1991). The increased light-harvesting efficiency in 495 nm region for spores is likely to extend their survival in dim light during this planktonic stage in the life cycle or as settled juveniles challenged with an environment with variable spectral quality and quantity.

Irradiance may change dramatically over depth in the water environment (Jerlov 1968). The spectral composition of the downwelling flux changes progressively with increasing depth. Depending on water quality, much of irradiance below about 15 m is enriched in blue-green, 400-550 nm, spectral region with peak occurring in the blue region at about 440-490 nm (Kirk 1983). According to Doty *et al.* (1974), *U. fasciata* and *U. reticulata* have appeared at over 51 and 128 m, even though they are common on the subtidal reef flat. If variable irradiance environments represent a physiological challenge while part of the planktonic community, one might expect reproductive cells to have more broad light-harvesting capacities than vegetative tissues. Novel light-harvesting carotenoids may also contribute to increased light-harvesting abilities as well as higher concentrations of expected light-harvesting components (Chl *a* : *b* ratios). The high concentrations of carotenoids (especially lutein) also has been documented in two deep water bryophytes, *Drepanocladus fluitans* and *Fontinalis antipyretica* (Boston *et al.* 1991).

According to Yokohama *et al.* (1992), for some members of the Cladophorales and Siphonocladales, the green light-harvesting pigment, siphonaxanthin, is found in thalli inhabiting deep or shaded sites, while lutein, a metabolic precursor of siphonaxanthin, is found in the algae inhabiting sunny sites in shallow water called a, "shallow-water type". A trace amount of lutein was detected in a deep-water species, "deep-water type", *Cladophora wrightiana*, that contained a large amount of siphonaxanthin. From their results it is clear that "deep-water types" contain a large amount of siphonaxanthin and "shallow-water types" contain a large amount of lutein. Lutein is thought by some to be essential to some shallow-water green algae; this xanthophyll might promote photoprotection in shallow sunny-sites (Yokohama 1983). As an exception, however, *Codium fragile* in shallow water sunny habitat contained siphonaxanthin (Yokohama *et al.* 1977) probably attributable to substantially self-shading by its a fairly thick and branched morphology. Spores in this study exhibit features similar to those of deep-water plants, while adult *U. fasciata* thallus exhibit features similar to shallow-water plant.

Upon release several important hydrodynamic processes influence the distribution of spores. Surface currents away from the sites of potential settlement and/or other compatible spores can reduce the probability of settlement (Roughgarden *et al.* 1988). Even at slow flow rates of 10 cm s^{-1}, a 6 h semi-diurnal tide cycle, water would cover 2.1 km. With the capability of satisfying metabolic and motility demands by efficient photosynthesis, the temporal window during which settlement may be accomplished could be extended. This possibility could increase the ecological range of a particular population and would increase potential for outcrossing among the local populations. Down welling currents would similarly alter spores distribution.

Spores must settle to continue the life-cycle; they can be expected to be tenacious in their settlement strategies. Pioneer weedy species can attribute part of their ecological success to a tenacious pool of motile cells that can rapidly colonize cleared or new substrate (Doty 1967). A longer-lived pool of motile cells that can contribute to their own metabolic demands insures this success. This ulvalean strategy exploits irradiance to enhance the success of weedy benthic algae as pioneer species.

Functional adjustments to the irradiance of coastal regions are likely to be regulated at the physiological level by changes of pigment concentrations providing near-optimal response for motile cells of pioneer species. Siefermann-Harms (1985) also suggested that the abundance of carotenoids in photosynthetic membranes have a role in photosynthetic reactions or in stabilizing the photosynthetic apparatus. However, it is less clear how carotenoids are involved in primary photochemistry or electron transfer although their function as collectors of light-energy used in photosynthetic processes and their role in protecting the photosynthetic apparatus against destructive effects of light and O_2 are well established. Efficient photosynthesis and distinct carotenoids in *U. fasciata* revealed a major ecological enhancement for photosynthetic performance, potentially leading to a broader ecological range of settlement sites, facilitating for ecological success a weedy species.

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