Chlorophyll *d* production in crushed algae in aqueous acetone

Shinya Akutsu^a, Hirohisa Komatsu^a, Daiki Fujinuma^a,
Hideaki Miyashita^b, Koji Iwamoto^c, Yoshihiro Shiraiwa^c, and Masami Kobayashi^{a*}

^aDivision of Materials Science, Faculty of Pure and Applied Science, University of Tsukuba, Japan;

^bGraduate School of Human and Environmental Studies, Kyoto University, Japan.

^cFaculty of Life and Environmental Sciences, University of Tsukuba, Japan;

(*Corresponding author. Tel: +81-298-53-6940; FAX: +81-298-53-4490; E-mail: masami@ims.tsukuba.ac.jp)

We for the first time report on the Chl *a* to Chl *d* conversion in crushed algae. *Synechocystis* sp. PCC6803, *Emiliania huxleyi*, *Gephyrocapsa oceanica*, *Isochrysis galbana*, *Helladosphaera cornifera*, *Pleurochrysis carterae*, *Chlorella vulgaris* and *Chlamydomonas acidophila*, incubated in aqueous acetone at 303 K for 2 days, where no Chl *a* was added externally to the system. The highest conversion of Chl *a* into Chl *d* was observed in *G. oceanica* (ca. 1.1%). The conversion yield was lower, when other algae were used: *S. sp.* PCC6803 (ca. 0.4%), *E. huxleyi* (ca. 0.3%), *C. vulgaris* (ca. 0.2%), *I. galbana* (ca. 0.1%), *H. cornifera* (ca. 0.1%), *P. carterae* (ca. 0.06%) and *C. acidophila* (ca. 0.04%). The well-known degradation reactions of Chl *a*, namely, pheophytinization (Chl *a* → Phe *a*) and epimerization (Chl *a* → Chl *a'*), were also observed. We should pay enough attention to the fact that Chl *d* was not detected at all in the initial algae examined here. It is interesting to note that Chl *d*, Chl *d'*, Chl *a'* and Phe *a* were also detected in processed algae food. *Chlorella* powder and dried laver. It is noteworthy that these artifacts function as key components in natural photosynthesis.

Introduction

In 1943, Chl *d* (Fig. 1) was first discovered in some species of red algae (Manning and Strain, 1943). The molecule structure was then identified to be 3-desvinyl-3-formyl-Chl *a* (Holt and Morley, 1959; Holt 1961). Since Chl *d* was not found in all red algae, Chl *d* had been hence thought to be an oxidative artifact of Chl *a* (Fig. 1).

A Chl *d*-dominated cyanobacterium, *Acarvochloris marina*, was accidentally discovered in 1993 (Miyashita *et al.*, 1996; Ohashi *et al.*, 2008). Chl *d* is expected to be oxidatively biosynthesized from Chl *a*, where oxidative cleavage of the C=C double bond of a vinyl group of Chl *a* at ring I (–CH=CH₂ → –CHO) is required, while the biosynthetic pathway of Chl *d* in *A. marina* has not yet been clarified. Chl *a* has many C=C bonds in the macrocycle (Fig. 1), and hence it is too difficult to oxidize only the C=C bond in the –CH=CH₂ moiety at ring I. Usually, both a special metal complex (OsO₄) and an oxidant (H₅IO₆) are needed for the selective oxidation (Mironov *et al.*, 2004).

Recently, we came across the formation of Chl *d* from Chl *a* with papain (EC 3.4.22.2) in several aqueous organic solvents (Kobayashi *et al.*, 2005; Koizumi *et al.*, 2005; Okada *et al.*, 2009; Ohashi *et*

Fig. 1 Molecular structures of chlorophylls, according to the IUPAC numbering system.

et al., 2010). The Chl *a* to Chl *d* conversion was not observed when Chl *a* was incubated with esterases

(esterase EC 3.1.1.1, cholesterol esterase EC 3.1.1.13, phosphatase EC 3.1.3.2) and other proteases (α -chymotrypsin EC 3.4.21.1, subtilisin carlsberg EC 3.4.21.14, ficin EC 3.4.22.3, bromelain EC 3.4.22.4) (Koizumi *et al.*, 2005; Okada *et al.*, 2009).

Quite recently, the same conversion was observed when Chl *a* was incubated with horseradish peroxidase (Fujita *et al.*, 2012). Further, we found that Chl *a* was nonenzymatically converted into Chl *d* in acetone/0.3% H₂O₂ (10/1, v/v) (Fujita *et al.*, 2012).

The Chl *a* into Chl *d* conversion was also observed when Chl *a* was incubated with several grated vegetables (Itoh *et al.*, 2011). In particular, Japanese radish (root) showed higher conversion than papain.

In order to clarify the conversion mechanisms *in vitro* and the birth of Chl *d* in nature, we incubated several crushed microalgae in acetone/H₂O (5/1, v/v) at 303 K for 48 h in the dark, and the Chl *a* into Chl *d* conversion was seen in all crushed algae examined, even no additional Chl *a* was added to the system.

Materials and Methods

Incubation of crushed algae in aqueous acetone

Eight species of microalgae, *Synechocystis* sp. PCC6803, *Emiliania huxleyi*, *Gephyrocapsa oceanica*, *Isochrysis galbana*, *Helladosphaera cornifera*, *Pleurochrysis carterae*, *Chlorella vulgaris* and *Chlamydomonas acidophila* were sonicated in acetone/water(5/1,v/v) for 30 s at 277 K, and then shaken gently at 303 K in the dark.

Pigment analysis

Samples were taken periodically and filtered by poly(tetrafluoroethylene) membrane filters. The filtrate was injected into a reversed-phase Senshupak PEGASIL-ODS HPLC column (4.6 mm ID x 250 mm) cooled to 277 K in an ice-water bath. The pigments were eluted isocratically with degassed ethanol/methanol/2-propanol/water(86/13/1/3,v/v/v/v) at a flow rate of 0.3 mL/min, and were monitored with a JASCO Multiwavelength MD-2015 detector (λ = 300 - 800 nm).

In the case of commercially available processed algae, *Chlorella* powder and dried laver, they were ground in a glass mortar for 1 min at 277 K. Pigments were extracted from the ground materials by sonication in an acetone/methanol (7/3, v/v) mixture for 2 min in the dark at room temperature. The extract was filtered and dried *in vacuo*. The thus-obtained solid material was immediately dissolved in 10 μ L of chloroform, and injected into

a silica HPLC column (YMC-pack SIL, 250 x 4.6 mm ID) cooled to 277 K in an ice-water bath. The pigments were eluted isocratically with degassed hexane/2-propanol/methanol (100/0.7/0.25, v/v/v) at a flow rate of 0.9 mL/min, and were monitored with a JASCO UV-970 detector (λ = 700 nm) and a JASCO Multiwavelength MD-915 detector (λ = 300 - 800 nm) in series.

Results and Discussion

Chl *d* produced from Chl *a* in crushed algae

Typical HPLC traces for crushed algae incubated in aqueous acetone at 303 K for 2 days are shown in Fig. 2. The conversion of Chl *a* into Chl *d* was observed in all algae examined here (Figs. 2A'-H'), even though no additional Chl *a* was added to the system. The results indicate that Chl *a* present originally in these algae was directly converted to Chl *d* by the reaction with something present in the algae. We should note that Chl *d* was not detected at all in the initial algae examined here (Figs. 2A-H).

The highest conversion of Chl *a* into Chl *d* was observed in *G. oceanica* (ca. 1.1%, Fig. 2A'). *G. oceanica* almost equals papain (ca. 2%, Kobayashi *et al.*, 2005; Koizumi *et al.*, 2005; Okada *et al.*, 2009; Ohashi *et al.*, 2010) in efficiency.

The conversion yields were lower in other crushed algae: *S. sp.* PCC6803 (ca. 0.4%, Fig. 2F'), *E. huxleyi* (ca. 0.3%, Fig. 2B'), *C. vulgaris* (ca. 0.2%, Fig. 2G), *I. galbana* (ca. 0.1%, Fig. 2C'), *H. cornifera* (ca. 0.1%, Fig. 2D'), *P. carterae* (ca. 0.06%, Fig. 2E') and *C. acidophila* (ca. 0.04%, Fig. 2H'), respectively.

Phe *a* and Chl *a'* produced as artifacts of Chl *a* in crushed algae

In our present study, familiar degradation reactions of Chl *a*, namely, pheophytinization (Chl *a* $\rightarrow$ Phe *a*) and epimerization (Chl *a* $\rightarrow$ Chl *a'*), were also observed (Figs. 2A'-H'). In the case of *C. vulgaris* and *C. acidophila*, degradation products of Chl *b*, Phe *b* and Chl *b'*, were also produced, although the corresponding peaks are not seen in Figs. 2G' and H', because the amount was very small and the detection wavelength was 700 nm.

Chl *d* detected in processed algae

In our present studies, an organic solvent, acetone, was used, but the natural world is not abundant in acetone. So we next examined the presence of Chl *d* in *Chlorella* powder and dried laver, because organic solvents including acetone are not

Fig. 2 Reversed-phase HPLC elution profiles for crushed algae incubated for 2 days in acetone water (10:1, v/v) in the dark at 303 K. Incubation times are 0 h (A ~ H) and 48 h (A' ~ H'), respectively. Detection wavelength is 700 nm

used to make such commercially available processed alga food.

Typical HPLC traces for *Chlorella* powder and dried laver are shown in Fig. 3. In *Chlorella* powder, a small amount of Chl *d* and Phe *d* was observed (Fig. 3A). By contrast, in dried laver a considerable amount of Chl *d* and Chl *d'* were clearly detected (Fig. 3B). Pheophytinization of Chl *a* seems to have proceeded in *Chlorella* powder processing (Fig. 3A). Chlorophyll *d*, Chl *d'*, Phe *a'* and Phe *d* seen in Fig. 3 are the artifacts of Chl *a* produced through processing. It is not clear whether Phe *d* seen in *Chlorella* powder had been produced from Chl *a* followed by pheophytinization or directly from Phe *a* produced by pheophytinization of Chl *a*. Anyway, it is well worth noting that Chl *d*, Chl *d'* and Phe *d* were produced from Chl *a* in the absence of acetone, meaning that organic solvents are not needed to convert Chl *a* into Chl *d*-type pigments.

The present findings exhibit that the Chl *a* to Chl *d* conversion is not a rare event in nature, and will provide new insight into the unsolved question as

Fig. 3 Normal-phase HPLC elution profiles for (A) *Chlorella* powder and (B) dried laver. $\lambda = 700$ nm.

to the birth of Chl *d* in photosynthesis. We want to emphasize that Chl *a* artifacts, Phe *a*, Chl *a'*, Chl *d* and Chl *d'*, function as key components in natural photosynthesis (Fig. 4). (Akiyama *et al.*, 2001; Kobayashi *et al.*, 2005; Ohashi *et al.*, 2008, 2010).

Fig. 4 Artifacts produced from Chl *a* and their function in natural photosynthesis.

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