Pigment composition and pigment-protein complex from *Symbiodinium* sp. strain Y106

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Abstract. Pigment composition and the pigment-protein complex of *Symbionidium* sp. strain Y106 isolated from the mantle lobe of *Tridacna crocea* were analyzed to investigate the photosynthetic machinery of this alga. High-performance liquid chromatography (HPLC) analysis was performed using a Waters Symmetry C_8 column and a programmed binary gradient elution with a photodiode array detector. The pigments were quantified and identified by comparison with standards. HPLC analysis showed the separation of 11 species of the pigments and 9 of them were identified. The pigment peridinin, was the most abundant carotenoid, consistent with *Symbiodinium* belonging to the group of dinoflagellates characterized by plastids containing peridinin rather than fucoxanthin. The separation of pigment-protein complexes from the membranes of this alga were performed by either nondetergent or detergent methods after solubilized with β-dodecyl maltoside. By using the nondetergent method involving hydrophobic chromatography, several color fractions were obtained. The fraction 41 (F41) was brick-red and most probably contained peridinin-chlorophyll protein complex (PCP). The fractions were characterized with respect to their pigment composition, spectroscopic characteristics and polypeptide composition. The F41 showed strong absorbance between 420 and 520 nm due to the carotenoids bound to the PCPs, mainly peridinin. Chlorophyll (Chl) *a* in the complexes appeared at 674 nm. The fraction contained five polypeptides including two known polypeptides of 15 and 30 kDa separated by gel electrophoresis.

Key words: Dinoflagellate, *Symbiodinium*, Peridinin-chlorophyll protein complex, Pigment composition.

Introduction

Recently, *Symbiodinium* pigments have frequently been analyzed because of their importance in coral bleaching, which is explained as a loss of color of corals due to a loss of symbiotic algal cells, a breakdown of algal pigments, or a combination of these two processes. The pigment composition of the largest group of photosynthetic dinoflagellates, including *Symbiodinium,* is distinct from every other alga in containing peridinin, and its isomer.

Symbiodinium contains one type of chlorophyll *c*, Chl c_2 and the xanthophylls, peridinin and its isomer, diadinoxanthin, and diatoxanthin. They 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 high-performance liquid chromatography (HPLC) studies of *Symbiodinium* (Kleppel et al. 1989; Ambarsari et al. 1997; Dove et al. 2006; Venn et al. 2006).

The light-harvesting pigments peridinin and Chl c_2 are associated with the various Chl *a*-protein complexes within the thylakoid lumen (Boczar and Prezelin 1987; Larkum and Howe 1997; Norris and Miller 1994). Two distinct light-harvesting systems were reported in dinoflagellates. One is an intrinsic Chl *a*-*c*-caroteno-complex with a 19 kDa polypeptide which is related to the other Chl *a*-*b* binding proteins. The other is a major unique water soluble peridinin-Chl *a* protein complex (PCP) and consists of either an apoprotein of 32 kDa or a dimer of 15 kDa apoproteins (Hiller et al. 1993). Molecular studies suggest that 32 kDa apoprotein arose by gene duplication or by the fusion of gene encoding the 15 kDa apoprotein (Norris and Miller 1994; Soon-Youl et al. 2005). PCP is known to transfer the gathered energy from the excited Chl *a* molecule towards the membrane bound light-harvesting and light energy conversion machineries. The exact *in vivo* function of PCP is, however, not yet experimentally resolved, although the species distribution, photo-physiology, and molecular topology of PCP have been extensively studied (Boczar and Prezelin 1987 and references there in).

In this study, we first analyzed the photosynthetic pigments from the *Symbiodinium* sp. strain Y106 by HPLC, and their pigment profiles were identified. The

biochemical features of PCP isolated from the algal cells were also characterized. Results indicate that *Symbiodinium* sp. strain Y106 has pigments characteristic of their non-endosymbiont types of dinoflagellates.

Materials and methods

Pigments standards

Chl *a*, pheophorbide *a*, β-carotene, and α-carotene were purchased from Wako (Osaka, Japan). Chl c_2, Chl c_3, divinyl Chl *a*, alloxanthin, diadinoxanthin, fucoxanthin, 19'-butanoyloxyfucoxanthin, 19'-hexanoyloxyfucoxanthin, lutein, peridinin, prasinoxanthin, violaxanthin, and zeaxanthin were obtained from DHI Water and Environment (Copenhagen, Denmark). Chls *a* and *b* were extracted from spinach (*Spinacia oleracea* L.) leaves and purified by sugar-column chromatography according to the method of Perkins and Roberts (1962) as described (Shioi 2006). Pheophytins *a* and *b* were prepared by acid treatment of the respective Chls as described previously (Shioi et al. 1983).

Symbiodinium *and growth of algae*

Symbiodinium sp. strain Y106 isolated from the mantle lobe of *Tridacna crocea* was obtained from Dr. M. Hidaka (University of the Ryukyus) and used in this study. Algal cells were grown in f/2 medium (Guillard and Ryther 1962) at 24°C for 21 days under a continuous light (60 μE m^{-2} s^{-1}).

Extraction of pigments

The cells were collected by centrifugation, and pigments of the symbiotic cells were extracted with 1 ml of cold 95% (v/v) methanol. The extraction was repeated twice and the combined extracts were filtered through a syringe filter (0.2 μm, Millex-LG, Millipore, Bedford, MA, USA) to remove cell debris. To avoid the shape distortion of earlier eluting peaks, the 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). Aliquots of extracts (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 deterioration.

HPLC analysis

Although there are many methodologies for HPLC analyses of photosynthetic pigments (Shioi 1991; Garrido and Zapata 2006), the method used in this investigation was the technique reported by Zapata et al. (2000), which is developed especially for the separation and analysis of various photosynthetic pigments from marine phytoplanktons. This method is simple and allows the separation of pigments into more than 80 peaks with high resolution. The HPLC system was a model LC-10A equipped with a degasser and column oven, using a Waters Symmetry C_8 column (4.6 x 150 mm). All components were from 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

Separated pigments were detected spectrophotometrically with a Shimadzu SPD-M10A photodiode array detector with an optical resolution of 1.2 nm, measuring from 320 to 720 nm, and monitoring five channels of representative wavelengths. The wavelengths used to indicate the pigments were 410 nm for pheophorbide/pheophytin *a* derivatives, 430 nm and 663 nm for Chl *a* species, 440 nm for carotenoids including neoxanthin and violaxanthin, and 450 nm for Chl *b*, Chl *c* species, and other carotenoids. Each peak was identified by comparison with HPLC retention times and absorption spectra of standards and the data from the photodiode array detection. The standard pigment was coeluted with a sample to assay it more precisely, if necessary. Concentrations of each pigment were calculated from the standard curves, which were created for those 20 pigments from the relationships between concentrations and peak area of HPLC using the appropriate wavelengths described above. Chromatogram peaks were quantified by a LC workstation, Class-LC10/M10A (Shimadzu).

Isolation of pigment-protein complex

Pigment-Chl protein complexes were isolated either by nondetergent method or detergent method. All procedures were carried out at 4ºC. For nondetergent method, the algal cells were suspended with 20 mM tris (hydroxymethyl) aminomethane (Tris)-HCl buffer (pH 8.0), and disrupted by ultrasonication for 2 min at an output of 140-W. Unbroken cells and large debris were removed by centrifugation at 20,000 x g for 30 min at 4ºC. The supernatant was filtrated through a Miracloth (Calbiochem, Darmstadt, Germany). Then $(NH_4)_2SO_4$ was added to the crude extract with stirring to 35% saturation. The stirring was continued for 30 min, and the mixture was left to stand for 1 h. The solution was centrifuged at 20,000 x g for 15 min, and the pellet was discarded. The resulting supernatant was applied to a column (2.5 x 8 cm) of butyl-Toyopearl 650M (Tosoh) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 35%

saturation of $(NH_4)_2SO_4$. Unbound proteins were washed with the equilibration buffer and the bound proteins were eluted with 20 mM Tris-HCl buffer (pH 8.0) containing a reverse linear gradient of $(NH_4)_2SO_4$ from 35% saturation to 0. To determine the pigment-protein complex, absorbance of peridinin at 447 nm was monitored and high absorbance fractions were collected. A flow chart of the isolation of PCPs is shown in Fig. 1. For the detergent method, the pigment-protein complexes from the membranes of this alga were solubilized with 10 mM β-dodecyl maltoside (DDM/Chl *a* = 41:1, w/w) for 1 h and then subjected to centrifugation with a sucrose-density gradient of 5-20% (w/v) at 200, 000 x g for 16 h, according to the previous methods (Büchel 2003).

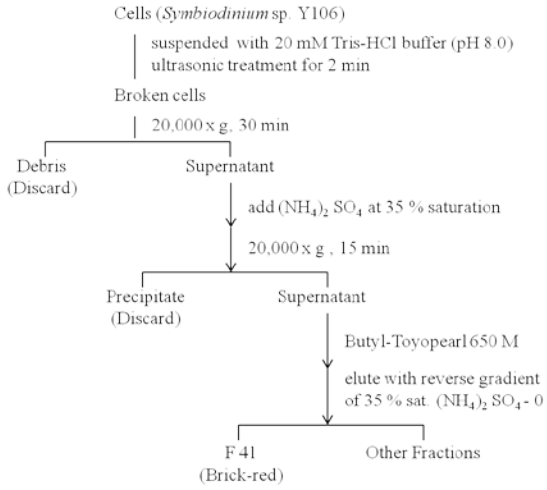

Fig. 1. Flow chart for isolation of PCP from *Symbiodinium* sp. strain Y106.

Electrophoresis

SDS-PAGE was performed by the method of Laemmli (1970) using 12% (w/v) polyacrylamide gel under reducing conditions. Heat treatment was performed at 95ºC for 3 min. Fixation and staining were done in aqueous methanol (25%, v/v) containing acetic acid (7.5%, v/v) and 0.25% (w/v) Coomassie brilliant blue R-250. Molecular markers used were SDS-PAGE molecular weight standards, broad range (Bio-Rad, Tokyo, Japan).

Protein determination

Protein concentrations were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard. Protein was assayed spectrophotometrically by measuring absorbance at 280 nm at room temperature.

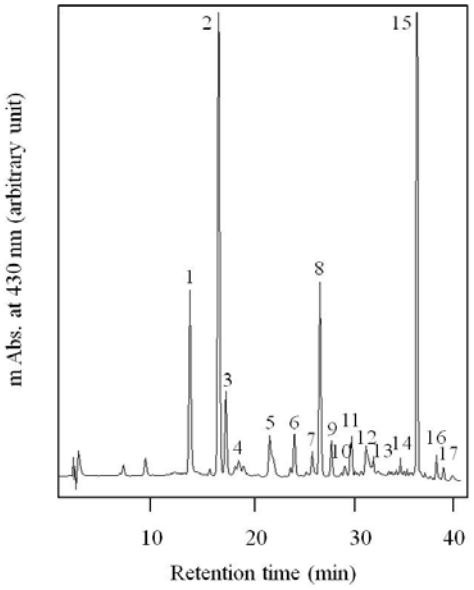

Fig. 2. Elution profile of the photosynthetic pigments in *Symbiodinium* sp. strain Y106. HPLC conditions are described in the text. Peak numbers in elution profile correspond to those in the identification table (Table 1).

Results and discussion

As shown in the elution profiles of the pigments extracted from the cells of isolated *Symbiodinium* sp. Y106 (Fig. 2), HPLC separated more than 17 pigment peaks from the algal cells. Among them, 12 pigment species were identified by comparison with HPLC retention times and absorption spectra of standards and spectral data from photodiode array detection. Peak 5 is putatively identified as the carotenoid pyrrhoxanthin. The results of pigment identification from the samples are summarized in Table 1. A variety of main pigments including Chl c_2 (peak 1), peridinin species (peaks 2 and 3), diadinoxanthin (peak 8), Chl *a* (peak 15), pheophytin *a* (peak 16), and β-carotene (peak 17) were detected. The absorption specta of representative pigments are shown in Fig. 3. The results of pigment analysis show that the isolated symbiotic cells have the pigments characteristic of peridinin containing dinoflagellates rather than those with fucoxanthin. The absorption specta of representative pigments are shown in Fig. 3.

Table 1. Identification of photosynthetic pigments in *Symbiodinium* sp. strain Y106.

Peak No.	*t*R (min)	Pigment	Absorption maxima in eluent (nm)		
1	13.66	Chlorophyll *c*2	452	583	635
2	16.49	Peridinin	473		
3	17.22	Peridinin isomer	473		
5	21.60	Pyrrhoxanthin	465		
7	25.86	Diadinochrome	408	430	
8	26.63	Diadinoxanthin	421	446	456
9	27.71	Dinoxanthin	441	465	475
10	29.06	Antheraxanthin	421	442	
12	29.65	Diatoxanthin	428	452	470
15	36.24	Chlophyll *a*	430	617	478
16	38.23	Pheophytin *a*	409		663
17	38.96	β-carotene	453	478	665

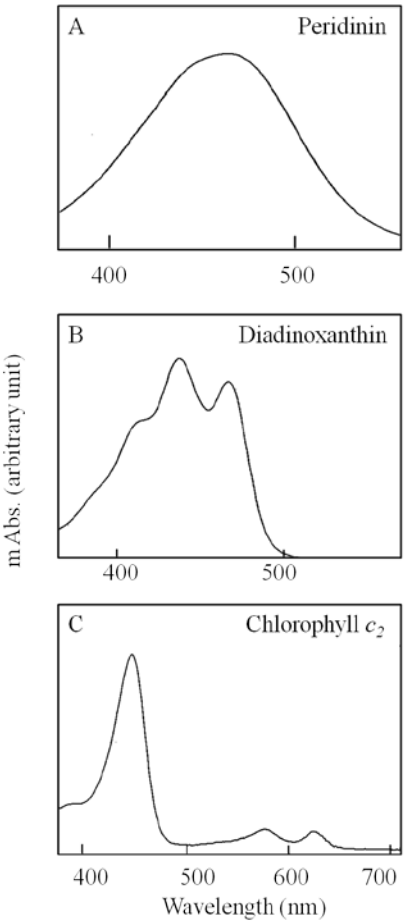

Fig. 3. Diode array absorption spectra of representative pigments in elution profile of the pigments extracted from *Symbiodinium* sp. strain Y106. Separation and analysis of the pigments are described in the text. For absorption maxima, see Table 2.

With respect to the carotenoid pigments, algae have peridinin and their isomer (peak 2 and 3) in addition to diadinoxanthin (peak 8), dinoxanthin (peak 9), and diatoxanthin (peak 12). Peridinin was the mostabundant carotenoid and represented about 60% of the total carotenoids of the cells. Next was diadinoxanthin of about 25%.

Subsequently, we analyzed the pigment-protein complex after separating by nondetergent method using hydrophobic column chromatography. By this method, three color bands were resolved from strain Y106 and main brick-red color band was analyzed primarily in this study. The other bands were brownish and pale green and most probably contained photosystems. They were not considered further. The absorption spectrum of the brick-red band at room temperature is shown in Fig. 4. This spectrum is typical for PCPs isolated from other dinoflagellates, although peak maxima are slightly different from preparation methods (Prezelin and Haxo 1976, Polivka et al. 2007). The Q_y band of Chl *a* in the complex absorb at 674 nm, whereas absorption of Chl *c* band was not detected. A strong absorption between 420 and 520 nm is due to carotenoid of peridinin bound to the PCPs.

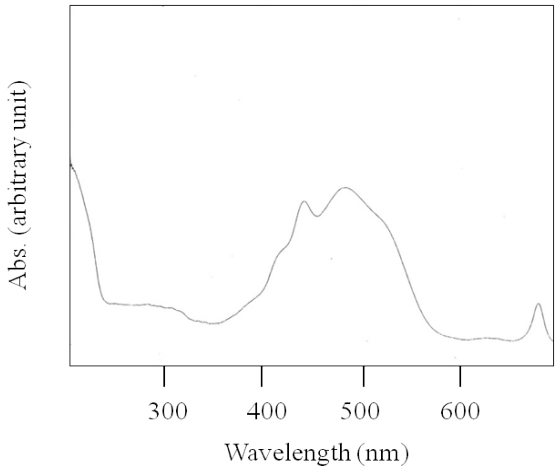

Fig. 4. Absorption spectrum of PCP (F41) isolated from *Symbiodinium* sp. strain Y106. Isolation of PCP is described in the text.

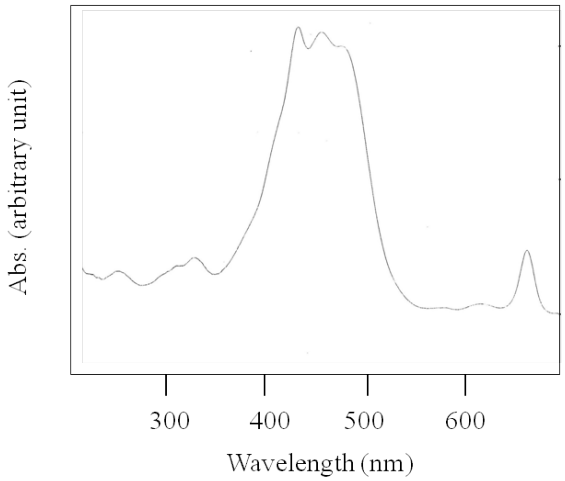

Fig. 5. Absorption spectrum of the pigments extracted from PCP (F41) of *Symbiodinium* sp. strain Y106.

Fig. 5 shows an absorption spectrum of the pigments extracted from the orange-red fraction. As expected, there are strong absorption peaks at 662 and 432.5 nm due to Chl *a* and peaks at 478 and 457 nm mainly due to carotenoid peridinin. This is evident from the results of HPLC analysis of the pigments extracts as shown in Fig. 6 and Table 2. Several peaks are separated. By comparison with spectral and analytical parameters of the standards, peaks 1 and 2 were identified to be peridinin and its isomer and peaks 4 and 5 were Chl *a* and its degradation pigment probably produced during extraction and analysis (Table 2). Unidentified carotenoid, peak 3, is putatively identified as pyrrhoxanthin, but minor peak

between peaks 2 and 3 was not identified at present study.

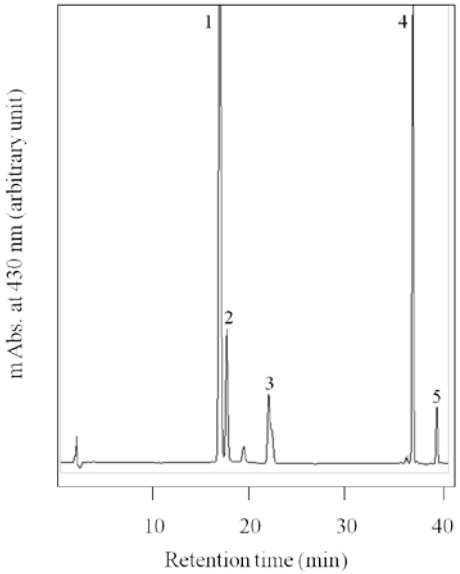

Fig. 6. Elution profile of the photosynthetic pigments extracted from isolated PCP of *Symbiodinium* sp. strain Y106. HPLC conditions are described in the text. Peak numbers in elution profiles correspond to those in the identification table (Table 2).

Table 2. Identification of photosynthetic pigments extracted from isolated PCP of *Symbiodinium* sp. strain Y106. Pigment analysis and identification were carried out as described in the text.

Peak No.	*tR* (min)	Pigment	Absorption maxima in eluent (nm)		
1	16.99	Peridinin	473		
2	17.72	Peridinin isomer	473		
3	22.06	Pyrrhoxanthin	461		
4	36.93	Chlorophyll *a*	430	617	663
5	39.47	Pheophytin *a*	409		665

A denaturating gel electrophoresis of PCPs showed that the fraction contained five polypeptides including 15 and 30 kDa, whereas two polypeptides of 15 and 22 kDa were observed in the fraction obtained from detergent method (data not shown). The size of 15 and 30 kDa polypeptides are known for the apoproteins of PCPs as reported previously (Prezelin and Haxo 1976; Sharples et al. 1996).

Conclusion

The pigment composition of the isolated *Symbiodinium* sp. Y106 was characterized in the present study. The pigment profile obtained is consistent with previous research which has shown *Symbiodinium* has pigments typical of other peridinin containing dinoflagellates. The PCPs were isolated by one-step nondetergent method using a hydrophobic column chromatography. The isolated PCPs contained mainly peridinin and Chl *a* by HPLC analysis. The procedure used in this study is a convenient and useful method for isolation of PCPs. A study is now in progress to elucidate the other fractions obtained and the relationship between PCP concentration and light intensity in *Symbiodinium.*

Acknowledgements

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