Journal of Photoscience (2002), Vol. 9(2), pp. 74–77

A Novel Chlorophyll *d*-containing Organism: Discovery and its Significance

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Chlorophyll (Chl) *d* was assigned to an antenna pigment of red algae in 1943, but its presence and function in red algae have not been necessarily clear for a long time. In 1996, it was shown that Chl *d* functioned as a major antenna pigment in a peculiar oxygenic photosynthetic prokaryote, *Acaryochloris marina*, isolated as a symbiont of a colonial ascidian from coral reefs. This finding evoked the necessity for reexamination of the presence and function of Chl *d* in red algae. We found Chl *d* in methanol-extract from several marine red algae, and the relative content was high in one species, *Ahnfeltiopsis flabelliformis*. Absorption and fluorescence spectra, HPLC analysis, and NMR and mass spectroscopy characterized Chl *d* extracted from the red algal thalli, and those were essentially identical to those of Chl *d* isolated from *A. marina*. However, micro-spectrophotometric analysis suggested that Chl *d* was not an actual constituent of the red algae but came from epiphyte(s) attached to surface of red algal thalli.

Key words: *Acaryochloris marina*, antenna pigment, chlorophyll *d*, epiphytic algae, red algae

INTRODUCTION

Photosynthetic pigments such as chlorophylls and carotenoids are essential to harvest sunlight for photosynthetic energy conversion and are vital to survival of phototrophs in the underwater habitat. Distribution of pigments in photosynthetic organisms is the primary criterion for classification of the organisms. Chlorophyll (Chl) *a* is a common and indispensable pigment in all oxygenic phototrophs, from cyanobacteria to land plants, except for photosynthetic prokaryote *Prochlorococcus*, which contains divinyl-Chl *a*. Chl *b* is present in all green algae and land plants, and a few prokaryotes, *Prochloron* and *Prochlorothrix*. Chl *d* (Fig. 1) was discovered for the first time in marine red algae by Manning and Strain in 1943 [1]. However, Chl *d* was reported to be an artifact during the extraction [2], and its content was fluctuated [3]. It was not necessarily clear for a long time that Chl *d* was

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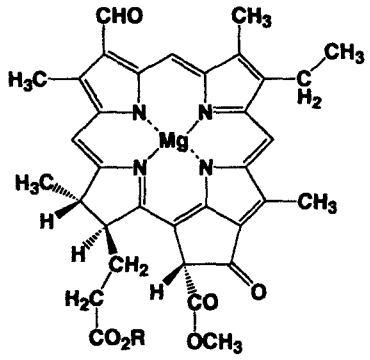

Fig. 1 Structure of chlorophyll *d*. (R: Phytol)

an actual component of red algae, even though the presence of Chl *d* in red algae had been reported by some other groups [4-7]. In 1996, it was shown by Miyashita et al. [8] that Chl *d* was functional in a symbiotic cyanobacterium, *Acaryochloris marina*, found in a colonial ascidian from coral reefs in Palau, the Caroline Islands. In this unusual organism, Chl *d* acts as a major antenna pigment in both photosystems (PSs) and the primary electron donor in PS I [9, 10]. This finding caused us to wonder whether Chl *d* is an actual component in red algae or not. Through our survey of marine algae around Awaji Island, Japan, we found that Chl *d* was present in the methanol extract from a red alga, *Ahnfeltiopsis flabelliformis*. However, it was suggested that Chl *d* was not present in red algal thalli themselves but came from epiphyte(s) on the red alga [11]. For further study on the generality of distribution of Chl *d*, we established the methodology to identify Chl *d* and surveyed other algal species.

MATERIALS AND METHODS

Samples collection: Red seaweeds were collected in the intertidal zone of the rocky seashore in Awaji Island, Japan. Algal thalli were washed many times with natural filtrated seawater, and used for following analyses.

Extraction of pigments: Thalli were wiped with paper to reduce water content on the surface. Methanol was added to samples and kept in the dark for 1 hr. After decanting the extracts, further analyses were performed. When necessary, the extracts were concentrated with an evaporator under the reduced pressure. The reservoir was not warmed during evaporation.

Spectroscopy: Absorption and fluorescence spectra were measured at room temperature with a Hitachi 557 spectrophotometer and a Hitachi F-4500 spectro-fluorometer, respectively.

HPLC analyses: Pigments were analyzed by HPLC using reverse phase column (Nova-Pack C_{18} column, Waters) and a photodiode array detector (MD-915, JASCO). Pigments were extracted with methanol, and extracts were evaporated, dissolved in acetone, and filtrated through cotton fiber that had been washed with acetone. Carrier solvent was dehydrated methanol, and a flow rate was 1 ml/min. Temperature was maintained at 22℃. Throughout the analyses, Chl *d* isolated from a cyanobacterium, *A. marina*, was used as the standard of Chl *d*.

RESULTS AND DISCUSSION

Absorption spectrum: In methanol, Chl *a* and *d* showed the absorption peak at 665 and 697 nm, respectively. The extinction coefficient of Chl *a* at 697 nm was less than 3% of the peak, and so Chl *d* content was able to be detected on the slope at approximately 700 nm. When it was present, a small shoulder was observed. By the absorption spectrum, Chl *d* content relative to 5% of Chl *a* was detectable.

Figure 2 showed absorption spectrum of one extract from species listed in Table 1. The Chl *d* content varied from 0% to 40% of Chl *a*.

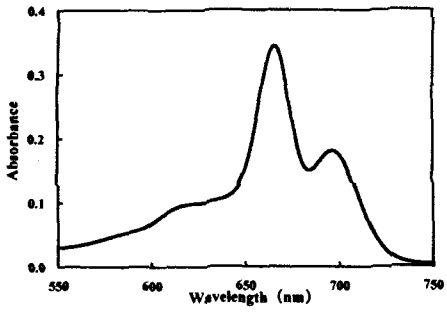

Fig. 2 Absorption spectrum of methanol extract from thalli of the red alga *Anfeltiopsis flabelliformis*

Fluorescence spectrum: When a Chl *d* content was low, Chl *d* was detectable by fluorescence spectrum. When an extract was excited at 435 nm, Chl *a* and Chl *d* were excited simultaneously, so that the peaks were observed at 669 and 708 nm, respectively. When excitation wavelength was changed to 465 nm, Chl *d* was preferentially excited. Under this condition, even a small amount of Chl *d* was detected. Figure 3 showed an example. In this case, the Chl *d* content relative to Chl *a* was less than 15%, but it was detected as a peak at approximately 708 nm. Either by absorption or fluorescence spectra, Chl *d* content was detectable even in a concentration of 5% of Chl *a*.

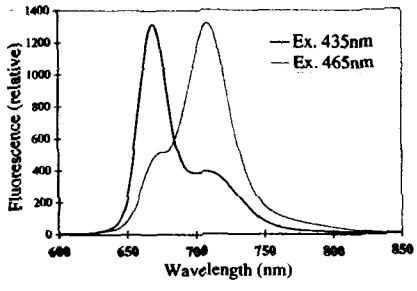

Fig. 3 Fluorescence spectra of methanol extract from *Callophyllis japonica*.

HPLC analysis: Figure 4 showed an elution pattern of Chl *a* and Chl *d* on a Nova-Pack C_{18} column with methanol as a carrier solvent. Chl *d* was eluted at the retention time of 6.25 to 6.37 min. and separated from Chl *a* whose retention time was 9.76 to 9.88 min. An authentic Chl *d* purified from *A. marina* was also analyzed; its retention time and absorption spectrum on photodiode array were just the

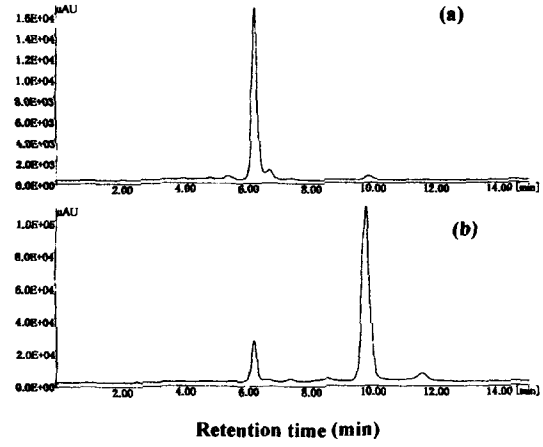

Fig. 4 HPLC chromatograms of Chl's extracted from (a) *Acaryochloris marina* and (b) *Anfeltiopsis flabelliformis*.

same as those of Chl *d* isolated from marine red algae, and both Chl *d* samples were identical in terms of chemical species.

Survey of algal species: We surveyed the presence of Chl *d* in species listed in Table 1 by the method described above. We found Chl *d* in 6 species out of 18 species, though variation of Chl *d* content was very large; relative content to Chl *a* was between zero and 0.4.

Optical properties of Chl *d* are very different from those of Chl *a*, and so the detection method is rather easily established. There are several degradation products of Chl *a*; all of them showed the absorption or fluorescence maximum at wavelengths shorter than that of Chl *d*, so that

Table 1 Survey of Chl *d* in red algae of Awaji Island

Species	Presence of Chl *d*
Ahnfeltiopsis flabelliformis	+++
Callophyllis japonica	++
Carpopeltis prolifera	++
Gelidium elegans	+
Pterocladiella tenuis	+
Grateloupia lanceolata	+
Grateloupia okamurae	-
Chondrus giganteus	-
Champia parvula	-
Prionitis ramosissima	-
Plocamium telfairiae	-
Hypnea flexicaulis	-
Lomentaria catenata	-
Gracilaria chorda	-
Gracilaria textorii	-
Amphiroa zonata	-
Porphyra yezoensis	-
Amphiroa zonata	-

it was rather easy to detect Chl *d*. An authentic Chl *d* is easily obtained from *A. marina*, and it is easy to establish the analytical method in any kind of solvents. This is very critical for survey of Chl *d* in a wide range of algal samples.

Several species of red algae contained Chl *d*. They were classified in some taxa, but not limited to one specific taxon. This indicated that distribution of Chl *d* was not limited to specific species. Therefore, if Chl *d* in these algae comes from epiphytes attached to the surface of the algal thalli as in the case of *A. flabelliformis*, specific interaction between surfaces of thalli and epiphytes will not occur. The epiphyte(s) seems cyanobacteria-like on the basis of fluorescence image and electron micrographs, even though it has not been identified yet. Two Chl *d*-containing species are very different in their habitats. *A. marina* was isolated from a colonial ascidian living in tropical coral reefs [8] and the novel organism reported in this paper was grown on the surface of red algae in the Temperate Zone. These extremely different and restricted habitats of the two Chl *d*-containing organisms seemed to be important to consider the phylogenic lineage of these organisms and the ecological and physiological significance of Chl *d*.

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