Lactonization of Chl *a* catalyzed by grated pineapple in aqueous acetone

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Conversion of Chl *a* into 15[1]-OH-lactone Chl *a* was observed when Chl *a* was incubated with grated pineapple (skin) in aqueous acetone at 303 K in the dark, while the formation of Chl *d* was not detected.

Introduction

Chlorophyll (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=CH_2 → -CHO ; Fig. 1) is required, while the biosynthetic pathway of Chl *d* in *Acaryochloris marina* (Miyashita *et al.* 1996; Ohashi *et al.* 2008) has not yet been clarified. We, however, serendipitously came across the formation of Chl *d* from Chl *a* with papain (EC 3.4.22.2) in several aqueous organic solvents at room temperature in the dark (Kobayashi *et al.* 2005, Koizumi *et al.* 2005, Okada *et al.* 2009, Ohashi *et al.* 2010); papain is a proteolytic and thiol protease with a relatively low selectivity which is widely used in food and medical fields. The same conversion was also observed when Chl *a* was incubated with several grated vegetables (Itoh *et al.* 2011).

In contrast, the Chl *a* →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 and phosphatase EC 3.1.3.2) and other proteases (α-chymotrypsin EC 3.4.21.1, subtilisin carlsberg EC 3.4.21.14 and ficin EC 3.4.22.3) (Koizumi *et al.* 2005, Okada *et al.* 2009).

Bromelain (EC 3.4.22.4) is also a proteolytic and thiol protease present in a familiar fruit, pineapple. In order to clarify the conversion mechanism *in vitro* and the origin of Chl *a* → Chl *d* conversion in nature, we incubated Chl *a* with grated pineapple in acetone/H_2O (10/1, *v/v*) at 303 K for 97 hours in the dark. The expected conversion was not observed, but a new peak was detected by HPLC analysis, and the pigment was found to be 15[1]-OH lactone Chl *a*.

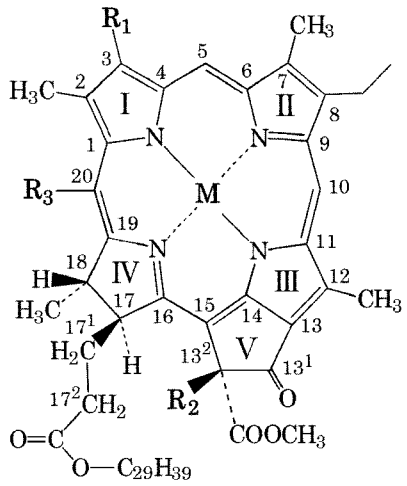
Materials and Methods

Pigment preparation

Chl *a* and Chl *d* were extracted from spinach (*Spinacia oleracea* L.) and from *Acaryochloris marina* MBIC11017, respectively, and then they were purified by normal-phase HPLC as described previously (Akiyama *et al.* 2001).

Reaction of Chl *a* with grated pineapple.

A commercially available fresh pineapple was cut to 1 g, and was then grated in a glass mortar. The ground material was transferred into a small glass beaker, to which 0.5 mL of water was added. The mixture was then added to 5 mL of acetone containing Chl *a* (ca. 2 x 10^{-5} M). The reaction mixture was sonicated for 20 s at 277 K, and then shaken gently for 97 hours at 303 K in the dark.



	M	R_1	R_2	R_3
Chl *a*	Mg	CH=CH_2	H	H
Phe *a*	2H	CH=CH_2	H	H
Cl-Chl *a*	Mg	CH=CH_2	H	Cl
Chl RC I	Mg	CH=CH_2	OH	Cl
13^2-OH-Chl *a*	Mg	CH=CH_2	OH	H
Chl *d*	Mg	CHO	H	H

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

Pigment analysis.

Samples of the reaction mixture 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 isocratically eluted with degassed ethanol/methanol/2-propanol (86/13/1, *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).

Results and Discussion

HPLC analysis

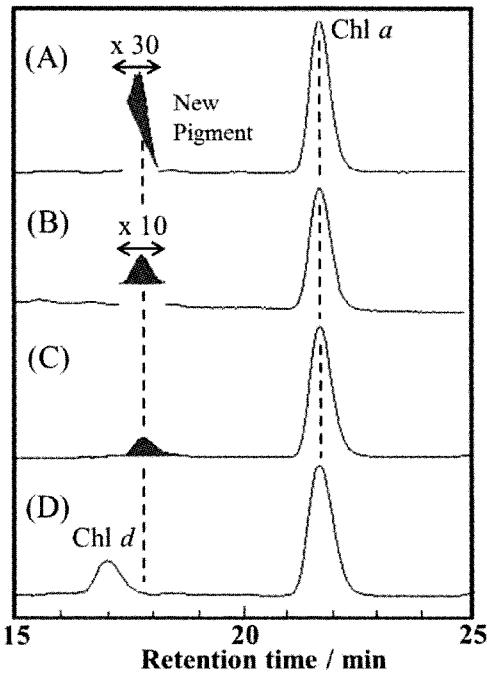


Fig. 2 Reversed-phase HPLC elution profiles for the reaction mixtures of Chl *a* incubated with grated (A) core, (B) flesh, (C) skin of a pineapple in acetone/water(10/1, *v/v*) at 303 K for 97 hours in the dark and (D) authentic standards. Detection wavelength is 656 nm.

Typical HPLC traces for the reaction mixture of Chl *a* incubated in an acetone/water (10/1, *v/v*) solution with grated pineapple (skin, flesh or core) in the dark at 303 K for two days are shown Fig. 2. Expected conversion of Chl *a* into Chl *d* was not observed at all, but a small new peak at the retention time of 18 min was detected. It is noteworthy that the conversion was part-dependent. As seen in Fig. 2, the production yield of new pigment was highest when the skin of pineapple was used (Fig. 2C), and the lowest when the core was used (Fig. 2A).

Absorption spectra

The absorption spectrum of the new chlorophyll in an HPLC eluent is shown in Fig. 3, and its absorption characteristics are compared with those of Chl *a* and Chl *d*. The new pigment displayed an extremely characteristic spectrum (Soret max. 416 nm, Q_Y max. 656 nm, Soret/Q_Y intensity ratio = 1.67), which are completely different from those of Chls *a* and *d*. The Soret- and Q_Y-bands of the pigment were significantly blue-shifted by 12 nm, as compared with those of Chl *a* (428 and 668 nm), and the splitting of the Soret band clearly seen in Chl *d* was not observed in this pigment.

The new pigment exhibited large Soret/Q_Y intensity ratio (1.67) in Fig. 3, and such the blue-shift of Soret band and the features are often seen in Mg-free chlorophylls, pheophytins (Phes). For example, Phe *a* shows characteristic two absorption bands, Q_X, in the region from 480 to 560 nm, whereas the Q_X-bands peak was not remarkable in this new pigment (Fig. 4), suggesting that the new pigment is not pheophytins but chlorophylls possessing Mg as

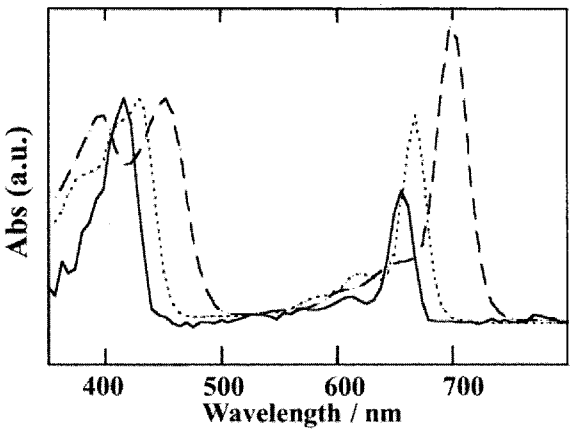


Fig. 3 Absorption spectra in an HPLC eluent of authentic Chl *a* (···), Chl *d* (– – –), and a new pigment (—) at the retention time of 18 min in Fig. 2. The Soret peaks are normalized to a common height.

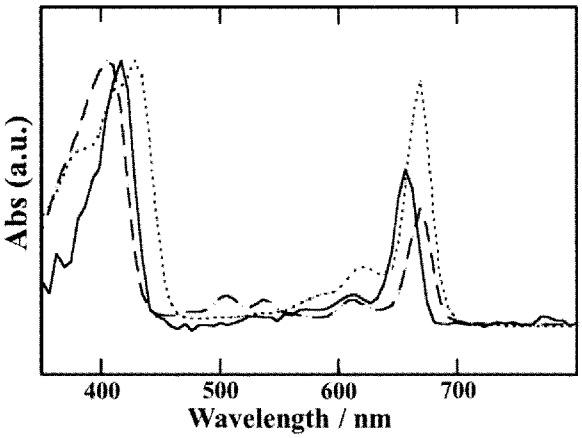


Fig. 4 Absorption spectra in an HPLC eluent of authentic Chl *a* (···) and Phe *a* (– – –), and a new pigment (—).

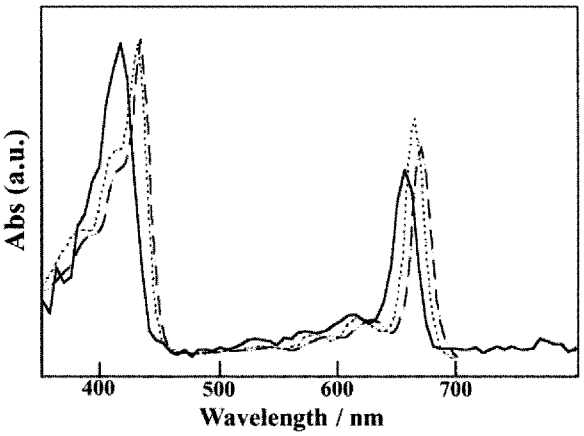


Fig. 5 Absorption spectra in 20-Cl-Chl a (– – –) and authentic Chl *a* (···) in benzene (Kobayashi *et al.* 1988), and a new pigment (—) in an HPLC eluent.

the central metal.

Chl *a* is known to be chlorinated yielding 20-Cl-Chl *a* (Fig. 1) in the wet medium of crushed plant tissue (Kobayashi *et al.* 1988, Senge and Senger 1988, 1989). Compared to Chl *a*, the absorption peaks of 20-Cl-Chl *a* are slightly red-shifted and the blue/red absorption ratio is a little larger in new pigment (Fig. 5). A significant change seen in the visible absorption spectrum in going from Chl *a* to 20-Cl-Chl *a* is rationalized by invoking the (steric)

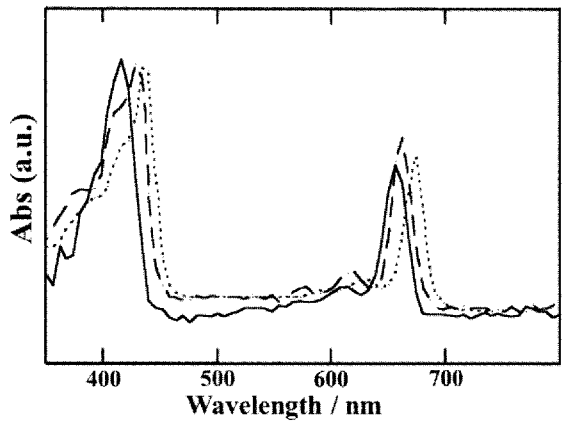


Fig. 6 Absorption spectra of Chl RC I (···) in acetone (Dörnemann and Senger, 1981), 13^2-OH-Chl *a* (— — —) in 1.5% (*v*/*v*) 2-PrOH in hexane (Kuronen *et al.* 1993) and a new pigment (—) in an HPLC eluent.

perturbation on the π -conjugated system of the chlorin macrocycle (Kobayashi *et al.* 1988). In the spectrum of new pigment, the corresponding two peaks were blue-shifted, and the blue/red ratio is significantly larger than that of 20-Cl-Chl *a*. These spectroscopic features of Cl-Chl *a* are far from those of new pigment. In addition, the retention time of 20-Cl-Chl *a* was reported to be shorter than that of Chl *a* on the normal-phase HPLC (Kobayashi *et al.* 1981), indicating 20-Cl-Chl *a* should have the longer retention time than Chl *a* on the reversed-phase HPLC, while the new pigment showed a shorter retention time than Chl *a* (Fig. 2). Hence there remain at least three possibilities as to the nature of new pigment; 13^2-OH-20-Cl Chl *a*, 13^2-OH-Chl *a* and 15^1-OH-lactone Chl *a*.

A novel Chl *a* derivative, 13^2-hydroxy-20-chloro-Chl *a* (13^2-OH-20-Cl-Chl *a*, Fig. 1), had been thought to be a building block of the photosystem (PS) I reaction center (P700), and called Chl RC I (Fig. 1) (Dörnemann and Senger 1981, 1986, Katoh *et al.* 1985, Senger *et al.* 1987). Unhydroxylated Chl *a*, namely 20-Cl-Chl *a*, is oxidatively converted into Chl RCI during its handling under aerobic conditions (Kobayashi *et al.* 1988). Compared to Chl *a* (428 and 668 nm), the absorption peaks of Chl RCI (433 and 672 nm) are slightly red-shifted and the blue/red absorption ratio is a little larger in new pigment (Fig. 6). The spectral features of Chl RCI in Fig. 6 were essentially the same as those of 20-Cl-Chl *a* (Fig. 5) as described by Senger and collaborators (Dörnemann and Senger 1981, 1986, Katoh *et al.* 1985, Senger *et al.* 1987); the site of 13^2-OH is out of theπ-conjugated system, and hence hydroxylation on carbon 13^2 would exert little effect on these spectroscopic properties (Kobayashi *et al.* 1988). As expected, 13^2-hydroxy-Chl *a* (13^2-OH-Chl *a*, Fig. 1) showed almost the same spectrum as that of Chl *a* (Fig. 6). Both 13^2-OH-20-Cl-Chl *a* and 13^2-OH-Chl *a* are expected to elute before Chl *a*, alike a new pigment, on the reversed-phase HPLC, and indeed 13^2-OH-Chl *a* was reported to elute before Chl *a* (Roca *et al.* 2007), while their absorption spectral characteristics are completely different from those of new pigment.

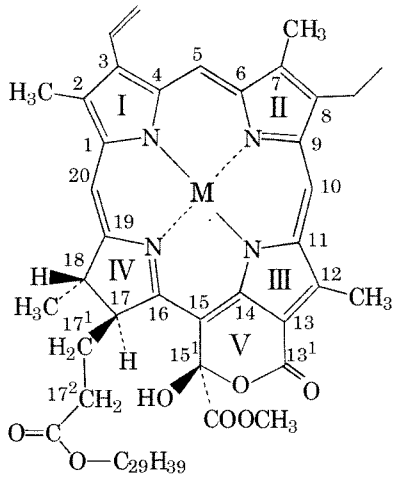


Fig. 7 Molecular structures and carbon numbering of 15^1-OH-lactone Chl *a*.

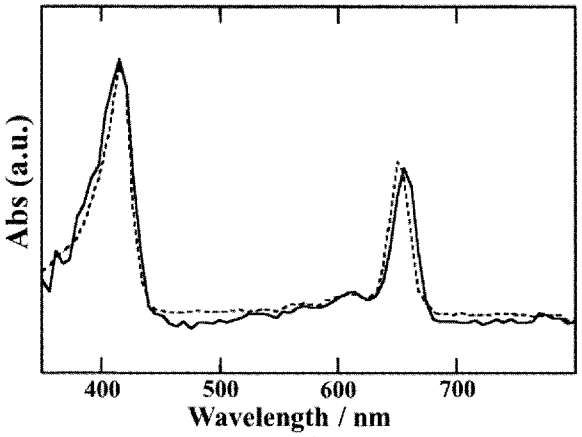


Fig. 8 Absorption spectra of 15^1-OH-lactone Chl *a* (···) in 1.5% (*v*/*v*) 2-PrOH in hexane (Kuronen *et al.*, 1993) and a new pigment (—) in an HPLC eluent.

One of the further oxidized Chl *a* derivatives is 15^1-OH-lactone Chl *a* (Fig. 7). As seen in Fig. 8, UV-VIS absorption spectrum of 15^1-OH-lactone Chl *a* is quite similar to that of new pigment. The retention time of 15^1-OH-lactone Chl *a* was reported to be shorter than that of Chl *a* (Roca *et al.* 2007), alike new pigment detected in Fig. 2. Consequently, we find as the most likely candidates for new pigment 15^1-OH-lactone Chl *a* on the basis of HPLC elution and UV-VIS absorption spectral characteristics. In the proposed mechanism for the 15^1-OH-lactone Chl *a* production from Chl *a* (Hynninen 1991), an hydroxyl radical reacts with the Chl *a* radical produced by molecular singlet oxygen in a termination step to produce the OH allomer, and another hydroxide ion reacts with Chl *a* hydroperoxide to form the OH-lactone allomer as the final product, where water is the source of the hydroxyl species (Wooley *et al.* 1998). Cleavage of ring V of Chl *a* during the formation of 15^1-OH-lactone Chl *a* leads to the characteristic changes in UV-VIS absorption spectrum as seen in Fig. 8.

Conclusion

In this study, the conversion of Chl *a* into Chl *d* was not performed. However, novel conversion of Chl *a* into 15^1-OH-lactone Chl *a* was observed in the presence of crushed pineapple in aqueous acetone. Allomerization including the conversion of Chl *a* into 15^1-OH-lactone Chl *a* has been implicated as an early stage reaction in the breakdown of chlorophylls in the natural environment (Hendry *et al.* 1987, Brown *et al.* 1991), which is important in food science where there is a need to control the stability of chlorophyll in foods subjected to modern processing methods (Wooly *et al.* 1998). Currently under way are studies aimed at revealing the mechanisms of Chl *a* lactonization under the present conditions.

Acknowledgements

This work was supported in part by Special Project of Organization for the Support and Development of Strategic Initiatives (Green Innovation) (Univ. Tsukuba) to M.K.

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