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Separation of chlorophyll c_1 and c_2 by reversed-phase high-performance liquid chromatography

Koichi Saitoh*, Izumi Awaka and Nobuo Suzuki

Department of Chemistry, Faculty of Science, Tohoku University, Sendai 980 (Japan)

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ABSTRACT

The separation of chlorophyll c_1 and c_2 was performed using an octadecyl-bonded polymer column with an aqueous methanolic mobile phase, *e.g.* a 90:10 (v/v) mixture of methanol and pH 3 buffer. The retention of both types of chlorophyll *c* increases with decreasing methanol content and also with decreasing pH (particularly lower than about pH 6) of the aqueous component of the mobile phase. Chlorophyll c_1 is eluted from the column in preference to chlorophyll c_2. UV–visible spectral data were obtained for the chlorophylls.

INTRODUCTION

Chlorophylls are the photosynthetic pigments contained in certain plant tissues. Every plant contains chlorophyll *a* (CHL-*a*) and, in addition, chlorophyll *b* (CHL-*b*) is contained in green plants and chlorophyll *c* (CHL-*c*) in brown algae. CHL-*c* occurs in two different forms [1,2], chlorophyll c_1 (CHL-c_1) and chlorophyll c_2 (CHL-c_2), which are magnesium complexes of tetra- and hexahydropheoporphyrin a_5 monomethyl esters, respectively (Fig. 1) [3].

The determination of chlorophylls and their degradation products in natural samples from the sea, lakes, rivers and other sources gives valuable information about the biological activity in different environments. In oceanography, the determination of particulate chlorophylls is an important measurement to estimate biomass. Traditional and popular analytical methods for chlorophylls are based on spectrophotometry or fluorimetry. Neither of these methods can cope with the difference in form of CHL-*c*, results being obtained only for the total of CHL-c_1 and CHL-c_2.

High-performance liquid chromatography (HPLC) is a promising technique for chlorophyll determination [4–6]. The separation of CHL-*a*

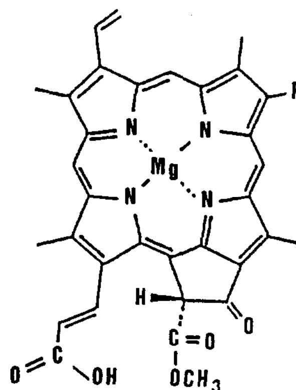

Fig. 1. Structure of CHL-c_1 (R = ethyl) and -c_2 (R = vinyl).

* Corresponding author.

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and CHL-*b* is possible by reversed-phase HPLC [7]. However, no clear evidence has been reported on the feasibility of HPLC for the resolution of CHL-*c* into its different forms, except for particular instances of thin-layer chromatography [1,2] and column liquid chromatography [8] developed by Jeffrey using a specially prepared polyethylene power as an adsorbent.

In our previous studies on the HPLC of chlorophylls and related compounds, the retention characteristics of CHL-*a* and CHL-*b* [7] and also various metal complexes of pheophorbide-*a* and -*b* [9] were investigated. In preference to the extension of such studies to CHL-*c*s and their derivatives, it was urgently required to develop a reliable HPLC method for separation of CHL-c_1 and -c_2. This paper describes the successful resolution of CHL-*c* into the c_1 and c_2 forms by reversed-phase HPLC with a commercially available octadecyl-bonded vinyl alcohol copolymer gel (polymer) column.

EXPERIMENTAL

HPLC

A Twincle liquid chromatograph (JASCO, Tokyo, Japan) was equipped with a UV–visible photodiode-array detector (Shimadzu Model SPD-M6A) for real-time recording of the spectra of the eluate. An octadecyl-bonded silica gel (ODS) column (particle diameter 5 μm; 15 cm × 4.6 mm I.D.) (Inertsil ODS-2; GL Science, Tokyo, Japan) and an octadecyl-bonded polymer (ODP) column (particle size 5 μm; 15 cm × 6.0 mm I.D.) (Asahipak ODP-50; Asahi Kasei, Kawasaki, Japan) were tested. The mobile phase was a 90:10 (v/v) mixture of methanol and pH 3 phosphate buffer (containing 1.24 m*M* phosphoric acid and 8.76 m*M* sodium dihydrogenphosphate). The flow-rates were 1.0 and 1.7 ml min^{-1} with the ODS and the ODP column, respectively. All chromatographic experiments were carried out in a thermostated room at 25°C.

Mass spectrometry

A JEOL Model JMS-HX2100 mass spectrometer was equipped with a fast atom bombardment (FAB) ionization system (JEOL, Tokyo, Japan). Nitrobenzyl alcohol was used as a matrix.

Preparation of CHL-c

Algal pigments were extracted with acetone from the fresh brown alga *Undaria pinnatifida* obtained at Onagawa Bay, Miyagi, Japan. The acetone extract was agitated with both light petroleum (b.p. 30–60°C) and a saturated aqueous solution of NaCl in order to remove the majority of co-extracted pigments, such as CHL-*a* and carotenoids. The resulting aqueous acetone phase was shaken with ethyl acetate. The ethyl acetate solution was then passed through a cellulose column, followed by concentration under a stream of nitrogen. The pigments in the concentrated ethyl acetate solution were resolved by preparative liquid chromatography using an octadecyl-bonded silica gel column (36 cm × 2.4 cm I.D.) (Fuji Gel RQ-2; Fuji, Tokyo, Japan) with methanol–water (90:10, v/v). The desired pigment passed easily through the column, whereas co-existing pigments, such as CHL-*a* and pheophytin *a* (demetallated form of CHL-*a*), were strongly retained.

RESULTS

The UV–visible spectral characteristics, such as the shape and the absorption maximum wavelengths, of the pigment separated from the alga agreed closely with that of CHL-*c* [1,2,10]. In the mass spectrum of the pigment, significant signals were detected around m/z 608–610, which matched the molecular ion peaks of CHL-c_1 and -c_2 (the molecular masses of CHL-c_1 and -c_2 are 610 and 608, respectively).

The feasibility of HPLC and high-performance TLC (HPTLC) was examined for resolving this pigment into CHL-c_1 and -c_2 by using various stationary phase substances, such as cellulose, silica gel and chemically bonded silica gels possessing cyano, amino, octyl and octadecyl groups, in the normal- or reversed-phase separation mode. No successful resolution was obtained, but all results implied that the pigment was a pure substance. The pigment, after being treated with 0.1 *M* hydrochloric acid for demetallation, gave two resolved peaks in the chromatogram obtained with an ODS column, as shown in Fig. 2. The early- and late-eluted peaks were assigned to pheoporphyric c_1 (PHEO-c_1)

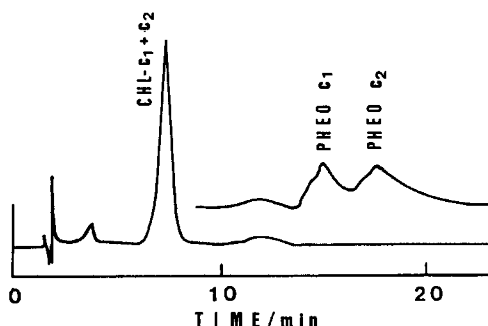

Fig. 2. Resolution of CHL-*c* and PHEO-*c* on an ODS column. Column: Inertsil ODS-2 (5 μm) (15 cm × 4.6 mm I.D.). Mobile phase: methanol–pH 3 buffer (90:10, v/v); flow-rate, 1.0 ml min^{-1}. Detection at 440 nm.

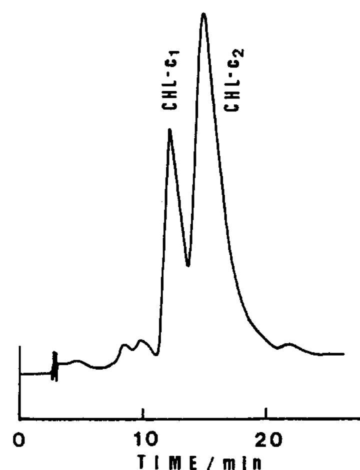

Fig. 3. Separation of CHL-c_1 and CHL-c_2 on an ODP column. Column: Asahipak ODP-50 (5 μm) (15 cm × 6.0 mm I.D.). Mobile phase: methanol–pH 3 buffer (90:10, v/v); flow rate, 1.7 ml min^{-1}. Detection at 440 nm.

and pheoporphyrin c_2 (PHEO-c_2), respectively, by comparing the UV–visible spectra of the peak fractions of the eluate with those of the pure substances [7].

According to the above results, the pigment of interest was regarded as a mixture of CHL-c_1 and CHL-c_2 at this stage. The pigment is hereafter denoted CHL-*c* for convenience.

Resolution of CHL-c

It was found that CHL-*c* could be resolved into two species by use of an ODP column with aqueous methanolic mobile phase, as shown in Fig. 3. The resolved CHL-*c* species were crystallized from corresponding fractions of the eluate. The early- and the late-eluted fractions of CHL-*c* gave intense mass spectral signals at *ca.* m/z 610 and 608, respectively (see Fig. 4), which matched the molecular ion peaks of CHL-c_1 and CHL-c_2. Accordingly, the early- and late-eluted species from the ODP column were identified as CHL-c_1 and CHL-c_2, respectively.

The UV–visible absorption spectral data for CHL-c_1 and -c_2 in methanol and 1% pyridine–methanol are given in Table I.

Effect of mobile phase solvent

Fig. 5 shows the effect of the methanol content of the mobile phase on the capacity factors (k') of CHL-c_1 and -c_2. For calculation of k', the column void volume was determined by injection of a nearly saturated solution of sodium nitrate. The plots of log k' *versus* methanol content for CHL-c_1 and -c_2 are almost parallel to

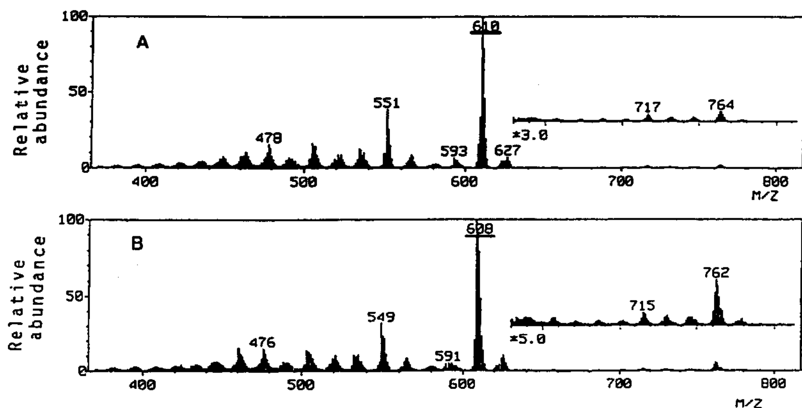

Fig. 4. Mass spectra of (A) CHL-c_1 and (B) CHL-c_2.

TABLE I

ABSORPTION MAXIMUM WAVELENGTHS (λ_{max}) AND MOLAR ABSORPTION COEFFICIENTS (ε) AT λ_{max} OF CHL-c_1 AND -c_2

Compound	In methanol		In 1% pyridine in methanol		
	λ_{max} (nm)	$\varepsilon(10^3\,l\,mol^{-1}\,cm^{-1})$	λ_{max} (nm)	$\varepsilon(10^3\,l\,mol^{-1}\,cm^{-1})$	Literature λ_{max} (nm) [2]
CHL-c_1	631	8.6	634	13	634
	588	4.9	584	7.6	584
	451	85	449	94	445
CHL-c_2	635	6.1	635	7.9	635
	588	5.8	587	6.6	587
	454	58	452	74	452

each other, and CHL-c_1 always gives a smaller k' than CHL-c_2 within the range of methanol contents tested.

It was found that both PHEO-c_1 and -c_2, which are the magnesium-free forms of CHL-c_1 and -c_2, were more strongly retained on the column than the respective chlorophylls, as shown in Fig. 5. A mobile phase containing 90% (v/v) of methanol was effective for the separation of CHL-c_1 and -c_2. Such a methanol content, however, was not practical if PHEO-c_1 and -c_2 were also present in the same HPLC run because of their much higher retentions. The plots for PHEO-c_1 and -c_2 at methanol contents 90% and 92% are not given in Fig. 5 owing to their large retentions.

Effect of pH of aqueous mobile phase component

The effect of the pH of the aqueous component of the mobile phase on the capacity factors of CHL-c_1 and -c_2 was examined using methanol–buffer (90:10, v/v). The buffers (about 10 m*M* solutions) were prepared at pH 3 as described under Experimental, at pH 4–6 with acetic acid and sodium acetate and at pH 8 with Tris and hydrochloric acid. The results are shown in Fig. 6, in which the results for PHEO-

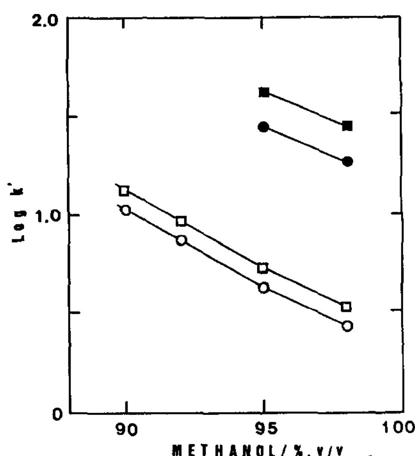

Fig. 5. Effect of the methanol content of the mobile phase on the capacity factors. Column: Asahipak ODP-50. Mobile phase: methanol–buffer (pH 3). Compounds: ○ = CHL-c_1; □ = CHL-c_2; ● = PHEO-c_1; ■ = PHEO-c_2.

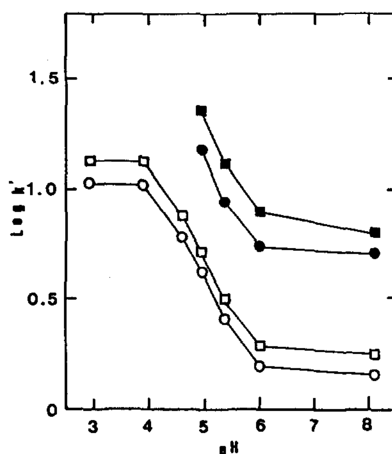

Fig. 6. Effect of the pH of the aqueous component of the mobile phase on the capacity factors. Column: Asahipak ODP-50. Mobile phase: methanol–buffer (90:10, v/v). Symbols as in Fig. 5.

c_1 and -c_2 are also plotted for comparison. A significant change in the retention of CHL-c_1 and -c_2 occurs in the pH range *ca.* 4–6, and a similar tendency is shown also for the corresponding metal-free forms, *i.e.*, PHEO-c_1 and -c_2. When the pH is lower than 5, both PHEO-c_1 and -c_2 are retained so strongly that they cannot be eluted in a short separation time. Such a pH dependence of the retention is attributable to the weakly acidic characteristics of the carboxylic groups possessed by these compounds. A pH lower than 4 is recommended for the separation of CHL-c_1 and -c_2 with a high reproducibility of the retention time, because their capacity factors are almost independent of pH.

ODP appears to be a promising reversed-phase column packing for chlorophyll analysis, particularly for the separation of CHL-c_1 and -c_2, because these pigments could not be resolved by the use of popular reversed-phase columns such as ODS. A 90:10 (v/v) mixture of methanol and a buffer solution of pH 3 is recommended as the mobile phase for this purpose. Application of ODP column to the determination of chlorophylls in real algal samples will be dealt with elsewhere.

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