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Separation of chloroplast pigments on Sephadex LH-20

A method has been developed for separating chloroplast pigments by Sephadex LH-20 (Pharmacia, Uppsala, Sweden) column chromatography. Column chromatographic procedures for the isolation of chlorophylls, carotenes and xanthophylls, using many kinds of adsorbent (*e.g.*, sugar, cellulose powder, magnesia, alumina etc.), have been established for many years[1,2]. However, each of these methods is only effective for a limited number of pigments and cannot be used for a wide range of pigments. For example, the author was unable to obtain a good separation of chlorophyll from its derivatives, *viz.*, pheophytin, pheophorbides and chlorophyllides, with the powdered sugar column chromatography which is reported to be the routine procedure for separating chlorophylls *a* and *b* (ref. 3).

For the purpose of this investigation, the molecular sieve nature of Sephadex LH-20 could not be relied upon to separate these chlorophyll derivatives, because there is only a small difference in molecular weight among these compounds. In fact, when the chromatogram was developed with 80% methanol instead of chloroform, they were so slightly separated from each other that they appeared as only one broad band. This difficulty could not be overcome by changing the eluent from methanol to acetone, ether, benzene or a mixture of them. DOWNEY *et al.*[4] reported the separation of chloroplast pigments of *Poa tivialis* by means of a Sephadex LH-20 column equilibrated with petroleum ether and eluted with petroleum ether–diethyl ether (80:20). They failed to separate the individual pigments, and β-carotene and pheophytin were eluted as the first band followed by two wide bands of carotenoid and chlorophyll compounds.

It has been reported that the particles of Sephadex LH-20 show a specific affinity towards compounds having a –COOH group, in chloroform[5]. Among the chlorophyll compounds which are commonly observed in plant extracts, those which have a –COOH group are the chlorophyllides and pheophorbides and they seem to be produced by enzymic hydrolysis of chlorophylls and pheophytins, respectively. When laminae of green plants are homogenized with a sufficient amount of magnesium carbonate in the cold, there is no detectable release of chlorophyllides, pheophytins or pheophorbides (except in a few cases such as some tobacco variants[6]).

In this investigation, the potential of Sephadex LH-20 for the separation of chlorophyll compounds and carotenoids was demonstrated and some of the results are briefly reported.

Experimental

Plastid pigments were extracted from the chloroplasts prepared from laminae of *Ambrosia artemisiaefolia* L. according to a method reported previously[7]. The chloroplasts were suspended in 80% acetone and stirred gently at room temperature under dim light. We had found that the leaves of this plant have a markedly high chlorophyllase activity. Under the above mentioned conditions, the chlorophyllase located in the isolated chloroplasts appears to react on the chlorophyll and organic acids also react on the chlorophyll itself and on its enzymically hydrolyzed products. After 60 min, the suspension was filtered through filter paper and the extraction was

repeated until the residue became colourless. The extracts were combined and transferred to a separating funnel containing a half volume of ethyl ether. After shaking well and separating into two distinct layers, the upper green layer was washed well with 10 % aqueous NaCl solution. The washed ether layer was dried with anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The remaining pigments were dissolved in a small volume of chloroform.

Sephadex LH-20 was swollen in chloroform–methanol (70:30) for 24 h and poured into a column (800 × 20 mm) with stirring to a height of about 60 cm. The suspension medium was replaced with chloroform–methanol (95:5) and finally with chloroform only. When the medium was completely replaced with chloroform the colour of the column changed from white to semi-translucent white and the height of the column bed increased to about 70 cm. After the sample had been applied on the top of the column bed, it was necessary to add a small volume of eluent (chloroform) gradually, because the specific gravity of chloroform is greater than that of the Sephadex particles (sp.gr. = 1.3) and the upper part of the column bed floats at the surface of the eluent. Development with chloroform was continued until the last yellow band was eluted from the column. The second solvent was the mixture chloroform–methanol (97:3); this was used to remove the slow moving green band. The green band remaining at the top of the column bed was developed with chloroform–methanol (70:30). The elution patterns of the chloroplast pigments were monitored spectrophotometrically. Consecutive fractions corresponding to selected regions of the elution diagrams were evaporated to dryness under reduced pressure, and dissolved in 0.5 ml of chloroform. The individual pigments of these pooled fractions were identified by TLC on silica gel or cellulose powder layers. The developing solvents and adsorbents used for TLC are as follows: (1) hexane–acetone (60:40)/silica gel[8]; (2) petroleum ether (60–80°)–acetone–*n*-propanol (90:10:0.45)/cellulose powder[9]; (3) ligroin (100–140°)–chloroform–isopropanol (90:70:10)/silica gel[10].

The separated pigments on the adsorbent layers were detected under UV light and the adsorbent on which the compounds were located was scraped from the glass plate. The compounds were eluted from the adsorbent with appropriate solvents (*i.e.*, ethyl ether for chlorophyll compounds; benzene, chloroform, carbon disulphide and ethanol for carotenoids). The absorption spectra of each pigment were determined in two or three kinds of solvent and compared with those of known pigments[1,2].

Results and discussion

As shown in Fig. 1, the four chlorophyll compounds — chlorophyll, chlorophyllide, pheophytin and pheophorbide — were separated very well. The clear separations observed between chlorophyll and pheophytin, and also chlorophyllide and pheophorbide suggest that the presence or absence of a Mg atom in the molecule causes the difference in the readiness to pass through the molecular sieve of Sephadex LH-20 in chloroform. Chlorophylls *a* and *b* could not be made to emerge as separate peaks. However, the examination of the component of the fractions by TLC indicated that the mobility of chlorophyll *a* was greater than that of *b*, suggesting that it should be possible to obtain a fraction containing a very large proportion of chlorophyll *a* (Fig. 2). Pheophytin and carotene (mainly *β*-carotene) were eluted from the column in this order, but when the flow rate of the eluent was greater than 2 ml per 10 min, these two compounds were eluted in the same fraction or in the reverse order. The

three orange-yellow compounds were identified as crysanthemaxanthin, auroxanthin and lutein from the top to the bottom. The two other minor compounds could not be identified.

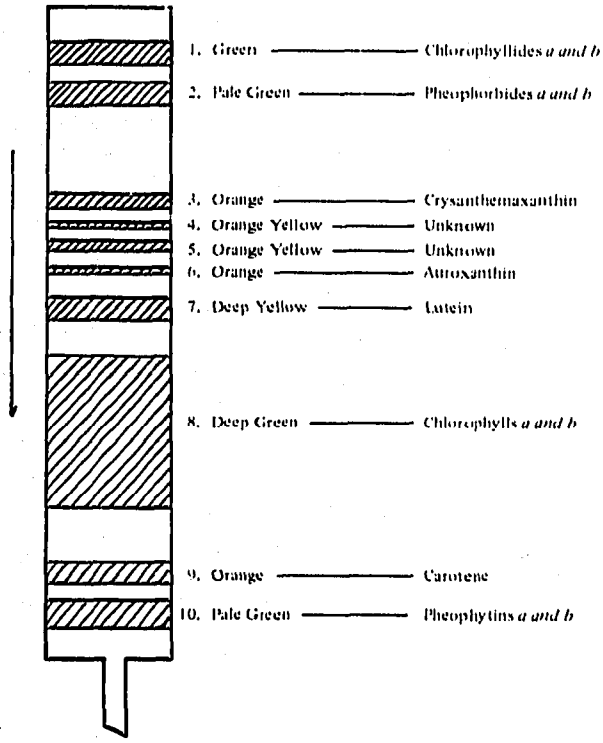

Fig. 1. Chromatographic separation of chloroplast pigments from *Ambrosia artemisiaefolia* L. on a Sephadex LH-20 column equilibrated and eluted with chloroform.

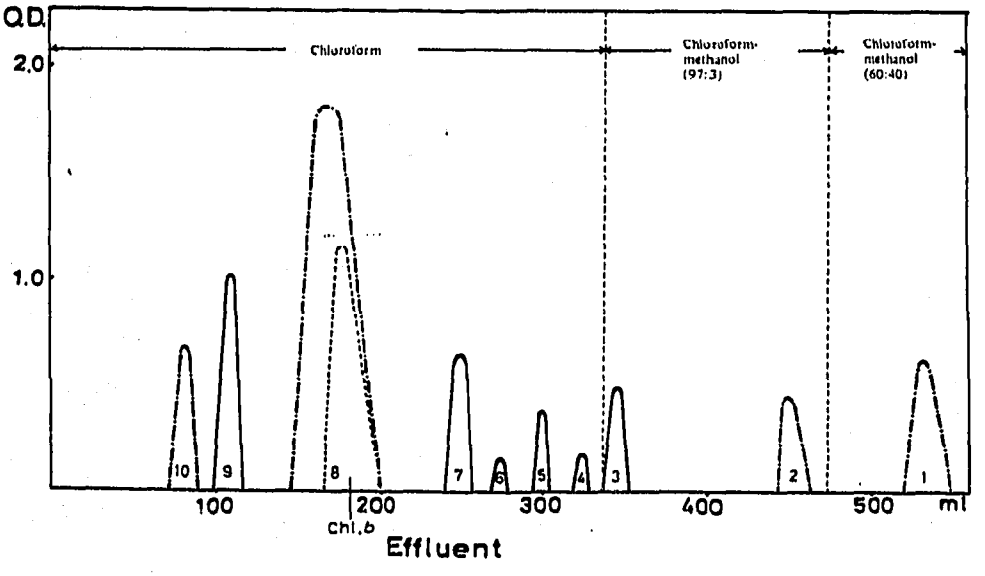

Fig. 2. Fractionation of chloroplast pigments from *Ambrosia artemisiaefolia* L. on a Sephadex LH-20 column equilibrated with chloroform. (–·–·–) absorbance due to carotenoid pigments at 455 mμ. (———) absorbance due to chlorophyll compounds at 655 mμ. Further details are given in the text.

When the period of stirring the chloroplast was prolonged to 10–24 h, three or four green bands were observed between the band of chlorophyll and that of lutein. The compounds corresponding to these bands had absorption spectra which were similar to those of chlorophylls *a* and *b* but clearly differed from them. These chlorophyll compounds seemed to be the chlorophylls *a'* or *b'* of STRAIN[3] or "changed chlorophyll" of BACON AND HOLDEN[9].

It is considered that this method of separating chlorophyll pigments may be suitable for measuring the chlorophyllase activity of plant materials. The enzymic activity has been expressed, in general, in terms of the amount of chlorophylls hydrolyzed (or that of chlorophyllides formed) during the reaction period. Unfortunately chlorophylls and chlorophyllides have the same absorption spectrum[11]; therefore in order to determine the amount of the latter spectrophotometrically, it is necessary to separate these two compounds completely. This has been carried out on the basis of the differences in their solubility towards organic solvents and their mobility on paper chromatograms[7]. These procedures, however, are of questionable accuracy in quantitative terms and troublesome to carry out. The present method is therefore considered to be particularly appropriate for this purpose. In fact, it seemed to be better to use a mixture of chloroform and methanol (95 : 5) as eluent instead of chloroform and also a shorter column (about 10 cm) for measuring chlorophyllase activity. Development with this chloroform–methanol mixture gave a rather incomplete separation of xanthophylls, but the time consumed in separating the chlorophylls from their enzymic products and eluting the latter from the column was shortened to a certain degree.

Department of Biology, Faculty of Science, Ochanomizu University, Ostuka, Bunkyo-ku, Tokyo (Japan) SEKI SHIMIZU

1 M. HOLDEN, in T. W. GOODWIN (Editor), *Chemistry and Biochemistry of Plant Pigments*, Academic Press, London, 1965, p. 462.
2 B. H. DAVIES, in T. W. GOODWIN (Editor), *Chemistry and Biochemistry of Plant Pigments*, Academic Press, London, 1965, p. 489.
3 H. H. STRAIN, in *Chloroplast Pigments and Chromatographic Analysis, 32nd Annual Priestley Lectures*, Penn. State Univ., Univ. Park, Pa., 1958.
4 W. K. DOWNEY, R. F. MURPHY AND M. K. KEOGH, *J. Chromatogr.*, 46 (1970) 120.
5 *Technical Bulletin of Sephadex LH-20*, Pharmacia Fine Chemicals, Uppsala, Sweden, 1966.
6 S. SHIMIZU, R. HOTTA AND E. TAMAKI, *Bot. Mag. (Tokyo)*, 79 (1966) 560.
7 S. SHIMIZU AND E. TAMAKI, *Bot. Mag. (Tokyo)*, 75 (1962) 462.
8 C. P. BOND, *J. Sci. Food Agr.*, 18 (1967) 161.
9 M. F. BACON AND M. HOLDEN, *Phytochem.*, 6 (1967) 193.
10 E. KESSLER AND F. C. CZYGAN, *Ber. Deut. Bot. Ges.*, 78 (1966) 342.
11 S. SHIMIZU AND E. TAMAKI, *Arch. Biochem. Biophys.*, 102 (1963) 152.

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