Preparation of Chlorophyll-a and Chlorophyll-b by Column Chromatography with Sephasorb HP Ultrafine

Masahiko YOSHIURA, Keiji IRIYAMA,* Masaru SHIRAKI, and Akira OKADA

Research Institute for Polymers and Textiles, Sawatari 4-1, Kanagawa-ku, Yokohama 221

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A method for the preparation of chlorophyll-a (Chl-a) and chlorophyll-b (Chl-b) by column chromatography with Sephasorb HP Ultrafine has been developed. The partially purified chlorophyll preparation containing Chl-a, Chl-b and nonsorbed carotenes was applied to a Sephasorb HP Ultrafine column and good separation of Chl-a and Chl-b achieved with a solvent program of (I), a diethyl ether/hexane mixture (1 : 9, volume ratio) followed by 0.2 and 0.5 vol % isopropyl alcohol in (I). 13.1 mg of Chl-a and 7.6 mg of Chl-b were obtained from 24.7 mg of the partially purified chlorophyll using the above method.

One of the methods for separation and isolation of chlorophyll in quantities of 0.01—1 g is column chromatography with powdered sugar. Recently, a method has been developed for the partial purification of chlorophyll-a (Chl-a) and chlorophyll-b (Chl-b) extracted from spinach leaves using dioxane[1] and subsequent washing with 80 vol % aqueous methanol[2] prior to chromatographic separation and isolation. Chl-a and Chl-b in the partially purified chlorophyll preparation were subsequently separated and isolated by passage through a powdered sugar column.[3] Special care is, however, required to prepare a powdered sugar column of good quality with good reproducibility, as powdered sugar has a strong tendency to adsorb moisture from the air and the presence of any trace water affects the separation of the pigments. Angapindu *et al.*[4] reported that cellulose was superior to powdered sugar as an adsorbent since passage through the cellulose columns was faster and the carrying capacity greater. Strain and Sato[5] found that the pigment bands were better defined on sugar rather than on cellulose columns. Shimizu[6] reported that the separation between Chl-a and Chl-b zones could not be performed by column chromatography with Sephadex LH-20 eluted with chloroform, although the mobility of Chl-a was greater than that of Chl-b. Sephadex LH-20 is a hydroxypropyl derivative of cross-linked dextran. Sephasorb HP Ultrafine is similar in structure to Sephadex LH-20, but has a higher matrix density making it suitable for the adsorption of a wide range of compounds soluble in organic solvents. The authors have found that the pigment bands are better defined on Sephasorb HP Ultrafine columns than on powdered sugar or cellulose.

Experimental

Materials. All solvents were of reagent grade further purified, by the methods described.[7] Sephasorb HP Ultrafine was purchased from Seikagaku Kogyo Co.

Preparation of Partially Purified Chl-a and Chl-b. The partially purified chlorophyll preparation was obtained according to the method of Iriyama *et al.*[1,2] All procedures for the preparation were conducted at 5 °C in total darkness or under dim green light. Spinach leaves (100 g fresh weight) were homogenized for 3 min in a Waring blender with chilled acetone (500 ml). The dark green extract obtained was filtered through a paper towel to remove the coarse material and the filtrate centrifuged at 10000×g for 5 min to remove the insoluble materials. The deep-green supernatant solution was employed in the partial purification of chlorophyll by the dioxane method.[1] The chlorophyll preparation, twice precipitated from acetone–dioxane solution by the drop-wise addition of water, was dissolved in methanol (500 ml) containing petroleum ether (125 ml, bp 20—40 °C) and then distilled water (250 ml) was added to the solution. The upper petroleum ether layer was washed with 80 vol % aqueous methanol several times to eliminate the remaining photosynthetic yellow pigments from the solution.[2] The solution was evaporated and dried in a vacuum desiccator to give dark-green microcrystals. Thin-layer chromatographic analysis according to the method of Shiraki *et al.*[8] revealed that the dark-green microcrystals (Ppt III) thus obtained contained Chl-a, Chl-b and nonsorbed carotenes. Ppt III was dissolved in a minimum volume of mixed solvent (diethyl ether: petroleum ether=1 : 9, volume ratio) and the solution (developing solution I) applied to the Sephasorb HP Ultrafine column.

Identification and Determination of Chl-a and Chl-b. The pigments were characterized by comparison of the visible absorption spectra with the literature values. The molar extinction coefficients of Comar and Zscheile[9] were used to determine the purity of the Chl-a and Chl-b preparations as standard values. In addition, the purity and chemical stability of Chl-a and Chl-b were examined by chromatography, since it has been recognized by Strain and Svec[10] that spectroscopic observations need to be supplemented by chromatographic tests to demonstrate that the chlorophyll molecules had not been altered. The purity and chemical stability of the pigments were examined by thin-layer chromatography[8] since the technique was simple and rapid. Where necessary, the thin-layer chromatographic observations were supplemented by high-performance liquid chromatographic tests according to the methods described.[11,12] High-performance liquid chromatography[11,12] is relatively complicated, but has the advantage of greater sensitivity. Qualitatively it was possible to detect the pigments in the order of 10^{-8} g and 10^{-10} g using thin-layer chromatography[8] and high-performance liquid chromatography,[11,12] respectively.

Preparation of a Sephasorb HP Ultrafine Column. A wet column was employed using a Pharmacia R25 chromatographic tube (2.5×45 cm) connected, through the UV monitor (JASCO UVIDEC-100), to a fraction collector. The bed volume (cm^3/g dry Sephasorb HP Ultrafine) in petroleum ether was 1.3. 97 g of Sephasorb HP Ultrafine suspended in petroleum ether. The suspension was poured into the chromatographic tube, providing a 26 cm column. The column thus prepared had a capacity of approximately 30 mg of Chl-a+Chl-b without being overloaded. The column was washed with petroleum ether (1000 ml) before use to eliminate the soluble materials from the Sephasorb HP Ultrafine.

Sephasorb HP Ultrafine Column Chromatography. Percolation of the pigment solution and the developing solvents were accelerated by the application of pressure from a rotary pump (SJ-1210, Mitsumi; flow speed: approximately 21.4 ml h^{-1} cm^{-2}) connected to the top of the chromatographic tube by way of a Teflon tube. Chromatograms were monitored by a monochromatic light of 380 nm and each of the pigment fractions collected by a fraction collector.

Results

All experiments were conducted at 20 °C in total darkness or under a dim green light unless otherwise stated.

The developing solution I containing Chl-a, Chl-b and nonsorbed carotenes was added to the top of the Sephasorb HP Ultrafine column and the column washed with the developing solvent system I (diethyl ether: hexane=1: 9, volume ratio) to adsorb the pigments at the top of the column and to elute the nonsorbed carotenes from the column. Subsequently the column was washed with 0.2 vol % isopropyl alcohol in the solvent system I to elute Chl-a from the column. After most of the Chl-a had been eluted, the column was washed with 0.5 vol % isopropyl alcohol in the solvent system I to accelerate the elution of Chl-b. The elution pattern is presented in Fig. 1, where the void volume of the column used here was about 59 ml. The chromatogram shows complete separation between Chl-a, Chl-b and nonsorbed carotenes. The Chl-a and Chl-b preparation thus obtained were evaporated and dried in a vacuum desiccator.

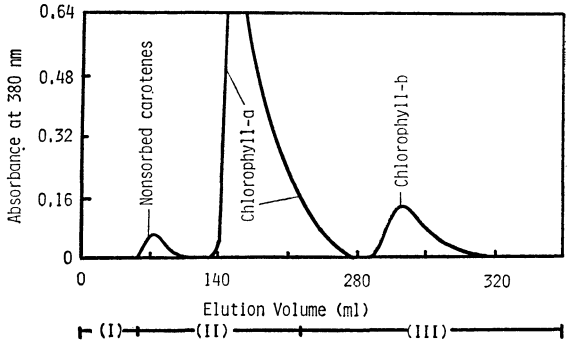

Fig. 1. Chromatogram for Ppt III.[a] Solvent systems for I, II, and III are a diethyl ether/hexane mixture (1: 9, volume ratio), 0.2 vol % isopropyl alcohol in I, and 0.5 vol % isopropyl alcohol in I.
a) For the explanation, see the text.

Thin-layer chromatographic examination of the Chl-a and Chl-b preparations on commercial silica gel sheets showed single spots, respectively. The purity of the Chl-a and Chl-b preparations were further examined by high-performance liquid chromatography[11,12] which supplemented the thin-layer chromatographic observations. The absorption spectra of the Chl-a and Chl-b preparations dissolved in diethyl ether did not show any significant differences in comparison with the literature within experimental error. The ratio of absorbance of the Soret peak at 427.0±1.0 nm to the red peak at 660.0±0.5 nm was 1.30±0.1 for the Chl-a preparation and the ratio of absorbance of the Soret peak at 452.8± 0.3 nm to the red peak at 642.3±0.3 nm was 2.83±0.1 for the Chl-b preparation. The purity of the Chl-a and Chl-b preparations obtained here was greater than 99% on a dry weight basis. 13.1 mg of Chl-a and 7.6 mg of Chl-b were obtained from 24.7 mg of Ppt III using the method developed here. 3.4 mg of nonsorbed carotenes were also isolated. Recovery of the chlorophylls in the column chromatography with Sephasorb HP Ultrafine was about 97%.

The chlorophyll preparations, when isolated in the solid state and stored in evacuated and sealed ampules in total darkness at −20 °C, could be preserved for at least three months without change or alteration. In a few cases, however, the chlorophyll molecules were degraded. For example, Chl-a was converted to chlorophyll-a′ (Chl-a′) and pheophytin-a. Chl-a and Chl-a′ are spectroscopically very similar as well as interconvertible[13] and subsequently spectroscopic observations provide no indication that Chl-a molecules had not altered. It should be noted that the purity of the chlorophyll preparations should be checked by thin-layer or high-performance liquid chromatography before use.

Table 1. Spectral properties of chlorophyll-a and chlorophyll-b with values reported by some authors[a]

	Red peak (nm)	Blue peak (nm)	Absorbance ratio (blue peak/red peak)	Authors
Chlorophyll-a	660.0	429.0	1.32	Comar and Zscheile[b]
	662.0	430.0	1.31	Smith and Benitez[c]
	660.5	428.5	1.30	Strain *et al.*[d]
	660.0 (±0.5)	427.0 (±1.0)	1.30 (±0.1)	This authors
Chlorophyll-b	642.5	453.0	2.82	Comar and Zscheile[b]
	644.0	455.0	2.82	Smith and Benitez[c]
	642.0	452.0	2.84	Strain *et al.*[d]
	642.3 (±0.3)	452.8 (±0.3)	2.83 (±0.1)	This authors

a) Solvent, diethyl ether; temperature, 25 °C.
b) C. L. Comar and F. P. Zscheile, *Plant Physiol.*, **17**, 198 (1942).
c) J. H. C. Smith and A. Benitez, "Modern Methods of Plant Analysis," ed by K. Paeck and M. V. Tracey, Springer-Verlag, Berlin (1955), p. 142.
d) H. H. Strain, M. R. Thomas, and J. J. Katz, *Biochim. Biophys. Acta*, **75**, 306 (1963).

Discussion

The spectral properties of Chl-a and Chl-b in diethyl ether with values reported by some authors are listed in Table 1. The extinction coefficients for Chl-a and Chl-b were not established due to the volatility of diethyl ether at room temperature (20 °C±5). In addition, as diethyl ether has a strong tendency to adsorb moisture from the air and the migration of trace water into chlorophyll solutions in diethyl ether influences the peak positions of Chl-a and Chl-b, the peak positions were shifted to the blue or red regions depending on the water content in solution. Nevertheless, the absorption spectra of the Chl-a and Chl-b preparations dissolved in diethyl ether did not show any significant differences in comparison with the literature values within experimental error as is shown in Table 1. The molar extinction coefficients of Comar and Zscheile,[9] which have been widely used, were used to determine the purity of the Chl-a and Chl-b preparations in this study. The values for the purity of the Chl-a and Chl-b preparations were greater than 99%. Subsequently, the presence of colorless substances as possible contaminants was examined. According to the method of Strain and Svec[10] the preparations were subjected to the test. The purity of the chlorophyll preparations, however, was not higher. In addition, it was found that the Chl-a and Chl-b molecules were degraded during the course of the chlorophyll purification. Thus, the procedure for the elimination of colorless substances from the chlorophyll preparations was not suitable in this case.

There have been no reports on the column chromatographic separation and isolation of chlorophylls with Sephasorb HP Ultrafine and Sephadex LH-20. Sephasorb HP Ultrafine was stable in the solvent systems used in this study as developing solvents. The bed volume obtained on swelling 1 g of dry Sephasorb HP Ultrafine in the solvent systems were relatively small (approximately 1.3 cm^3/g dry gel). No detectable and undesirable substances extracted from Sephasorb HP Ultrafine with the developing solvents used were found. The preparation of Sephasorb HP Ultrafine columns was relatively simple and the column could be used repeatedly for the separation of chlorophylls. In addition, good separation between Chl-a, Chl-b, and nonsorbed carotenes was achieved and Sephasorb HP Ultrafine did not react with chlorophyll molecules to form degradation products of the pigment molecules. For these reasons, Sephasorb HP Ultrafine may be one of the best adsorbents for separating chlorophylls.

The method developed here is suitable for the preparation of Chl-a and Chl-b from Ppt III, which was prepared from spinach leaves according to the method of Iriyama *et al.*[1,2]

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