Proceedings of the 11th International Coral Reef Symposium, Ft. Lauderdale, Florida, 7-11 July 2008
Session number 5

High-performance Liquid Chromatographic Analysis of Photosynthetic Pigments in Corals: An Existence of a Variety of Epizoic, Endozoic and Endolithic Algae

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Abstract. Photosynthetic pigments of corals were analyzed by high-performance liquid chromatography (HPLC) to investigate the interrelationship between corals and their symbiotic algae, zooxanthellae (a dinoflagellate of the genus *Symbiodinium*). Coral samples were collected from Sesoko, Okinawa, Japan. HPLC analysis achieved the separation of more than 60 peaks of the pigments from more than 20 species of corals and 31 pigment types were identified. In addition to marker pigments of dinoflagellates, a variety of pigments including chlorophyll *b*,chlorophyll *d*, chlorophyll c_1, zeaxanthin, and lutein were detected. After brushing the surface of one of the common species of coral in the study area, *Montipora digitata*, to remove epizoic algae, the pigment composition of the coral was analyzed. However, the brushing treatment was not sufficient to remove most of the algae attached. These findings suggest that in addition to the pigments from symbiotic zooxanthellae, some of the pigments belong to epizoic and/or endolithic algae that grow in association with corals, such as cyanobacteria, green algae and diatoms. From these facts, corals are not only the host of symbiotic zooxanthellae, but also a community of diverse algae including cyanobacteria. This means that the coral host together with its associated algae may have an important role in terms of energy production for the whole reef ecosystem.

Keywords Photosynthetic pigments, HPLC, zooxanthellae, epizoic and endolithic algae, symbiosis

Introduction

The coloration of many reef-building corals is mainly derived from the photosynthetic pigments of their endosymbiotic, dinoflagellate algae of the genus *Symbiodinium*. The pigment composition of dinoflagellates is distinct from terrestrial plants and chlorophyte algae in containing chlorophylls c_1 and/or c_2, and the major xanthophylls peridinin or fucoxanthin (Kirk 1994). Dinoflagellates including *Symbiodinium* also contain the xanthophylls diadinoxanthin and diatoxanthin (Ambarsari *et al*. 1997), which are functionally equivalent to the photoprotective xanthophyll cycle pigments violaxanthin, antheraxanthin and zeaxanthin in terrestrial plants (Demmig-Adams & Adams 1996). *Symbiodinium* pigment profiles are dominated by chlorophyll *a* and peridinin, both of which comprise isomers in the HPLC eluant appearing as two or more peaks in HPLC chromatograms (Venn et al. 2006). All *Symbiodinium* also contain the pigments β-carotene and pheophytin *a* and additionally the minor xanthophyll dinoxanthin and the alteration product of diadinoxanthin, diadinochrome. These minor xanthophylls have been reported in HPLC studies of *Symbiodinium* (Kleppel et al. 1989; Ambarsari *et al*. 1997; Dove *et al*. 2006). Photosynthetic endolithic algae and cyanobacteria live within the skeletons of many reef-building corals. Under normal conditions, the green endolithic algae grow under less than 5% of the ambient photosynthetically active radiation (PAR) because of the absorbance of light by the zooxanthellae, coral tissues and the carbonate skeleton (Fine *et al*. 2005). However, there is little information concerning the diversity of such endolithic algae in corals.

In this study, we analyzed photosynthetic pigments of corals by HPLC to investigate the

interrelationship between the coral host and its epizoic and endolithic algae in order to understand their ecological role in the whole coral ecosystem.

Materials and Methods

Sampling of corals

Coral samples (see Table 1 for species list) were collected from Sesoko Island (26°38'54"N, 127°51'16"E) and Bisezaki (26°42'03"N, 127°52'35"E) located at the northern part of Okinawa, Japan. The collected corals were put into sterilized plastic bags, brought back immediately to the laboratory and stored at –30°C until treatment and pigment measurements.

Standard pigments

Chlorophyll *a*, pheophorbide *a*, α-carotene and β-carotene were purchased from Wako (Osaka, Japan). Chlorophyll c_2, chlorophyll c_3, divinyl chlorophyll *a*, alloxanthin, diadinoxanthin, fucoxanthin, 19'-butanoyloxyfucoxanthin, 19'-hexanoyloxyfucoxanthin, lutein, peridinin, prasinoxanthin, violaxanthin, and zeaxanthin were obtained from DHI Water and Environment (Copenhagen, Denmark). Chlorophylls *a* and *b* were extracted from spinach (*Spinacia oleracea*) leaves and purified by sugar-column chromatography according to the method of Perkins and Roberts (1962). Pheophytins *a* and *b* were prepared by acid treatment of the respective chlorophylls as described previously (Shioi *et al*., 1983). Chlorophyll *d* was isolated and identified from the cells of *Acaryochloris* sp. or thalli of the red alga, *Carpopeltis crispate*, collected from Itoh City, Shizuoka, Japan.

Treatment of Montipora digitata

For removing the epizoics, living coral tissue surfaces were brushed gently using a toothbrush. Alternatively, coral tissues were removed by water pik treatment and centrifuged at 5000 xg for 10 min to separate zooxanthellae from the coral tissues.

Extraction of pigments from coral species

Frozen corals from field sampling were crushed into small pieces and then homogenized with 20 mL of cold 95% (v/v) methanol in a mortar for pigment extraction. After extracting the pigments with sonic treatment for 5 min, extracts were then filtered through a syringe filter (0.2 μm, Millex-LG, Millipore) to remove cell and skeleton debris. To avoid the shape distortion of earlier eluting peaks, methanol extract (1.0 mL) was mixed with 0.2 mL of distilled water just prior to injection according to the protocol described by Zapata et al. (2000). These extracted samples (200 μl) were immediately injected into the HPLC. All samples were prepared under subdued light and subjected to HPLC analysis within 5 min after extraction to avoid pigment destruction.

HPLC analysis

HPLC analysis was performed according to the method reported by Zapata et al. (2000). The HPLC system employed was model LC-10A equipped with degasser and column oven, using a Waters Symmetry C_8 column (4.6 x 150 mm). All apparatus was Shimadzu (Kyoto, Japan). Pigments were eluted at a flow rate of 1.0 mL per min at 25°C with a programmed binary gradient elution system according to the method. Solvents used were, A: methanol:acetonitrile: 0.25 M aqueous pyridine solution (50:25:25, by volume), and B: methanol:acetonitrile:acetone (20:60:20, by volume).

Pigment identification and quantification

Separated pigments were detected spectrophotometrically with a photodiode array detector, Shimadzu SPD-M10A, with an optical resolution of 1.2 nm, measuring from 320 to 720 nm and monitoring 5 channels of representative wavelengths at 410, 430, 440, 450 nm, and 663 nm. The wavelengths used to indicate the pigments were 410 nm for pheophorbide/ pheophytin *a* derivatives; 430 nm and 663 nm for chlorophyll *a* species; 440 nm for neoxanthin and violaxanthin; and 450 nm for chlorophyll *b*, chlorophyll *c* species and other carotenoids. Each peak was identified by comparison with HPLC retention times and absorption spectra of the standards and the data from photodiode array detection. A coelution of the standard pigment with a sample was done to assay more precisely, if necessary. Concentrations of each pigment were calculated from the standard curves, which were created for those 20 pigments from the relationships of concentrations and peak areas of HPLC using the appropriate wavelengths described above.

Results and Discussion

Pigment analysis of various coral species

As shown in elution profiles of representative corals (Fig. 1), HPLC analysis achieved the separation of more than 60 peaks of the pigments from more than 20 species of corals. Among them, 31 pigment species were

identified. The results of pigment identification from 20 coral species are summarized in Table 1. The pigment species extracted from corals were different in each coral, despite the fact that the corals used in this study were collected from similar environmental conditions. In addition to marker pigments of dinoflagellates, a variety of pigments including chlorophyll *b*, chlorophyll c_1, chlorophyll *d*, zeaxanthin, and lutein were detected. Interestingly, chlorophyll *d*, which is only present in prokaryotic *Acaryochloris*, was found from several species of corals. The absorption spectrum of chlorophyll *d* is shown in Fig. 2.

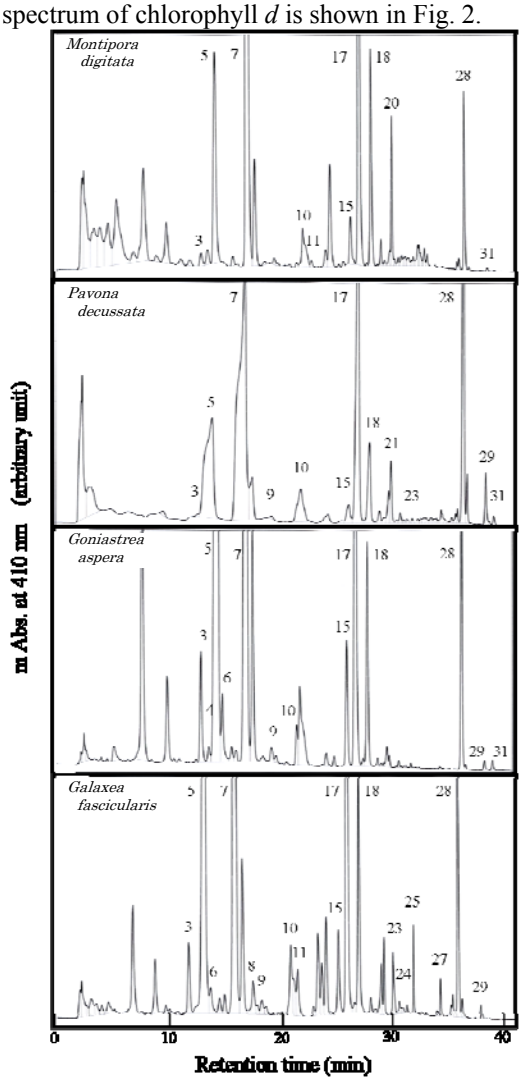

Fig. 1. Elution profiles of the photosynthetic pigments in representative coral species. HPLC conditions are described in the text. Peak numbers in elution profiles correspond to those in the identification table (Table 1).

Also, chlorophyll 684, which has a maximum absorption peak at 684 nm in the red band, was found in several species of corals. This is considered to be a degradation product of chlorophyll *a*. β-carotene is one of the marker pigments of dinoflagellates, but it was sometimes not detected. This is probably due to degradation of the pigment and it changing into its derivatives. As shown here, in addition to zooxanthellar pigments, various photosynthetic pigments from different algae were detected in corals. This finding shows that some of the pigments are probably due to the presence of epizoic and/or endolithic algae, growing on the coral surface or inside the coral skeleton, such as green algae, diatoms and cyanobacteria.

Pigment analysis of treated M. digitata

To examine whether algae are attached to the coral surface or not, brushing and water pik treatment of the coral were carried out. After brushing *M. digitata*, to remove epizoic algae, the pigment composition of corals was analyzed in the same manner. As shown in Table 2, even after brushing, similar types of photosynthetic pigments from different algae were detected in corals, in addition to zooxanthellar pigments, indicating that most of the epizoics could not be removed. Similar results were also obtained from water pik treatment. These facts show that epizoic algae are firmly attached and/or live inside the coral tissue.

Conclusion

In this study, we showed that some of the pigments detected by the HPLC technique in corals belong to various epizoic, endozoic and/or endolithic algae growing in association with the coral host. Moreover, these algae were unable to be removed by brushing treatment. Therefore, corals are not only hosts for their zooxanthellae, but also for various other algae. This means that the coral host and its algae might have an even more important role in the primary production of the whole ecosystem than otherwise suspected. Further research is necessary to investigate not only the relationship between corals and *Symbionidium*, but also interrelationships among coral hosts and their algal community.

Acknowledgements

This research is financially supported by grants from Mitsubishi Corporation and the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 19255002).

Table 1. Identification of photosynthetic pigments in 20 species of corals

Peak No.	t_R (min)	Maxima in eluant (nm)			Pigment	*Montipora digitata*	*Montipora aequituberculata*	*Montipora peltiformis*	*Montipora grisea*	*Montipora sammarensis*	*Acropora latistella*	*Pavona decussata*	*Favites chinensis*	*Favia favus*	*Goniastrea aspera*	*Cyphastrea serailia*	*Platygyra daedalea*	*Leptastrea purpurea*	*Leptastrea transversa*	*Astreopora myriophthalma*	*Symphyllia recta*	*Psammocora contigua*	*Galaxea fascicularis*	*Porites lutea*	*Porites cylindrica*
1	8.329	465	601	650	Chlorophyllide *b*			+	+					+	+	+	+		+	+					
2	11.050	451	584	633	MV Chlorophyll c_3																		+		
3	12.893	429	618	665	Chlorophyllide *a*	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+
4	13.425	439		629	Mg DVP																				
5	14.132	452	584	634	**Chlorophyll c_2**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	14.869	446	583	631	Chlorophyll c_1	+	+	+	+		+	+		+	+		+	+	+	+	+	+	+	+	+
7	17.019	474			**Peridinin**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	18.354	411		665	Pheophorbide *a*		+	+	+		+		+	+	+	+		+	+	+	+	+		+	+
9	19.463	454	473		Uriolide	+						+											+		
10	22.026	461			Fucoxanthin	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	22.799	418	438	467	Neoxanthin	+	+	+	+			+	+	+	+	+	+	+	+	+	+	+	+	+	
12	23.832	456			Prasinoxanthin																				
13	23.951	418	441	470	Violaxanthin			+	+					+	+	+	+	+						+	
14	24.000	446	469		19'-Hexanoyloxyfucoxanthin			+	+					+	+	+	+	+	+		+	+		+	
15	24.078	478			Astaxanthin	+						+													
16	26.302	405	429	457	Diadinochrome	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+
17	27.080	425	447	477	**Diadinoxanthin**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
18	28.135	417	442	470	**Dinoxanthin**	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+
19	29.535	428	451	478	Alloxanthin																				
20	30.002	427	452	480	Diatoxanthin	+			+											+				+	
21	30.187	420	448	472	Monadoxanthin							+													
22	30.506	427	453	480	Zeaxanthin		+	+	+	+	+		+	+	+	+	+	+	+	+	+	+		+	+
23	31.525	422	446	474	Lutein	+		+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
24	32.222	427	453	684	Chlorophyll 684				+										+				+		+
25	33.511	457			Siphonein			+	+					+	+	+	+	+	+	+			+	+	
26	34.806	458	646	691	Chlorophyll *d*				+	+	+														
27	35.115	461	608	649	Chlorophyll *b*			+	+				+	+	+	+	+	+	+	+	+	+	+	+	
28	36.492	431	616	662	**Chlorophyll *a***	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
29	38.536	406	503	665	Pheophytin *a*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30	39.012	422	446	475	α-Carotene				+																
31	39.195	425	451	478	**β-Carotene**				+	+		+		+			+							+	+

Bold, Marker pigments of dinoflagellates

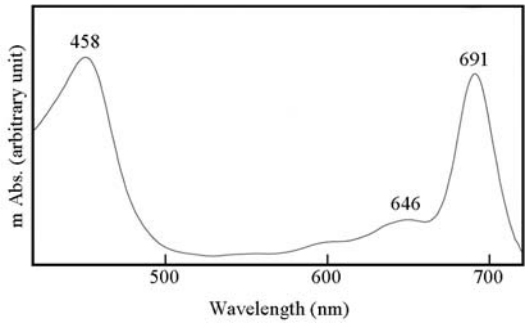

Fig. 2. Diode array absorption spectrum of chlorophyll *d* extracted from *Montipora sammarensis*. Separation and analysis of the pigment are described in the text.

Table 2. Identification of photosynthetic pigments in *Montipora digitata*

Peak No.	t_R (min)	Maxima in eluant (nm)			Pigment	No treatment n = 7	Wash n = 7	Water pick n = 7
3	12.599	431	622	665	Chlorophyllide *a*	7	7	0
5	13.906	452	585	634	**Chlorophyll c_2**	7	7	7
6	14.570	445	581	629	Chlorophyll c_1	7	7	7
7	16.740	474			**Peridinin**	7	7	7
8	18.980	410	507	668	Pheophorbide *a*	0	0	7
9	19.388	452	475		Uriolid	7	7	0
10	22.099	449			Fucoxanthin	7	7	7
11	22.749	413	439	468	Neoxanthin	7	7	0
16	26.324	408	429	457	Diadinochrome	7	7	7
17	27.151	422	446	474	**Diadinoxanthin**	7	7	7
18	28.228	418	441	469	**Dinoxanthin**	7	7	7
20	30.010	429	452	479	Diatoxanthin	7	7	7
23	31.680	422	448	477	Lutein	0	0	7
26	34.883	458	646	691	Chlorophyll *d*	2	1	0
27	34.970	458		646	Chlorophyll *b*	1	0	0
28	36.695	431	616	662	**Chlorophyll *a***	7	7	7
29	38.787	408	504	666	Pheophytin *a*	5	6	7
31	39.764	448	477		**β-Carotene**	0	0	5

Peak numbers correspond to those of Table 1. **Bold**, Marker pigments of dinoflagellates; Under line, Values changed.

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