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Separation of chlorophyll-c_1 and -c_2 by micellar electrokinetic capillary chromatography

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Abstract

Successful separation of chlorophyll c_1 (Chl-c_1), chlorophyll c_2 (Chl-c_2) and their demetallated forms (pheoporphyrins) was performed by the electrokinetic chromatographic mode of capillary zone electrophoresis in a running solution containing micelles of sodium dodecyl sulfate and dimethylformamide. Chl-c_1 migrated with higher velocity than Chl-c_2, and their migration times were shorter than those of the corresponding demetallated forms. Complete resolution was obtained for these compounds within 20 min under the conditions of migration distance (i.e., effective capillary length) 50 cm and electric field 429 V/cm. Application to the separation of chlorophylls in a typical brown seaweed was demonstrated.

1. Introduction

Chlorophylls are typical instances of naturally occurring metal (magnesium) chelate complexes that are well known as photosynthetic pigments. Different chlorophylls possessing a chlorin or porphyrin macrocyclic structure are found in accordance with the species of plants. For example, chlorin-type compounds, such as chlorophyll *a* (Chl-*a*) and chlorophyll *b* (Chl-*b*), are found in green algae (*Chlorophyta*) and higher plants, and porphyrin-type compounds, such as Chl-c_1 and Chl-c_2, in brown algae (*Phaeophyta*).

The determination of chlorophylls and their degradation products in natural samples from the sea, rivers, lakes and other sources gives considerable information about the biological activity in different environments. Traditional analytical methods for chlorophylls are based on spectrophotometry or fluorimetry, and high-performance liquid chromatography (HPLC) is today a more promising approach. HPLC is fairly effective for separation of Chl-*a* and Chl-*b* [1–3], but few such successful separations of Chl-c_1 and Chl-c_2 have been reported so far. Exceptional instances of the separation of Chl-c_1 and Chl-c_2 were developed by means of thin-layer chromatography [4] and conventional column liquid chromatography [5] by Jeffrey using a specially prepared polyethylene powder, and by HPLC using a commercially available octadecyl-bonded vinyl alcohol copolymer in this laboratory [6]. However, methods with a much higher resolving ability need to be developed for the accurate measurement of these chlorophylls and related compounds in real samples.

There is increasing interest in the high resolving ability of capillary zone electrophoresis (CZE) and also its expanded mode, micellar

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electrokinetic capillary chromatography (MECC) [7]. The applicability of MECC to the separation of metal chelates was previously confirmed for uncharged bis- and trisacetylacetonato complexes of di- and trivalent metal ions, respectively [8,9], and bioporphyrins, such as protoporphyrin and haematoporphyrin, in the free acid forms and the complexed forms with copper(II) and zinc(II) [10].

This paper describes the feasibility of applying MECC to the separation of chlorophylls, such as Chl-c_1 and Chl-c_2, and related compounds, in particular. A solution containing sodium dodecyl sulfate (SDS) micelles and dimethylformamide (DMF) is applied as the running solution. The effects of the composition of the running solution on the migration velocity and resolution of these chlorophylls are detailed.

2. Experimental

2.1. Chlorophyll-c

Algal pigments were extracted with acetone from fresh brown seaweed *Undaria pinnatifida* obtained from Onagawa Bay, Miyagi, Japan. The acetone extract was shaken with both light petroleum and a saturated aqueous solution of sodium chloride to remove Chl-*a* and carotenes. Chlorophyll *c* was transferred from the resulting water-rich phase to ethyl acetate, and then purified on an octadecyl-bonded silica gel column with methanol–water (90:10, v/v). The coloured fraction of the eluate was shaken with both ethyl acetate and a saturated aqueous solution of sodium chloride, and the ethyl acetate phase was passed through a cellulose column with pure ethyl acetate. A mixture of Chl-c_1 and Chl-c_2 (see Fig. 1) was obtained after removal of the solvent from the coloured fraction of the eluate. The mixture was resolved into Chl-c_1 and Chl-c_2 by reversed-phase HPLC as detailed previously [6].

Pheoporphyrin c_1 (Pheo-c_1) and pheoporphyrin c_2 (Pheo-c_2) were prepared by demetallation of Chl-c_1 and Chl-c_2, respectively, using 0.1 *M* hydrochloric acid.

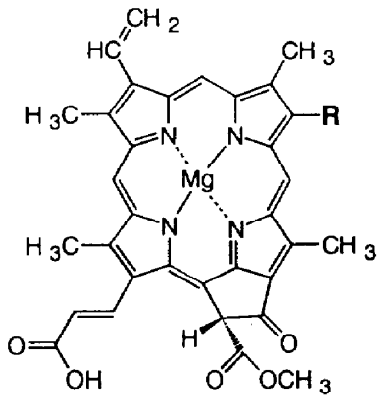

Fig. 1. Structure of Chl-c_1 (R = ethyl) and Chl-c_2 (R = vinyl).

2.2. Electrophoresis

A Jasco (Tokyo, Japan) Model CE-800 CZE system was used with a fused-silica capillary (70 cm × 50 μm I.D. × 375 μm O.D.) (Scientific Glass Engineering, Ringwood, Australia). The running solution filling the capillary was prepared to contain a certain amount of SDS and DMF in a solution adjusted to pH 11 with 20 m*M* N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) and 17 m*M* sodium hydroxide.

A methanol solution of chlorophyll(s) was diluted with the same composition as the running solution, and then was introduced into the positive end of the capillary by siphoning (10 cm, 5 s). Electrophoresis was run with an applied voltage of 30 kV (electric field 429 V/cm) and at 25°C. Detection was performed by on-column measurement of UV absorption at 430 nm through a 50 μm × 0.75 mm slit located 20 cm from the grounded end of the capillary.

3. Results

A simple buffer solution adjusted to pH 11 with CAPS and sodium hydroxide was not applicable to the medium (or running solution) for

the electrophoresis owing to the low solubility of the substances to be studied in the solution. The solubility should be enhanced by the addition of appropriate surfactant micelles and/or organic solvent to the buffer solution. In this work, SDS and DMF were taken as the former and the latter additives, respectively. SDS is popular as a surfactant in MECC. DMF was chosen from consideration of its high dissolving capability for chlorophylls, low viscosity, high dielectric constant and high miscibility with water. The solubilities of the chlorophylls and their demetallated forms of interest were sufficient for the following experiments in both solutions containing SDS micelles and DMF. DMF-containing micellar solutions were effective for performing successful MECC separations of porphyrins [10,11].

3.1. Formation of micelles of SDS

The formation of micelles of SDS in a DMF-containing solution was examined by conductimetry [10]. The critical micelle concentration (CMC) of SDS was estimated from the break of the proportionality between the specific conductivity change and the total concentration of SDS in the solution. The CMC values in different compositions of DMF-containing solution are given in Table 1.

Table 1
Critical micelle concentration (CMC) of SDS in DMF-containing solutions[a] at 25°C

DMF (%, v/v)	CMC (m*M*)
0	4.4
9.1	6.4
16.7	8.0
23.1	10.0
28.6	7.7
33.3	5.1
37.5	3.8

[a] Mixtures of DMF with an aqueous solution of 20 m*M* CAPS and 17 m*M* sodium hydroxide (pH 11).

3.2. Effects of SDS and DMF on electrophoretic migration

The migration behaviour of the chlorophyll compounds was studied in buffer solutions (pH 11) containing SDS and DMF at concentrations up to about 50 m*M* and 47% (v/v), respectively. The migration time of methanol was considered to be equal to the migration time of the electroosmotic flow of the solution (t_0).

When the running solution was a simple micellar solution of SDS (concentration 25 m*M*), all chlorophyll compounds, including Chl-c_1, Chl-c_2, Pheo-c_1 and Pheo-c_2, moved in the capillary and were detected at a migration time (t_S) identical with that of uncharged and hydrophobic compounds, such as Oil Yellow OB. The t_S value obtained for these compounds was larger than t_0. These results implied that all these compounds were completely solubilized in the negatively charged SDS micelles dispersed in the solution. Accordingly, the compounds could not be resolved on the basis of differential partitioning between the micelle pseudo-phase and bulk solution phase in the micellar solution.

The migration velocity of each chlorophyll compound varied with the addition of DMF to the running solution containing SDS micelles. When the running solution was a mixture of buffer solution (pH 11) and DMF containing 25 m*M* SDS, the t_S values of Chl-c_1, Chl-c_2, Pheo-c_1 and Pheo-c_2 increased with increasing proportion of DMF in the mixture, as shown in Fig. 2. A significant resolution was obtained for both the Chl-c_1–Chl-c_2 and the Pheo-c_1–Pheo-c_2 pairs when the DMF concentration was >20%. The t_0 value also increased with increasing DMF content. The electroosmotic velocity of a liquid in a capillary is, in general, directly proportional to the zeta potential on the capillary wall, the dielectric constant of the liquid and the electric field strength, but inversely proportional to the viscosity of the liquid. Therefore, the observed decrease in the velocity of the electroosmotic flow was probably caused by the diminution of the dielectric constant and/or increase in the viscosity of the solution due to the addition of

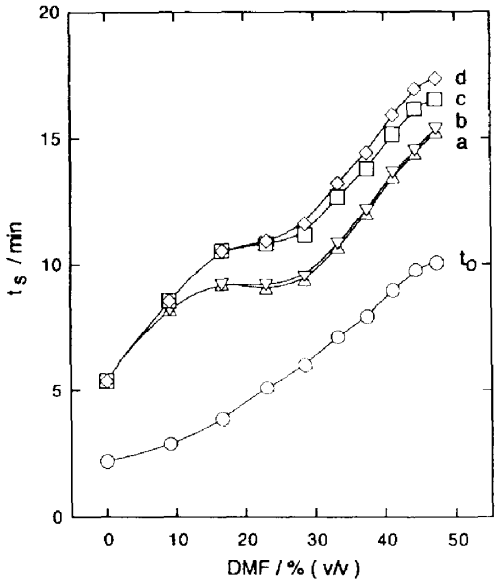

Fig. 2. Dependence of the migration time (t_S) on the DMF content of the running solution containing 25 m*M* SDS at pH 11. CZE conditions: migration distance, 50 cm; electric field, 429 V/cm. Compounds: (a) Chl-c_1; (b) Chl-c_2; (c) Pheo-c_1; (d) Pheo-c_2. t_0 = Migration time of methanol.

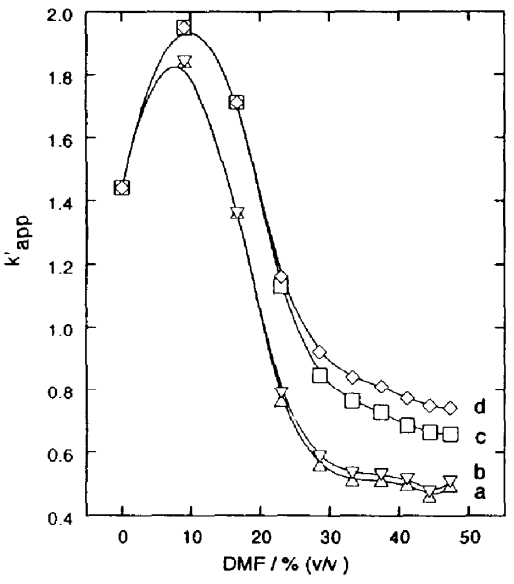

Fig. 3. Variation of apparent capacity factors (k'_{app}) of (a) Chl-c_1, (b) Chl-c_2, (c) Pheo-c_1 and (s) Pheo-c_2 with the addition of DMF to the running solution (pH 11). The SDS content was maintained at 25 m*M*. CZE conditions as in Fig. 2.

DMF to aqueous micellar solution (dielectric constants at 25°C, water 78.39 and DMF, 36.71 D; viscosities at 25°C, water 0.890 and DMF 0.802 cP).

The capacity factor could not be calculated for either chlorophyll in the running solution containing DMF, because a strict measurement of the velocity of the micelles was not carried out. The apparent capacity factor, k'_{app}, was calculated for each compound from the equation

$$k'_{app} = (t_S - t_0)/t_0 \quad (1)$$

as a qualitative measure of the interaction between the solute and the micelle (k'_{app} has no strict relationship to the capacity factor usually employed in MECC).

The k'_{app} value of each compound decreased with increasing DMF content, as shown in Fig. 3. The decrease in k'_{app} with increasing DMF content is considered to result from the enhancement of the solubility in the DMF-containing bulk solution phase in a micellar solution. The migration of each chlorophyll compound is af-

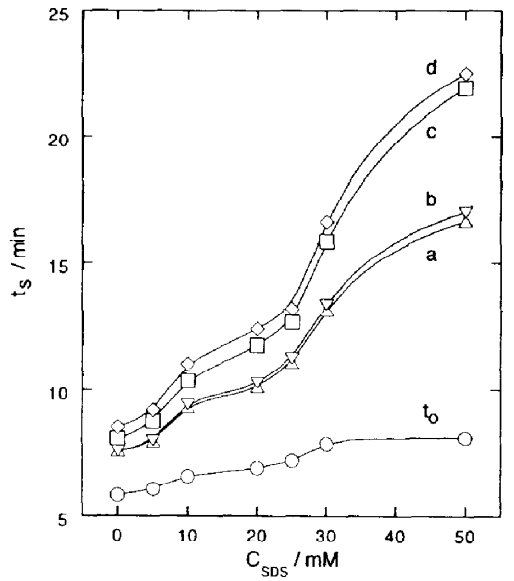

Fig. 4. Dependence of the migration times of Chl-c_1 and Chl-c_2 on the concentration of SDS in the running solution containing 33.3% (v/v) DMF. CZE conditions as in Fig. 2. Compounds: (a) Chl-c_1; (b) Chl-c_2; (c) Pheo-c_1; (d) Pheo-c_2. t_0 = Migration time of methanol.

fected by the partitioning between the SDS micelles and bulk solution.

Fig 4 shows the effect of the concentration of SDS (C_{SDS}) in the running solution on the migration time (t_S) of chlorophyll compounds, the DMF content being maintained at 33.3% (v/v). The t_S value of each chlorophyll compound increased with increasing C_{SDS}, which was considered to result from the increase in their distribution to the SDS micelles. The resolution for the Chl-c_1–Chl-c_2 pair was also enhanced with increasing C_{SDS}. The separation of Chl-c_1 and Chl-c_2 was successful with $C_{SDS} \geq 10$ mM.

3.3. Application

The applicability of the present CZE conditions was demonstrated for the analysis of an acetone extract from brown seaweed *Undaria pinnatifida*. The running solution contained SDS at 30 mM in a 10:6 (v/v) mixture of CAPS buffer (pH 11) and DMF (DMF content, 37.5%). The electropherograms obtained for the seaweed sample and a standard mixture are compared in Fig. 5. Three peaks are clearly found for the sample; they are assigned to Chl-*a*, Chl-c_1 and Chl-c_2, although no quantitative measurement was carried out.

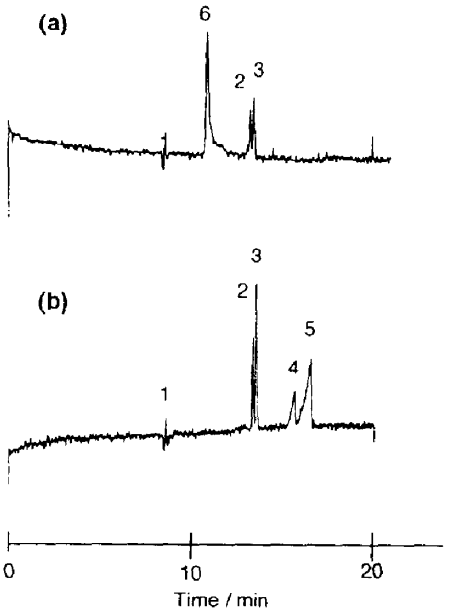

Fig. 5. MECC separation of (a) chlorophyll pigments in acetone extracts from *Undaria pinnatifida* and (b) standard mixture. Running solution, 30 mM SDS in a mixture of 20 mM CAPS buffer (pH 11) and DMF (10:6, v/v); capillary, 50 cm (effective length for separation) × 50 μm I.D.; electric field, 429 V/cm; detection at 430 nm. Peaks: 1 = methanol; 2 = Chl-c_1; 3 = Chl-c_2; 4 = Pheo-c_1; 5 = Pheo-c_2; 6 = Chl-*a*.

4. Conclusions

According to the migration studies on different forms of chlorophyll-*c*, the main contribution to the separation of Chl-c_1 and Chl-c_2 is their partitioning phenomena between the micelles and the bulk solution, and the addition of DMF to the solution is the key to the successful resolution of the chlorophylls. MECC using a DMF-containing micellar solution is a promising tool for the separation and determination of Chl-c_1 and Chl-c_2, in particular.

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