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Determination of copper(II) chlorophyllin by reversed-phase high-performance liquid chromatography

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Abstract

Copper(II) chlorophyllin, consisting of copper(II) pheophorbide *a*, copper(II) chlorin e_6, copper(II) rhodin g_7 and copper(II) chlorin e_4, was prepared and separated by semi-preparative high-performance liquid chromatography (HPLC). The components of copper(II) chlorophyllin were determined on a reversed-phase Inertsil ODS-2 column using a mobile phase of methanol–water (97:3, v/v) containing 1% (v/v) of acetic acid. Linear calibration plots were obtained for copper(II) chlorophyllin in the concentration range of 0–30 μg cm^{-3} with photometric detection at 407 or 423 nm. The detection limits of copper(II) pheophorbide *a*, copper(II) chlorin e_6, copper(II) rhodin g_7 and copper(II) chlorin e_4 were 3.5, 1.5, 3.3 and 1.4 ng cm^{-3} with relative standard deviations ($n = 10$) of 1.8, 1.6, 5.2 and 3.6%, respectively. The reversed-phase HPLC method proposed here was demonstrated to be useful for the determination of the components of sodium copper(II) chlorophyllin.

1. Introduction

In recent years metallochlorophyllins have received a great deal of attention because of their importance in food additives, pharmaceuticals and electrode materials of photoelectron conversion. Sodium copper(II) chlorophyllin is utilized as food additive due to its high light-stability and applied to medicine owing to its antioxidative effect [1,2]. Some kinds of copper(II) chlorophyllins, including copper(II) pheophorbides, are useful in the functionalized electrode for artificial photosynthetic systems [3]. Commercially available sodium copper(II) chlorophyllins consist of a few kinds of copper(II) chlorophyll derivatives, e.g. copper(II) pheophorbide *a*, copper(II) chlorin e_6, copper(II) chlorin e_4, copper(II) rhodin g_7 and their degradation products (cf. Fig. 1). Spectrophotometry, widely used for the determination of metallochlorophyllins, is insufficient for the accurate determination of each component of sodium copper(II) chlorophyllin, because all components have similar absorption spectra. Recently high-performance liquid chromatography (HPLC) without any time-consuming pretreatment techniques prior to the quantitation step has been exploited for zinc(II) chlorophyllin [4]. The potential usefulness of HPLC has been demonstrated for the separation and determination of metallochlorophylls such as iron(III) [5], nickel(II) [6], copper(II) [7] and zinc(II)

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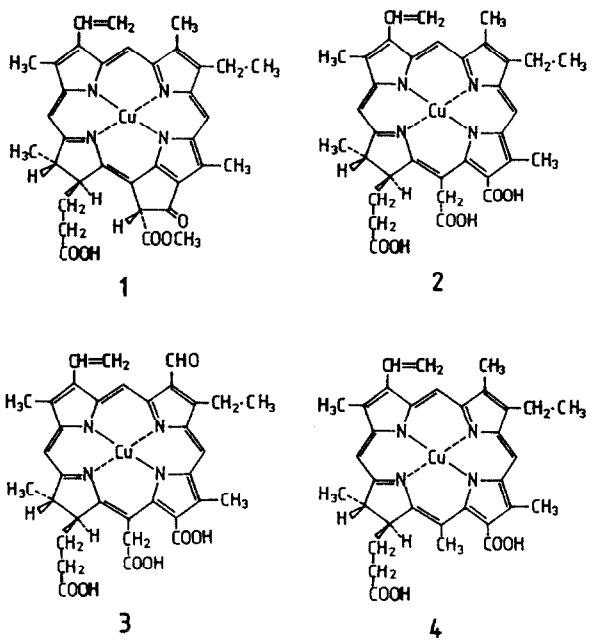

Fig. 1. Structures of copper(II) chlorophyllin components. 1 = Copper(II) pheophorbide *a*; 2 = copper(II) chlorin e_6; 3 = copper(II) rhodin g_7; 4 = copper(II) chlorin e_4.

chlorophylls [8]. The present study has focused on both the preparation of pure copper(II) chlorophyllin by semi-preparative HPLC and rapid and accurate determination of each component of copper(II) chlorophyllin by analytical HPLC. A development of the preparation and purification method of copper(II) chlorophyllin would provide a basis for a comparison of its spectroscopic and structural properties and reveal its previously unrecognized functions.

2. Experimental

2.1. Reagents and instruments

All chemicals were of analytical-reagent grade and used as supplied without further purification. Pheophorbides *a* and *b*, chlorin e_6 and rhodin g_7 were prepared according to the method of Hynninen [9]. Chlorin e_4 was prepared from chlorin e_6 by a modification of the literature method [10]. Organic solvents were purchased from Kanto Chemical and of LC quality. The JASCO BIP-1 liquid chromatograph, equipped with a Uvidec-1000 variable-wavelength UV–Vis detector or a JASCO MULTI-340 multichannel detector, a Shimadzu C-R3A chromatopac integrator, an Inertsil ODS-2 column (5-μm spherical ODS, 250 mm × 4.6 mm I.D., Gasukuro Kogyo), and a Rheodyne Model 7125 injector with a 20-mm^3 injection loop, was used for analytical HPLC. A semi-preparative Develosil ODS column (10-μm spherical octadecyl silica, 25 cm × 3 cm I.D., Nomura Chemical, Aichi, Japan) equipped with a Kusano-Kagaku KV-3W loop injector (0.59 cm^3) was used for semi-preparative HPLC. Each HPLC solvent or solvent mixture was filtered through a fresh 0.45-μm hydrophilic Millipore filter and degassed every time before use by ultrasonically vibrating the solvent container. The flow-rate of the analytical HPLC was 1.4 cm^3 min^{-1}, the inlet pressure was 5.0–10 MPa depending on the mobile phase and the analytes were usually detected at 407 nm. The column temperature was set at 35°C by dipping the analytical column in a thermostat bath. The electronic absorption spectra were recorded with a Hitachi U-2000 spectrophotometer using 1-cm quartz cells. The mass spectra of copper(II) chlorophyllin were measured with a JEOL JMS-AX505H fast atom bombardment (FAB) mass spectrometer.

2.2. Preparation of copper(II) chlorophyllin

Copper(II) pheophorbide a was prepared by adding a 5-fold molar excess of $Cu(CH_3COO)_2 \cdot H_2O$ (8.4 mg) to pheophorbide a (5 mg) in 10 cm^3 of glacial acetic acid. The reaction mixture was stirred at 20°C for 15 min and the extent of insertion reaction was monitored spectrophotometrically via the Soret band of copper(II) pheophorbide *a* (λ_{max}, 423 nm). The reaction mixture was allowed to cool, followed by addition of 20 cm^3 of chloroform. The mixed solution was washed five times with distilled water to remove the residual copper(II) acetate and acetic acid. The dark green chloroform solution was concentrated by rotary evaporation to obtain copper(II) pheophorbide *a*. The copper(II) pheophorbide a obtained was purified with a

semi-preparative Develosil ODS column at a flow-rate of 7.4 cm^3 min^{-1}. All operations were carried out in the dark under an argon atmosphere at an ambient temperature of 20°C. Copper(II) chlorin e_6, copper(II) rhodin g_7 and copper(II) chlorin e_4 were prepared in a manner similar to that for copper(II) pheophorbide *a*.

2.3. Analytical procedures

A 50 μg cm^{-3} stock solution of each copper(II) chlorophyllin component was prepared in glass-stoppered volumetric flasks, which were then sealed and stored at 5°C in the dark. Standard solutions for calibration graphs were prepared with a solvent composition as near to the sample solution as possible, by diluting the appropriate volume of the stock solution with a mixture of methanol–water (97:3, v/v) containing 1% (v/v) of acetic acid. All the samples of commercially available sodium copper(II) chlorophyllin were treated as follows. A certain amount of sodium copper(II) chlorophyllin (30 mg) was dissolved in 40 cm^3 of distilled water. After the pH value of the aqueous solution was adjusted to 2.3 with 0.1 *M* hydrochloric acid (30 cm^3), 40 cm^3 of diethyl ether was added to the copper(II) chlorophyllin solution. Copper(II) chlorophyllin was extracted by vigorously shaking and the ether layer was washed five times with distilled water. Then the extract was brought to dryness by rotary evaporation and subjected to the HPLC determination of each copper(II) chlorophyllin component. All sample solutions were filtered prior to injection into the chromatograph with a Toyo Roshi DISMIC-25JP filter (0.45 μm).

3. Results and discussion

3.1. Preparation and identification of copper(II) chlorophyllin

Copper(II) chlorophyllin is usually available as sodium salt and a mixture of copper(II) chlorin e_6, copper(II) rhodin g_7 and their degradation products. A preliminary study revealed that most of commercially available copper(II) chlorophyllin preparations consists of copper(II) pheophorbide *a*, copper(II) chlorin e_6 and copper(II) rhodin g_7 [11]. In fact a few chromatographic peaks were observed on a typical three-dimensional chromatogram measured for commercially available copper(II) chlorophyllin (cf. Fig. 2). In order to identify and determine each peak component on the high-performance liquid chromatogram, copper(II) pheophorbide *a*, copper(II) chlorin e_6, copper(II) rhodin g_7 and copper(II) chlorin e_4 were prepared according to the methods described under Experimental. Each component of copper(II) chlorophyllin thus prepared was purified by semi-preparative HPLC and then the absorption spectrum was measured in a solution of methanol–water (97:3, v/v) containing 1% (v/v) of acetic acid. The absorption spectra of the components of cop-

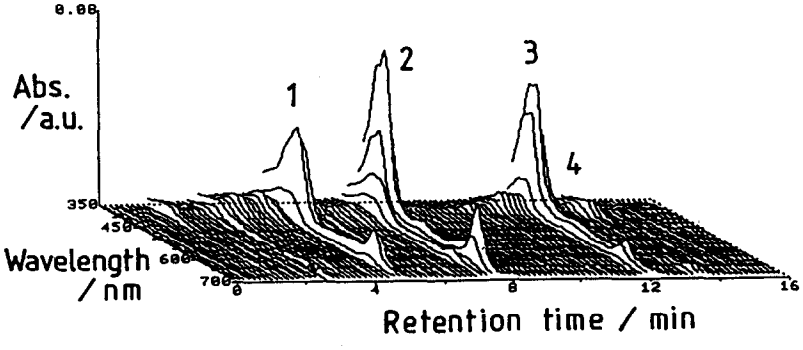

Fig. 2. Three-dimensional chromatogram of copper(II) chlorophyllin. Peaks: 1 = copper(II) rhodin g_7; 2 = copper(II) chlorin e_6; 3 = copper(II) chlorin e_4; 4 = copper(II) pheophorbide *a*.

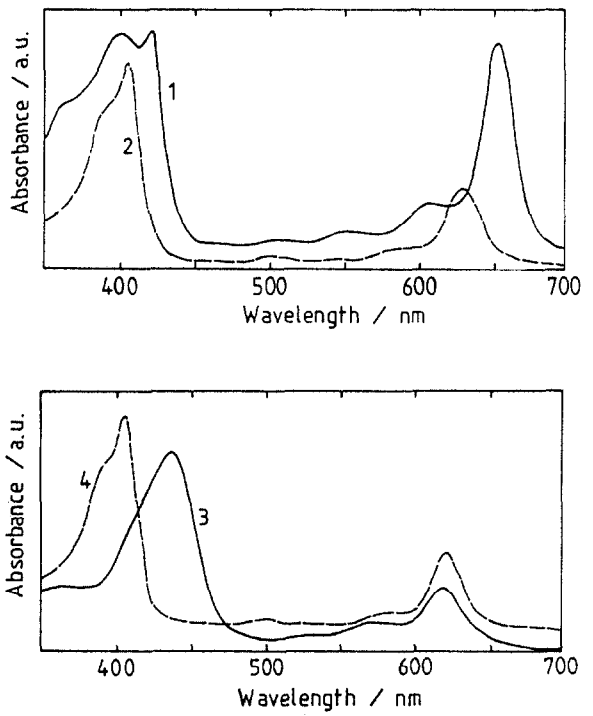

Fig. 3. Absorption spectra of copper(II) chlorophyllin components. 1 = Copper(II) pheophorbide *a*; 2 = copper(II) chlorin e_6; 3 = copper(II) rhodin g_7; 4 = copper(II) chlorin e_4.

per(II) chlorophyllin are shown in Fig. 3, and the spectral data are summarized in Table 1. All spectra are very similar to each other, but slightly different in the spectral shape and absorption maxima. Each peak component on the chromatogram of copper(II) chlorophyllin was fractionated and carefully concentrated by a rotary evaporator. The sample obtained by evaporating the residual acetic acid was subjected to FAB mass spectrometry to identify each peak component. The mass spectrum of each fraction showed the presence of the chlorophyllin component with a molecular ion M^+: copper(II) chlorin e_6, 657 (M^+, 45%); copper(II) chlorin e_4, 613 (M^+, 65%). Each peak component on the chromatogram was also identified by a comparison of the retention times with those of the corresponding pure compounds and the absorption spectra.

3.2. Chromatographic separation of copper(II) chlorophyllin

Commercially available sodium copper(II) chlorophyllin is soluble in water, but hardly distributed to octadecyl silica(ODS). Therefore, it is necessary to convert sodium metallochlorophyllins to their corresponding carboxylic acids by the release of sodium ions using hydrochloric acid. Octadecyl silica was selected as a stationary phase in combination with a mobile phase of methanol–water (97:3, v/v). The best separation and retention of copper(II) chlorophyllin were achieved using a mobile phase of methanol–water (97:3, v/v) containing 1% (v/v) of acetic acid. The small amount of acetic acid

Table 1
Absorption maxima and molar extinction coefficients (ϵ) of copper(II) chlorophyllin components

Compound	λ_{max} (nm)		$\epsilon \times 10^4$ (cm^{-1} mol^{-1} dm^3)	
	Soret band	Q band	Soret band	Q band
Copper(II) pheophorbide *a*	401, 423	653	5.14, 4.86	4.71
Copper(II) chlorin e_6	407	633	8.08	3.20
Copper(II) rhodin g_7	436	622	4.35	1.38
Copper(II) chlorin e_4	404	626	2.70	0.95

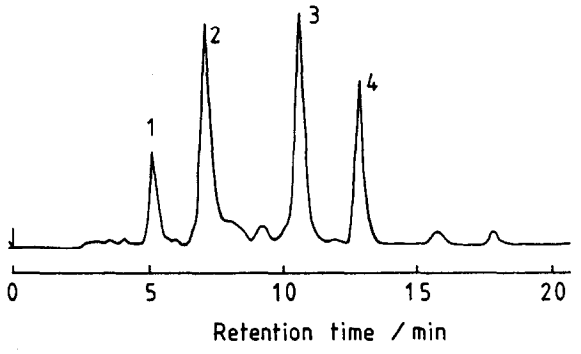

Fig. 4. Typical chromatogram of copper(II) chlorophyllin. Peaks: 1 = copper(II) rhodin g_7; 2 = copper(II) chlorin e_6; 3 = copper(II) chlorin e_4; 4 = copper(II) pheophorbide *a*.

was added to the mobile phase to prevent the liberation of protons at the carboxyl group. A typical high-performance liquid chromatogram of copper(II) chlorophyllin is shown in Fig. 4. Four well-resolved peaks were obtained using UV detection at 423 nm and copper(II) rhodin g_7, copper(II) chlorin e_6, copper(II) chlorin e_4 and copper(II) pheophorbide *a* eluted in the order given. The retention time of copper(II) chlorophyllin decreases with the increasing number of carboxyl groups, indicating that the carboxyl group attached to the chlorin ring allows greater interactions with the mobile phase. As expected, rhodin g_7 is eluted fast compared with chlorin e_6 due to less interaction of the former with the non-polar ODS stationary phase. This elution order agrees with the general tendency that the aldehyde group attached to the chlorin ring is more polar than the methyl group.

3.3. Determination of copper(II) chlorophyllin

The preparation of working standard solutions by diluting the stock solution in methanol–water (97:3, v/v) yielded a standard solution of copper(II) chlorophyllin which was stable over 24 h. The calibration graphs for four components of copper(II) chlorophyllin were constructed in the concentration range of 0–30 μg cm^{-3}. The minimum detectable concentrations of copper(II) pheophorbide *a*, copper(II) chlorin e_6, copper(II) rhodin g_7 and copper(II) chlorin e_4 were 3.5, 1.5, 3.3 and 1.4 ng cm^{-3} with relative standard deviations ($n = 10$) of 1.8, 1.6, 5.2 and 3.6% at a concentration level of 1 μg cm^{-3}, respectively. They were calculated from that amount of copper(II) chlorophyllin which yielded a signal-to-noise (S/N) ratio of 2. The HPLC method proposed here was applied to the assay of commercially available sodium copper(II) chlorophyllin. The analytical results obtained for samples A–D are summarized in Table 2. The sum total of the analytical values of individual components is less than 100% (w/w), because the samples assayed are contaminated by degradation products other than copper(II) chlorophyllin which was identified in the present study. It is noteworthy that one of the most abundant components in commercial sodium copper(II) chlorophyllin is chlorin e_4. The chlorophyll *a* in

Table 2
Analytical results on commercially available sodium copper(II) chlorophyllin

Sample	Content (%, w/w)			
	Copper(II) pheophorbide *a*	Copper(II) chlorin e_6	Copper(II) rhodin g_7	Copper(II) chlorin e_4
A	8.7 (0.1)	8.0 (0.2)	10.1 (0.2)	57.1 (1.1)
B	17.1 (0.2)	3.7 (0.2)	10.7 (0.2)	60.2 (2.4)
C	5.4 (0.1)	8.4 (0.3)	9.0 (0.2)	34.7 (1.0)
D	3.8 (0.1)	13.2 (0.1)	6.7 (0.1)	56.6 (0.6)

The number in parentheses is the standard deviation of the analytical values ($n = 5$).

the starting material of copper(II) chlorophyllin must be converted to copper(II) chlorin e_4 in the course of the production processes. This is supported by the fact that the starting material of copper(II) chlorophyllin contains much chlorophyll *a* compared with chlorophyll *b* and chlorin e_4 is easily derived from pheophorbide *a* or chlorin e_6. The HPLC method described above has been applicable to commercially available sodium copper(II) chlorophyllin. Consequently, it will be very useful for the evaluation of food additives and effective to the study of the antioxidative and antimutagenic components in metallochlorophyllins.

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