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Short communication

High-performance liquid chromatographic separation of iron(III) chlorophyllin

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Abstract

The components of iron(III) chlorophyllin were separated by high-performance liquid chromatography on a reversed-phase Inertsil ODS column. Good retention was achieved with acetonitrile–phosphate buffer (pH 2) (60:40, v/v) containing 0.01 *M* tetramethylammonium chloride (TMACl) in studies of the chromatographic behaviour with various elution solvents and their mixtures. Three kinds of iron(III) chlorophyll derivatives, i.e., iron(III) pheophorbide *a* (Fe-Phed *a*), iron(III) chlorin e_6 (Fe-Chl e_6) and iron(III) chlorin e_4 (Fe-Chl e_4), were detected as major components. Standard materials for qualitative and quantitative analysis were derived from chlorophyll *a* which was extracted from spinach. The required compounds were prepared under strict conditions by the insert reaction of iron(II) chloride with pheophytins to avoid molecular degradation by light or molecular oxygen.

1. Introduction

In recent years, metallochlorophyllins have received a great deal of attention because of their importance in food additives, pharmaceuticals and electrode materials for photoelectron conversion. Sodium iron(III) chlorophyllin and sodium copper(II) chlorophyllin, which are water-soluble green pigments derived from iron(III) and copper(II) chlorophylls by saponification, have been utilized as food additives, especially for confectionaries, owing to their bright colour and applied in medicine owing to their antioxidative effect [1].

Although the major components of metallochlorophyllins are known to be metallopheophorbide *a* (M-Phed *a*, Fig. 1a), metallochlorin e_6 (M-Chl e_6, Fig. 1b) and metallochlorin e_4 (M-Chl e_4, Fig. 1c) [2], no reliable analytical method has been established for metallochlorophyllins. In particular, the sepa-

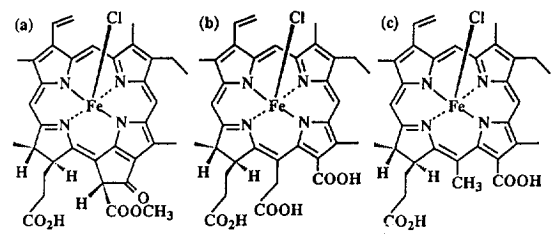

Fig. 1. Structure of iron(III) chlorophyllin. (a) Iron(III) pheophorbide *a* (Fe-Phed *a*); (b) iron(III) chlorin e_6 (Fe-Chl e_6); (c) iron(III) chlorin e_4 (Fe-Chl e_4).

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ration of their components is required for qualitative and quantitative analysis. The qualitative analysis of metallochlorophyllin has been carried out by thin-layer chromatography (TLC), but the resolution of TLC is insufficient for the separation and determination of the components of metallochlorophyllin [3]. The potential usefulness of high-performance liquid-chromatography (HPLC) has been demonstrated for the rapid separation and determination of copper(II) chlorophyllin [4]. However, it is more difficult to determine iron(III) chlorophyllin than copper(II) chlorophyllin because no sharp peak can be obtained for the former owing to the axial binding ability and standard materials are not available.

This study was focused on both the chromatographic separation of the components and the preparation of iron(III) chlorophyllin to facilitate the rapid and accurate determination of each component of iron(III) chlorophyllin.

2. Experimental

2.1. Reagents and instrumentation

All chemicals were of analytical-reagent grade and used as supplied. Organic solvents of LC quality were purchased from Kanto Chemical (Tokyo, Japan). The analytical HPLC instrument consisted of a Yanaco L-4000 W pump (Yanagimoto, Kyoto, Japan), a Rheodyne (Berkeley, CA, USA) model 7125 injector, a JASCO (Tokyo, Japan) MULTI-340 multi-channel detector and an Inertsil ODS column (5-μm spherical octadecylsilica, 250 × 4.6 mm I.D.), (Gasukuro Kogyo, Tokyo, Japan). The flow-rate of the mobile phase was 1.4 cm^3 min^{-1} and the column temperature was maintained at 35°C by immersing the column in a thermostated bath. A semi-preparative Develosil ODS column (10-μm spherical octadecylsilica, 250 × 30 mm I.D.), (Nomura Chemical, Aichi, Japan) equipped with a KV-3W loop injector (0.59 cm^3) (Kusano-Kagaku, Tokyo, Japan) was used for semi-preparative HPLC. Electronic absorption spectra were recorded in dichloromethane solutions with a JASCO V-550 spectrophotometer using 1-cm quartz cells. The mass spectra of iron(III) chlorophyllin were measured with a JEOL JMS-AX505H fast atom bombardment (FAB) mass spectrometer using *m*-nitrobenzyl alcohol as the matrix compound.

2.2. Preparation of iron(III) chlorophyllin

Sodium iron(III) chlorophyllin was obtained from Nacalai Tesque (Kyoto, Japan). Pheophorbide *a* (H_2-Phed *a*) and chlorin e_6 (H_2-Chl e_6) were prepared according to the method of Hynninen [5]. Chlorin e_4 (H_2-Chl e_4) was prepared from H_2-Chl e_6 by a modification of the literature method [6]. The standard materials of iron(III) chlorophyllin were prepared by the insert reaction of metal ion with pheophytin.

Iron(III) pheophorbide *a* chloride was prepared from H_2-Phed *a* as follows. To 2.0 mg of H_2-Phed *a* in 5 cm^3 of acetone were added 7.4 mg of iron(II) chloride tetrahydrate (Merck) and 7.5 mg of L-(+)-ascorbic acid (Wako, Osaka, Japan) in 5 cm^3 of methanol. The reaction mixture was stirred for 3.5 h at 60°C, 50 cm^3 of dichloromethane were added, then it was washed three times with saturated sodium chloride solution followed by 0.01 *M* hydrochloric acid and water. The organic phase was dried (Na_2SO_4) and evaporated to dryness. All operations were carried out in the dark under an argon atmosphere. The product was identified as the compound sought from the change in the UV–Vis absorption spectrum from that of H_2-Phed a and the dechlorinated ion $[M-Cl]^+$ of Fe-Phed *a*(Cl) in the FAB mass spectrum (yield 57%); λ_{max} 396 nm (ε $4.3 \cdot 10^4$), 623 ($1.8 \cdot 10^4$); mass spectrum, *m/z* 646 ($[M-Cl]^+$).

Iron(III) chlorin e_6 chloride was prepared from H_2-Chl e_6 by the following method. To 5.0 mg of H_2-Chl e_6 in 5 cm^3 of acetone were added 16.8 mg of iron(II) chloride tetrahydrate and 18.0 mg of L-(+)-ascorbic acid in 5 cm^3 of acetone. The reaction mixture was stirred at 60°C for 3.5 h and treated in a similar manner to that of Fe-Phed *a*(Cl) (yield 53%); λ_{max} 386 nm (ε $4.2 \cdot 10^4$), 622 ($1.3 \cdot 10^4$); mass spectrum, *m/z* 650 ($[M-Cl]^+$).

Iron(III) chlorin e_4 chloride was prepared from H_2-Chl e_4 as follows. To 5.0 mg of H_2-Chl e_4 in 5 cm^3 of acetone were added 15.4 mg of iron(II) chloride tetrahydrate and 17.2 mg of L-(+)-ascorbic acid in 5 cm^3 of acetone. This reaction mixture was stirred for 3.5 h at 60°C and treated in a similar manner to that for Fe-Phed *a*(Cl) (yield 48%); λ_{max} 389 nm (ε $4.2 \cdot 10^4$), 611 ($1.4 \cdot 10^4$); mass spectrum, *m/z* 606 ($[M - Cl]^+$).

2.3. Analytical procedures

Commercially available sodium iron(III) chlorophyllin (100 mg) was dissolved in distilled water (50 cm^3). After the pH of the aqueous solution had been adjusted to 2 with 0.1 *M* hydrochloric acid, iron(III) chlorophyllin was extracted three times with diethyl ether (25 cm^3), then the extract was evaporated and subjected to the HPLC separation of components. The sample solutions (15 $\mu g\ cm^{-3}$) of iron(III) chlorophyllin and its standard materials were prepared in glass-stoppered volumetric flasks with the HPLC mobile phase as solvent and stored at 5°C in the dark. Prior to injection into the chromatograph, all sample solutions were filtered with a DISMIC-25JP filter (0.45 μm) (Advantec Toyo, Tokyo, Japan). A suitable volume of the sample solution was injected to fill the 100-mm^3 injection loop.

3. Results and discussion

3.1. Chromatographic separation of iron(III) chlorophyllin

The well resolved separation of metal-substituted chlorophylls has been achieved by reversed-phase HPLC using an ODS column [4,7]. In the present case, some particular conditions are required to separate the components of iron(III) chlorophyllin because of their carboxyl groups and the axial binding ability of the central iron(III). A series of solvent mixtures, *i.e.*, methanol–acetone, acetronitrile–acetone, methanol–water–acetic acid and acetronitrile–water–acetic acid, were tested as the mobile phase, but shortening of the retention and peak tailing were observed. The peak tailing was prevented by the addition of 0.01 *M* tetramethylammonium chloride (TMACl), but the shortening of the retention was not improved. Therefore, a mixture of organic solvent with phosphate buffer was examined for the efficient retention of iron(III) chlorophyllin. When acetonitrile–phosphate buffer (pH 7) (60:40, v/v) was used as the mobile phase, the retention time of iron(III) chlorophyllin was too short. This is probably due to the high polarity originating from the free carboxylic group of iron(III) chlorophyllin. Therefore, the pH of the phosphate buffer was adjusted to 2 with hydrochloric acid to prevent the release of protons in the carboxylic groups. As a result, sufficient retention was attained and about five peaks appeared on the chromatogram (Fig. 2). These peaks were sharp enough to identify the individual components. Acetonitrile–phosphate buffer (pH 2) (60:40, v/v) containing 0.01 M TMACl was the most suitable HPLC mobile phase for separating iron(III) chlorophyllin components.

3.2. Identification of main components of iron(III) chlorophyllin

The components of peaks 2, 4 and 5 on the three-dimensional chromatogram (Fig. 2) were identified as iron(III) complexes of chlorophyll derivatives on the basis of the blue shift of their Q_y transition from pheophytins and the broadness of their UV–Vis absorption bands. In general, considerable broadening of UV–Vis absorption bands such as Q_x band is brought about and some vibronic bands disappear on insertion of iron ions to pheophytins [8]. The absorption spectrum of peak 3 has a Q_x band at *ca.* 500 nm, whereas that of peak 1 has a strong absorption band in the UV region but no absorption band in the Vis region. Therefore, peaks 3 and 1 are assigned as metal-free chlorophyll and a chromophore other than chlorophylls by means of the three-dimensional chromatogram. The molecular mass of each peak component was examined by FAB mass spectrometry. The mass spectrum of

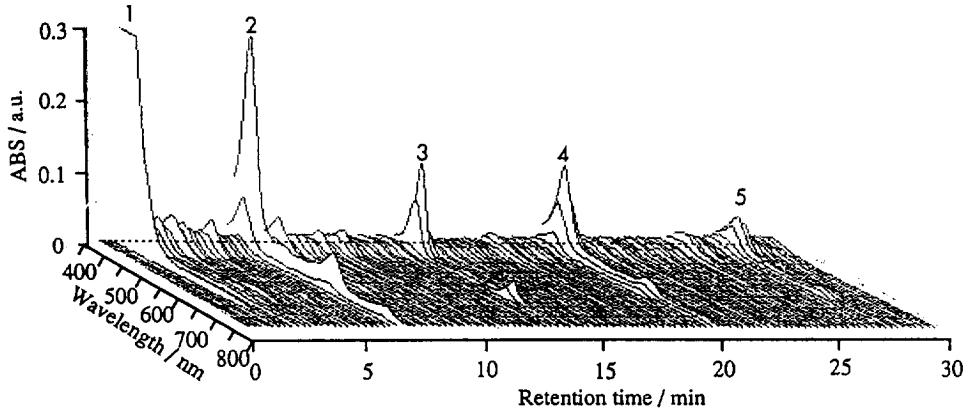

Fig. 2. Three-dimensional chromatogram of iron(III) chlorophyllin. Peaks: 1 = unknown compound; 2 = Fe-Chl e_6; 3 = pheophytin derivative; 4 = Fe-Chl e_4; 5 = Fe-Phed *a*. Column, Inertsil ODS (250 mm × 4.6 mm I.D.); mobile phase, acetonitrile–phosphate buffer (pH 2) (60:40, v/v) containing 0.01 *M* TMACl; flow-rate 1.4 cm^3min^{-1}.

each peak fraction showed the presence of the iron(III) chlorophyllin components with a dechlorinated ion $[M - Cl]^+$ at *ca. m/z* 650 (peak 2), 606 (peak 4) and 646 (peak 5). Consequently, peaks 2, 3 and 5 are identified as Fe-Chl e_6, Fe-Chl e_4 and Fe-Phed *a*, respectively. This is consistent with the assignment based on their chromatographic behaviour such as the elution order of peaks 2, 3 and 4, i.e., the relationship between the number of carboxylic groups and the hydrophobicity of iron(III) chlorophyllin components.

3.3. Preparation of standard materials

It is difficult to prepare iron(III) chlorophyllin in pure form because it is contaminated by its degradation products. Typical degradation processes of M-Phed *a* are allomerization, which is the substitution reaction of a proton with a hydroxyl or methoxyl group on the C-10 position of a cyclopentanone ring, and pyrorization, which is the substitution reaction of a methoxycarbonyl group with a proton on the same position as allomerization [9]. The chromatogram of a reaction mixture is shown in Fig. 3a, where H_2-Phed *a* was reacted with an excess of iron(II) chloride in acetone–methanol (50:50, v/v) at 60°C. Some peaks are observed which might be degradation products, as mentioned above. The use of a smaller amount of the metal salt prevents these degradation reactions (Fig. 3b). When H_2-Chl e_6 was reacted with iron(II) chloride in an acetone–methanol mixture, an

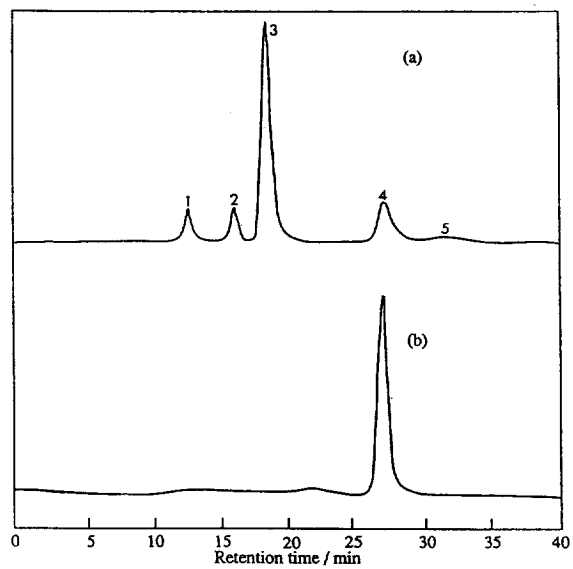

Fig. 3. Chromatograms of Fe-Phed *a*. (a) An excess of iron(III) chloride tetrahydrate (200 molar excess) was added to the H_2-Phed *a* solution. Peaks: 1–3 and 5 = by-products of Fe-Phed *a*; 4 = Fe-Phed *a*. (b) An excess of iron(III) chloride tetrahydrate (10 molar excess) was added to the H_2-Phed *a* solution. HPLC conditions as in Fig. 2.

unknown peak appeared at 13.2 min, but it disappeared when reacted in acetone. The definite assignment of the by-products will be reported in a future publication.

Acknowledgements

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