Journal of Chromatography, 177 (1979) 154–156

CHROM. 11,934

Note

Separation of chlorophyll *a* and chlorophyll *b* by column chromatography with Sephadex LH-20 or powdered sugar

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(First received January 12th, 1979; revised manuscript received April 17th, 1979)

In 1970, Downey *et al.*[1] suggested that chromatography on Sephadex LH-20 (Pharmacia, Uppsala, Sweden) may be used as a preliminary step in the purification of chloroplast pigments. Croft and Howden[2] purified chlorophyll *c* on a Sephadex LH-20 column. Subsequently, successful separation of chlorophylls and carotenoids in a short time has been achieved on Sephadex LH-20 columns by Shimizu[3] and Sato and Murata[4], although chlorophyll *a* (Chl-*a*) and chlorophyll *b* (Chl-*b*) were not separated. Recently, we have been able to separate Chl-*a* and Chl-*b* on a Sephadex LH-20 column. In addition, a new solvent program has been found to separate Chl-*a* and Chl-*b* on a powdered sugar column. In this note, both separations are reported.

MATERIALS AND METHODS

All solvents were analytical-reagent grade and were used without further purification, unless otherwise stated.

The partially purified chlorophyll (Ppt III) containing Chl-*a*, Chl-*b* and non-sorbed carotenes was prepared from fresh spinach leaves by the method of Iriyama *et al.*[5,6].

The purity of the chlorophyll preparations and the chemical stability of the chlorophyll molecules during the course of preparation were monitored by thin-layer chromatography (TLC)[7] and spectrophotometry[8]. The elution patterns of the pigments were monitored at a wavelength of 380 nm. Consecutive fractions corresponding to selected regions of the elution diagrams were evaporated to dryness under reduced pressure, and then dissolved in diethyl ether. The individual pigments of these pooled fractions were identified by TLC.

A Sephadex LH-20 column (26 × 2.5 cm I.D.) was equilibrated with hexane and pre-washed with 200 ml of hexane, to stabilize the column and to elute possible contaminants. A powdered sugar column (65 × 3 cm I.D.), prepared according to the method previously described[9], was washed with *ca.* 300 ml of hexane before use. Percolation of the pigment solutions and of the developing solvents was accelerated by slight pressure from a rotor pump connected to the top of the chromatographic tube by a PTFE tube.

All the experiments were carried out at 15° in total darkness or under dim green light, unless otherwise stated.

RESULTS AND DISCUSSION

Separation on Sephadex LH-20

A 9.3-mg amount of Ppt III was dissolved in 5 ml of diethyl ether–hexane (1:9) and was put on the Sephadex LH-20 column and developed with hexane (I). Chl-*a* and Chl-*b* were adsorbed on top of the column, and the non-sorbed carotenes were separated. On subsequent elution with 0.1% isopropyl alcohol in (II), non-sorbed carotenes were eluted as a single peak and Chl-*a* was separated from Chl-*b*. On continued washing with II, Chl-*a* was eluted as a large peak. By changing the solvent system from II to 0.5% isopropyl alcohol in I, (III), the elution of Chl-*a* and Chl-*b* was accelerated. In addition, after the complete elution of Chl-*a*, the elution of Chl-*b* was further accelerated by washing with diethyl ether (IV). Fig. 1 shows the elution pattern of Ppt III. Good resolution of the pigments in Ppt III was obtained. TLC revealed that the chromatographic fractions from 382 ml to 402 ml contained Chl-*a* and Chl-*b*. In this case, 5.2 mg of Chl-*a*, 2.0 mg of Chl-*b*, 0.3 mg of Chl-*a* + Chl-*b* and 1.7 mg of non-sorbed carotenes were obtained from 9.3 mg of Ppt III. In the column chromatography with Sephadex LH-20, Chl-*a* and Chl-*b* were almost completely recovered.

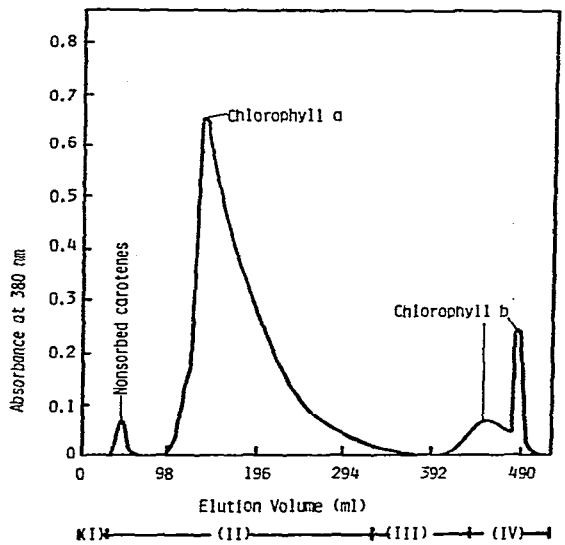

Fig. 1. Chromatogram of Ppt III on a Sephadex LH-20 column monitored at 380 nm with a flow-rate of 16 ml/h · cm². Fractions of 9.8 ml were collected. TLC revealed that the fractions from 98 ml to 382 ml and the fractions from 402 ml to 509.6 ml contained chlorophyll *a* and chlorophyll *b*, respectively. Eluents: I = hexane; II = 0.1% isopropyl alcohol in hexane; III = 0.5% isopropyl alcohol in hexane, IV = diethyl ether.

Separation on powdered sugar

A 23.1-mg amount of Ppt III was dissolved in *ca.* 10 ml of diethyl ether–hexane mixture (1:9) and the solution was added to the top of a powdered sugar column.

The column was washed with a solvent program 0% (V), 6% (VI), 7% (VII), 7.5% (VIII) and 8% (IX) acetone in diethyl ether–hexane (1:9). Fig. 2 shows a typical chromatogram for the pigments in Ppt III. Good resolution of all the

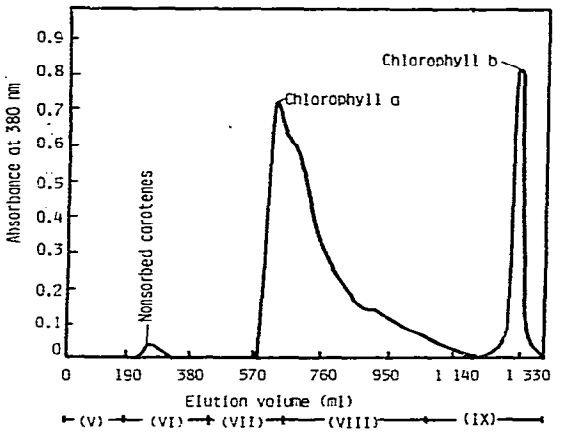

Fig. 2. Chromatogram of Ppt III on a powdered sugar column monitored at 380 nm with a flow-rate of 21.4 ml/h · cm^2. Fractions of 19 ml were collected . TLC tests of the column chromatographic fractions from 589 ml to 1045 ml yielded a single spot corresponding to chlorophyll *a*. Eluents: V = 0%, VI = 6%, VII = 7%, VIII = 7.5% and IX = 8% acetone in diethyl ether–hexane (1:9).

pigments was obtained. Nonsorbed carotenes were eluted as a single peak near the void volume (V_e, 230 ml). Solvent system V was used to dissolve Ppt III and enable the pigments to adsorb at the top of the column. Solvent system was used as an eluent to investigate the possible presence of degradation products of Chl-*a* formed in Ppt III. Subsequent elution of the column with the solvent systems VII and VIII, eluted Chl-*a* as a large peak with two shoulders. The appearance of two shoulders may be due to changing the developing solvent system twice. TLC tests revealed that the chromatographic fractions from 589 ml to 1045 ml gave only one spot corresponding to Chl-*a*. The elution of Chl-*a* fractions was accelerated by washing with IX. TLC tests showed that the fractions from 1045 to 1235 ml contained both pigments, Chl-*a* and Chl-*b*, and that the fractions from 1235 ml to 1425 ml contained only Chl-*b*. In this case, 13.0 mg of Chl-*a*, 4.4 mg of Chl-*b* and 2.3 mg of Chl-*a* + Chl-*b* were obtained from 23.1 mg of Ppt III.

The presence of degradation products of chlorophylls and yellow leaf pigments other than nonsorbed carotenes in Ppt III interfered with the column chromatographic separation and the isolation of Chl-*a* and Chl-*b* in both methods developed here. For this reason, Ppt III should be checked by TLC before attempting chromatographic separation of Chl-*a* and Chl-*b*. Chl-*a* and Chl-*b* in Ppt III stored at −20° in total darkness were chemically stable for at least one week.

The methods reported here may be recommended as routine for the preparation

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