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## **HPLC determination of chlorophyll *a'* and phylloquinone in photosystem I of higher plants and cyanobacteria**

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### **Introduction**

Chlorophyll (Chl) *a'* is the C13<sup>2</sup>-epimer of Chl *a*, and was detected by ourselves in photosystem (PS) I of higher plants and cyanobacteria by HPLC analysis of pigment extracts (Kobayashi (1988), Nakamura (1998)). The existence of one Chl *a'* molecule in P700, the primary electron donor of PS I, has recently been confirmed by X-ray crystallographic analysis of *Synechococcus elongatus* PS I at 2.5 Å resolution (Jordan (2001)).

Chl *a'* can be determined solely by HPLC analysis of pigment extracts, since its absorption and fluorescence spectra are almost the same as those of Chl *a*. However, in organic solvents, Chl *a* is readily epimerized to Chl *a'*, and may bring ambiguities into the results of HPLC analysis. This has long hampered the development of analytical conditions which can precisely determine Chl *a'*. Development of analytical conditions for precise determination of Chl *a'* is a prerequisite for examining universal existence of Chl *a'* within PS I of oxygenic photosynthetic organisms, and further studies of functional roles of Chl *a'* in P700.

To determine the stoichiometry of Chl *a'* within PS I, the PS I concentration must be quantified precisely. Though PS I is usually determined from oxidized minus reduced spectrum of P700, the differential extinction coefficient of P700 is known to be affected by detergent treatments during sample preparation (Sonoike(1990)). Thus, determination of other functional ingredient of PS I, such as phylloquinone (PhQ; two molecules per PS I), the secondary electron acceptors of PS I, in combination with P700 would yield more precise PS I concentration.

In this work, we developed HPLC conditions which can compare Chl *a'*, Chl *a* and PhQ on a single HPLC trace with minimal alteration of Chls, and applied the conditions for pigment composition analysis of PS I isolated from *S. elongatus*, *Synechocystis* PCC 6803 and spinach. The results show that one molecule of Chl *a'* is present in PS I of not only *S. elongatus*, but also of *S. PCC 6803* and spinach, indicating universal existence of one Chl *a'* in PS I of oxygenic photoautotrophs.

### **Materials and Methods**

*Synechococcus elongatus* and *Synechocystis* PCC6803 were provided by Prof. M. Ikeuchi (Univ. Tokyo), and grown in BG11 medium by aerating 5% CO<sub>2</sub> in air at 54 °C and 33 °C, respectively. Thylakoid membranes and PS I trimers of cyanobacteria were isolated according to Kruij (1997) with modifications. PS I-60 was prepared by Triton X (TX)-100 treatment and hydroxyapatite chromatography. Thylakoid membranes and PS I of spinach were prepared according to Wynn (1988) with modifications.

Conditions for pigment extraction and HPLC analysis were detailed in Nakamura (2001). P700 was determined by light-induced absorbance change at 808 nm for cyanobacteria and at 820 nm for spinach by using differential extinction coefficients of  $7.8 \text{ mM}^{-1}\text{cm}^{-1}$  at 808 nm and  $6.5 \text{ mM}^{-1}\text{cm}^{-1}$  at 820 nm (Mathis (1981)). P700 of SDS-treated PS I was determined from the maximum of  $Q_Y$ -bleaching band of chemically oxidized minus reduced spectrum by using the extinction coefficients of  $84 \text{ mM}^{-1}\text{cm}^{-1}$  (Sonoike (1990)). Chl *a* concentration was determined after extraction with acetone/water = 4/1 according to Porra (1989).

## Results and Discussion

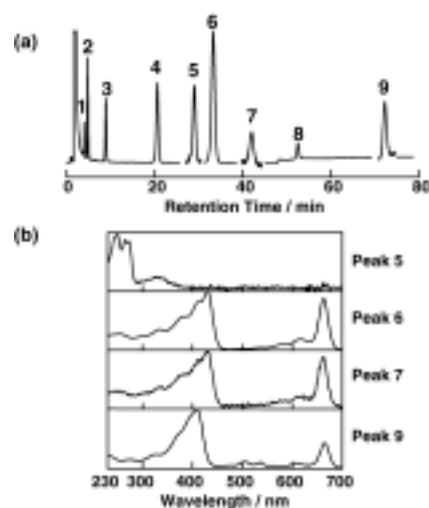
**Development of analytical conditions** We developed reversed-phase HPLC conditions which can resolve Chl *a*, Chl *a'*, PhQ, Pheo *a*, Chl *b*, and carotenoids (Fig. 1). Absorption spectra of Chl *a'* and PhQ peaks showed that these peaks were free from contaminations from other pigments. Though more than several percents of Chl *a* allomers were observed in many previous reversed-phase HPLC traces, allomerization of Chl *a* during HPLC was suppressed to very low levels (less than 0.1% of Chl *a* in highly purified Chl *a* sample) in our HPLC conditions. To examine whether the conditions developed here are sufficiently inert for determination of Chl *a'*, pigment composition of LHC II, which would contain neither Chl *a'* or Pheo *a*, was determined by the conditions developed here (Fig. 2). The Chl *a'*/Chl *a* and Pheo *a*/Chl *a* ratios thus determined were very low as expected (0.03 to 0.04 % and 0.04 to 0.05%, respectively), showing the sufficient inertness of the conditions developed here for precise determination of Chl *a'*.

**Pigment composition analysis of cyanobacterial PS I** Thylakoid membranes, PS I trimers and

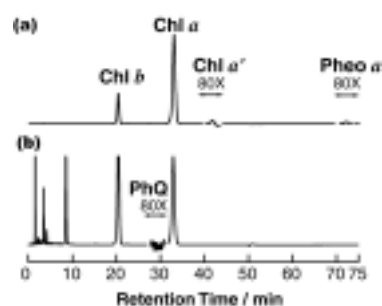
TX-100-treated PS I of *S. elongatus* and *S. PCC 6803* were subjected to pigment composition analysis by HPLC. Fig. 3 shows HPLC traces of pigments extracted from the preparations of *S. elongatus*. The peak area of Chl *a'* was not largely increased from thylakoid membranes to

PS I trimer, because the amount of PS II and the number of Chl *a* associated with it were small relative to that of PS I. The peak area of Chl *a'*

increased from PS I trimer to PS I-60, due to removal of antenna Chl *a* molecules by TX-100 treatment. The Chl *a*/Chl *a'* ratio of PS I trimer was  $91 \pm 1$  ( $n = 5$ ), in good agreement with the result of X-ray crystallography, Chl *a*/Chl *a'* ratio = 96 (Jordan (2001)). This demonstrates the accuracy of analytical conditions developed here. The peak area of PhQ also increased from thylakoid membranes to PS I-60 by keeping the Chl *a'*/PhQ ratio at 0.5 (Fig. 3). The Chl *a'*/P700 ratios were about 1.1 for all the samples. The results of HPLC



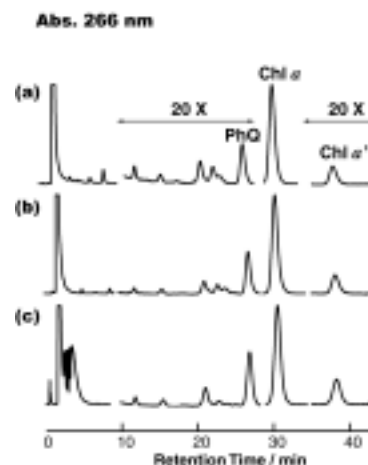
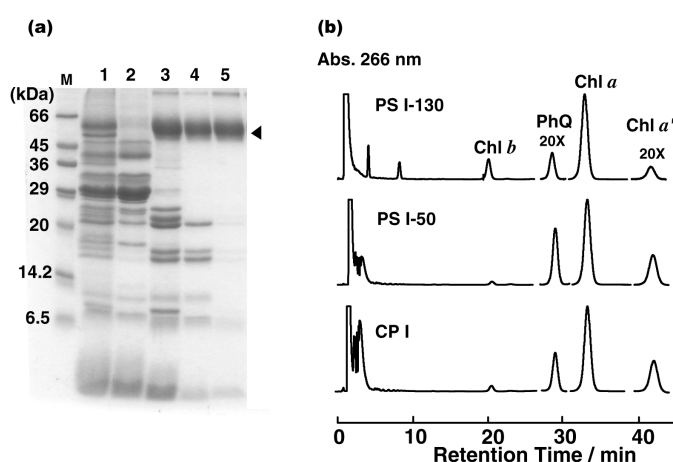
**Fig. 1.** (a) HPLC trace of pigments extracted from spinach thylakoid membranes detected at 266 nm. Peak 1-3, xanthophylls; peak 4, Chl *b*; peak 5, PhQ; peak 6, Chl *a*; peak 7, Chl *a'*; peak 8,  $\beta$ -carotene; peak 9, Pheo *a*. (b) absorption spectra of peaks 5, 6, 7 and 9.



**Fig. 2.** HPLC traces of pigments extracted from LHC II. (a) detected at 660 nm, (b) detected at 266 nm.

analysis and P700 determination established the 1:2:1 stoichiometry between P700, PhQ and Chl *a*' in PS I of *S. elongatus* (Fig. 5), in line with the X-ray crystallographic analysis.

In *S. PCC6803*, though extra PhQ molecules were observed in thylakoid membranes, the stoichiometry between P700, PhQ and Chl *a*' in purified PS I was almost the same as in *S. elongatus* (Fig. 5). The 1:1 stoichiometry between Chl *a*' and P700 in mesophilic cyanobacterium *S. PCC6803* shows that the existence of one Chl *a*' molecule in PS I is not a special case in thermophilic *S. elongatus*.

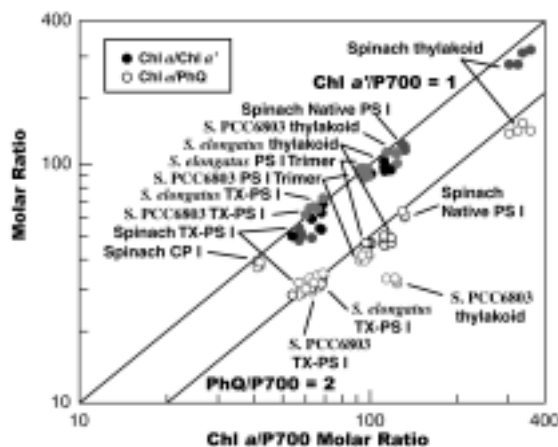


**Fig. 3.** HPLC traces of pigments extracted from (a) thylakoid membranes, (b) PS I Trimer and (c) PS I-60 of *S. elongatus*. The peak areas of Chl *a* are arbitrarily scaled to a common intensity. Slight difference of the retention times with Fig. 1. is the effect of column temperature.

**Fig. 4** (a) SDS PAGE profile of thylakoid membranes (1), PS II membranes (2), Native PS I (3), PS I-50 (4), CP I (5), molecular weight marker (M). Polypeptides were separated with 14% polyacrylamide gel containing 4 M urea. (b) HPLC traces of pigments extracted from Native PS I, PS I-50 and CP I detected at 266 nm. The peak areas of Chl *a* are arbitrarily scaled to a common intensity.

**Pigment composition analysis of spinach PS I** To examine the stoichiometry and location of Chl *a*' in higher plant PS I, PS I complexes with different subunit compositions were prepared from spinach, and their pigment compositions were examined by HPLC. Native PS I (PS I-130), containing PS I and LHC I, was almost free from contamination of PS II and Cyt *b<sub>6</sub>/f* (Fig. 4(a)). PS I-50 prepared by TX-100 treatment lacked LHC I and several peripheral subunits, and CP I prepared by SDS treatment contained only PsaA/B proteins (Fig. 4(a)). The peak areas of Chl *a*' and PhQ increased from PS I-130 to PS I-50, while the peak area of Chl *b* decreased. The Chl *a*'/PhQ ratio of about 0.5 remained the same after removing LHC I, showing the absence of Chl *a*' in LHC I (Fig. 4(b)). In CP I complex, the peak area of PhQ decreased from PS I-50 to CP I, and the peak area of Chl *a*' slightly increased, yielding the Chl *a*'/PhQ ratio of 1 (Fig. 4(b)). The Chl *a*'/PhQ ratio of 1 is due to detachment of one PhQ molecule by harsh SDS treatment; the PhQ/P700 ratio also reduced to 1. The stoichiometric comparison between the Chl *a*/Chl *a*' and the Chl *a*/P700 ratios showed the Chl *a*'/P700 ratio of about 1 for Native PS I, PS I-50 and CP I. The results of HPLC and P700 determination confirm the presence of one Chl *a*' in PsaA/B complex of spinach.

To summarize, we developed analytical conditions for precise determination of Chl *a'*. Application of the technique to pigment analysis of LHC II and PS I trimer of *S. elongatus* showed that the analytical conditions developed here can determine Chl *a'* precisely with minimal alteration of Chls. Pigment analysis extended to *S. PCC6803* and spinach indicate that one molecule of Chl *a'* universally exists in the core part of PS I as the constituent of P700.



**Fig. 5.** Dependence of Chl *a*/Chl *a'* and Chl *a*/PhQ ratios on the Chl *a*/P700 ratio in *S. elongatus*, *S. PCC 6803* and spinach PS I.

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