Journal of Chromatography, 134 (1977) 359–364

CHROM. 9788

THIN-LAYER CHROMATOGRAPHY OF CHLOROPHYLLS AND THEIR DERIVATIVES ON CELLULOSE LAYERS

GUNNEL SIEVERS
Department of Biochemistry, University of Helsinki, Unioninkatu 35, SF-00170 Helsinki (Finland)
and
PAAVO H. HYNNINEN
Department of Biochemistry, University of Kuopio, P.O.B. 138, SF-70101 Kuopio (Finland)
(Received September 17th, 1976)

SUMMARY

A method for the separation and identification of chlorophyll *a* and *b*, pheophytin *a* and *b*, pheophorbide *a* and *b*, chlorin e_6, rhodin g_7 and the corresponding chlorophyll *a'* and *b'* derivatives, as well as the saponification products of pheophorbide *a* and *b*, by thin-layer chromatography on commercial cellulose layers on the micro-scale has been developed. Two solvent systems were used: light petroleum (b.p. 60–80°)–pyridine (9:1, v/v) and *n*-heptane–pyridine (7:3, v/v). The former was suitable for chlorophylls, pheophytins and pheophorbides, and the latter for pheophorbides, chlorin, rhodin and their esters. The separation of the derivatives was good and no chemical alteration of the derivatives could be observed. The method is rapid and easy to use and is therefore suitable for checking the purity of derivatives during the preparation of chlorophylls and their derivatives.

INTRODUCTION

Thin-layer chromatography (TLC) has been widely used for several years for the separation of chloroplast pigments[1,2]. Inorganic adsorbents, such as silica gel and Kieselguhr, however, cause chemical alterations of chlorophyll derivatives[2–5]. Using specially prepared organic thin layers of sucrose[6–8], glucose[9,10] or cellulose[11,12], the recovery of chlorophylls is over 90%, showing that fewer alterations occur. The reversed-phase TLC of chlorophylls on oil-impregnated Kieselguhr layers is also a mild method for the separation of chlorophylls[13–15], but it is laborious and cannot be used in preparative separations as the impregnation oil is eluted with the chlorophyll derivatives.

In this study, a method for the separation of chlorophylls and their derivatives on commercial cellulose layers has been developed. The method is rapid and can be used on the micro-scale for checking the purity of the derivatives during their preparation and can also be used for preparative separations.

EXPERIMENTAL

Chlorophylls and derivatives

Chlorophylls *a* and *b* were isolated from frozen clover leaves by the improved two-phase extraction method[16] followed by separation on a sucrose column[16]. A single fractionation yielded chlorophyll *a*, which had the following spectroscopic properties in diethyl ether: 660.0 (1.28), 613.0 (8.18), 575.5 (15.0), 531.0 (25.2), 500.5 (46.7), 428.0 (1.00), 408.5 (1.49), 380.5 (2.42) nm, where the numbers in parentheses are the *R* values (*R* = quotient of absorbance at Soret band divided by absorbance at wavelength indicated). Chlorophyll *a'* was obtained by re-fractionation of chlorophyll *a* on a sucrose column. The visible absorption spectrum of chlorophyll *a'* was virtually identical with that of chlorophyll *a*.

The first sucrose column fractionation yielded chlorophyll *b*, which was contaminated with lutein. Chlorophyll *b* was further purified by first extracting lutein from the effluent solution of the pigments with 70% (w/w) aqueous methanol (6 × 300 ml). The light petroleum solution was then washed three times with distilled water and evaporated approximately to dryness at reduced pressure. The residue was dissolved in 3 ml of the eluent (light petroleum, b.p. 60–80°, containing 0.5% of 1-propanol) and the solution was again evaporated approximately to dryness. The final sample for re-chromatography was obtained by dissolving the residue in 3 ml of the eluent. The second sucrose column fractionation yielded chlorophyll *b*, which had the following spectroscopic properties in diethyl ether: 642.0 (2.81), 593.5 (13.8), 452.5 (1.00), 429.0 (2.51) nm. The second sucrose column fractionation also yielded chlorophyll *b'*, which was spectroscopically identical with chlorophyll *b*.

Pheophytin and pheophorbide *a* and *b* were prepared from chlorophyll *a* and *b* by shaking an ethereal solution of the chlorophyll with 13 and 30% (w/w) hydrochloric acid, respectively, for 5 min[17,18]. The corresponding *a'* and *b'* compounds were prepared from chlorophyll *a'* and *b'* in a similar manner.

Saponifications of pheophorbide *a* and *b* with 0.5 and 30% (w/w) methanolic potassium hydroxide solutions were performed as described previously[18].

Chlorin e_6 was an old preparation isolated by multiple liquid–liquid partition[18] (component A in Fig. 3, ref. 18). The preparation had been standing in the dark at 4° in diethyl ether solution exposed to air.

Rhodin g_7 was also an old preparation isolated by multiple liquid–liquid partition[18] (component A in Fig. 4, ref. 18). It had been standing under conditions similar to those for chlorin e_6.

Reagents

All reagents were of analytical grade and used as received, except for pyridine, which was redistilled over potassium hydroxide pellets and stored at —16°, and formamide, which was distilled *in vacuo* according to Verhoek[19].

Thin-layer chromatography

Commercial cellulose sheets (TLC aluminium sheets without fluorescence indicator, E. Merck, Darmstadt, G.F.R.) were cut into smaller plates (10 × 10 cm). Solutions of chlorophyll derivatives in diethyl ether or light petroleum were spotted with a 2-μl micropipette 1 cm from the lower edge. The chromatograms were develop-

ed in thin-layer chambers lined with chromatography paper, the atmosphere in the chamber being pre-equilibrated with the developing solvent for 15 min before the plates were inserted. The developing solvents used were (I) light petroleum (b.p. 60–80°)–pyridine (9:1, v/v) and (II) *n*-heptane–pyridine (7:3, v/v). After the solvent front had ascended 8–9 cm (requiring about 10 min) at room temperature, the plates were dried under a fan and photographed under ultraviolet light (365 nm). Excellent separation was also achieved on smaller plates (5 × 5 cm), which were developed in small museum jars.

RESULTS AND DISCUSSION

Fig. 1 shows the thin-layer chromatogram of chlorophyll, pheophytin and pheophorbide *a* and *b* developed in solvent system I. In neither series are the chlorophylls and pheophytins separated completely from each other, but they are easily distinguished by their colour in white light (chlorophyll *a* is blue-green, chlorophyll *b* is yellow-green and pheophytins are grey). Pheophytin *b* also contains some faster moving material (R_F 0.86), which has not been identified. Pheophorbide *a* and *b* (both containing trace amounts of the corresponding pheophytins) are separated excellently from the other compounds. In solvent system I, chlorin e_6 and rhodin g_7 move very slowly (Table I) and it was therefore necessary to develop another system for separating chlorin and rhodin from the corresponding phorbins.

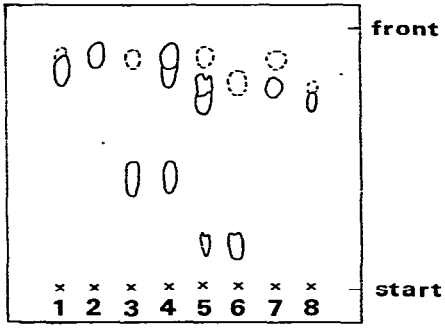

Fig. 1. Thin-layer chromatogram of chlorophyll *a* and *b* and their derivatives on a cellulose layer. Solvent, light petroleum–pyridine (9:1, v/v); plate size, 10 × 10 cm; distance of solvent front from start, 8 cm. 1 = Chlorophyll *a*; 2 = pheophytin *a*; 3 = pheophorbide *a* (containing a trace amount of pheophytin *a*); 4 = mixture of chlorophyll *a*, pheophytin *a* and pheophorbide *a*; 5 = mixture of chlorophyll *b*, pheophytin *b* and pheophorbide *b*; 6 = pheophorbide *b* (containing a trace amount of pheophytin *b*); 7 = pheophytin *b* (containing a small amount of an unknown component); 8 = chlorophyll *b*. The dotted spots are minor components.

In solvent system II, containing more of the polar solvent, the chlorophylls and pheophytins move almost with the solvent front, but good separations of pheophorbide *a* from chlorin e_6 (Fig. 2) and pheophorbide *b* from rhodin g_7 (Fig. 3) are achieved. Fig. 2 shows that the products from the saponification of pheophorbide *a* in 30% potassium hydroxide solution in methanol are also separated. The two slowest moving derivatives were separated only partially from each other, but they could be clearly distinguished on account of their different colours under ultraviolet light

TABLE I

R_F VALUES OF CHLOROPHYLL *a* AND *b* AND THEIR DERIVATIVES IN SOLVENT SYSTEMS I (LIGHT PETROLEUM–PYRIDINE, 9:1, v/v) AND II (*n*-HEPTANE–PYRIDINE, 7:3, v/v)

The R_F values are mean values from several chromatograms.

Derivative	*Solvent system*	
	I	*II*
Chlorophyll *a'*	0.85	
Chlorophyll *a*	0.84	
Chlorophyll *b'*	0.69	
Chlorophyll *b*	0.68	
Pheophytin *a'*	0.90	
Pheophytin *a*	0.88	0.90
Pheophytin *b'*	0.82	
Pheophytin *b*	0.76	0.94
Pheophorbide *a'*	0.49	
Pheophorbide *a*	0.42	0.59
Pheophorbide *b'*	0.16	
Pheophorbide *b*	0.14	0.49
Purpurin-18		0.56
b-Purpurin-18		0.46
Chlorin e_6	0.02	0.34
Rhodin g_7	0	0.16

(366 nm). The slower moving compound (R_F 0.32) appeared as a blue spot while the slightly faster migrating compound (R_F 0.34) had a red colour similar to that of chlorin e_6. The blue component presumably represents chlorin k[18] while the red component is obviously free chlorin e_6. The principal derivative from this saponification moves in the middle (R_F 0.56) and represents purpurin-18 while the fastest moving compound (R_F 0.84) is possibly 10-methoxypheophorbide *a*[18]. The products from the saponification of pheophorbide *a* with 0.5% potassium hydroxide solution in methanol

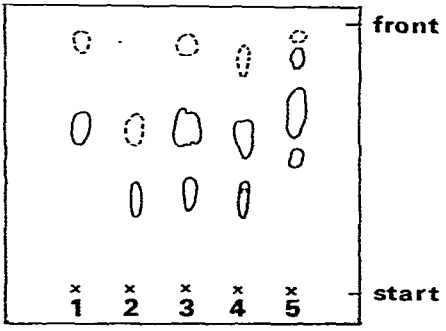

Fig. 2. Thin-layer chromatogram of chlorophyll *a* derivatives on a cellulose layer. Solvent, *n*-heptane–pyridine (7:3, v/v); plate size, 10 × 10 cm; distance of solvent front from start, 8.5 cm. 1 = Pheophorbide *a* (containing some pheophytin *a*); 2 = chlorin e_6 (containing some pheophorbide *a*); 3 = mixture of pheophorbide *a* and chlorin e_6; 4 = products of the saponification of pheophorbide *a* in 30% potassium hydroxide solution in methanol; 5 = products of the saponification of pheophorbide *a* in 0.5% potassium hydroxide solution in methanol.

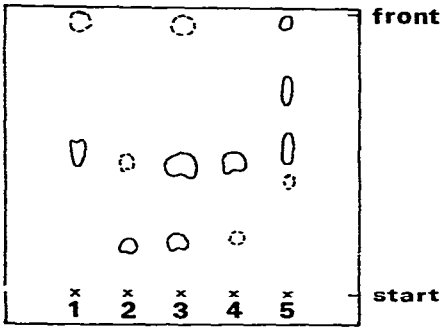

Fig. 3. Thin-layer chromatogram of chlorophyll *b* derivatives. Conditions as in Fig. 2. 1 = Pheophorbide *b* (containing some pheophytin *b*); 2 = rhodin g_7 (containing a small amount of pheophorbide *b*); 3 = mixture of pheophorbide *b* and rhodin g_7; 4 = products of the saponification of pheophorbide *b* in 30% potassium hydroxide solution in methanol; 5 = products of the saponification of pheophorbide *b* in 0.5% potassium hydroxide solution in methanol.

give chlorin e_6 methyl esters[18], which are separated as four spots according to the degree of esterification.

The corresponding chromatography of chlorophyll *b* derivatives in solvent system II is shown in Fig. 3. As expected, pheophorbide *b* (containing a small amount of pheophytin *b*) and rhodin g_7 move less than the *a* derivatives (Table I), but are separated well. The saponification of pheophorbide *b* in 30% potassium hydroxide solution in methanol gives one major product (R_F = 0.46), which probably represents *b*-purpurin-18[18], and trace amounts of rhodin g_7. The products from the saponification with 0.5% potassium hydroxide solution in methanol yields the methyl esters of rhodin g_7[18], which are separated into four spots, according to the degree of esterification.

TLC of chlorophyll *a* and *a'* (numbers 1 and 2 in Fig. 4) and chlorophyll *b* and *b'* (numbers 1 and 2 in Fig. 5) shows that the *a'* and *b'* derivatives are not separated from the original chlorophylls in solvent system I. However, chlorophyll *a'* and *b'* each yield two spots, the faster moving of which represents pheophytin *a'* and *b'*. This result agrees well with the earlier observation[20] that chlorophyll *a'* has a great

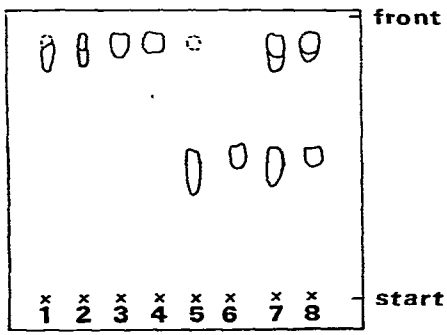

Fig. 4. Thin-layer chromatogram of chlorophyll *a* and *a'* derivatives. Conditions as in Fig. 1. 1 = Chlorophyll *a*; 2 = chlorophyll *a'*; 3 = pheophytin *a*; 4 = pheophytin *a'*; 5 = pheophorbide *a*; 6 = pheophorbide *a'*; 7 = mixture of chlorophyll *a*, pheophytin *a* and pheophorbide *a*; 8 = mixture of chlorophyll *a'*, pheophytin *a'* and pheophorbide *a'*.

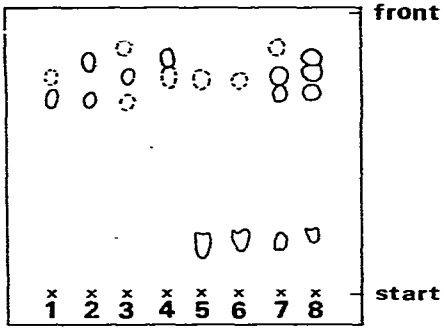

Fig. 5. Thin-layer chromatogram of chlorophyll *b* and *b'* derivatives. Conditions as in Fig. 1. 1 = Chlorophyll *b*; 2 = chlorophyll *b'*; 3 = pheophytin *b*; 4 = pheophytin *b'*; 5 = pheophorbide *b*; 6 = pheophorbide *b'*; 7 = mixture of chlorophyll *b*, pheophytin *b* and pheophorbide *b*; 8 = mixture of chlorophyll *b'*, pheophytin *b'* and pheophorbide *b'*. The dotted spots are minor components.

tendency to be converted into a pheophytin *a*-like pigment. Pheophytin *b* contains a small amount of chlorophyll *b* and also some faster moving material (R_F 0.86), which is not identical with pheophytin *b'* (numbers 3 and 4 in Fig. 5). Pheophorbide *a'* and *b'*, obtained from chlorophyll *a'* and *b'*, appear to move slightly faster than pheophorbide *a* and *b*. It remains to be shown whether these differences in the R_F values (Table I) result from differences in concentration or whether they are caused by epimerization at C-10[21] and/or hydrogen chelation[20].

The results in this study show that TLC on commercial plates on the microscale with the two solvent systems used is a rapid and mild method for separating chlorophylls and their derivatives. It does not require laborious sample or TLC plate handling and is easy to use for checking the purity of chlorophyll derivatives in preparative work.

REFERENCES

1 Z. Sesták, *Photosynthetica*, 1 (1967) 269.
2 H. H. Strain and W. A. Svec, *Advan. Chromatogr.*, 8 (1969) 119.
3 M. F. Bacon, *Biochem. J.*, 101 (1966) 340.
4 H. H. Strain, J. Sherma and M. Grandolfo, *Anal. Chem.*, 39 (1967) 926.
5 J. Sherma, *J. Chromatogr.*, 61 (1971) 202.
6 B. Colman and W. Vishniac, *Biochim. Biophys. Acta*, 82 (1964) 616.
7 D. Nutting, M. Voet and R. Becher, *Anal. Chem.*, 37 (1965) 445.
8 S. W. Jeffrey, *Biochim. Biophys. Acta*, 162 (1968) 271.
9 J. C. Madgwick, *Deep-Sea Res.*, 12 (1965) 233.
10 J. C. Madgwick, *Deep-Sea Res.*, 13 (1966) 459.
11 M. F. Bacon, *J. Chromatogr.*, 17 (1965) 322.
12 H. A. W. Schneider, *J. Chromatogr.*, 21 (1966) 448.
13 K. Egger, *Planta*, 58 (1962) 664.
14 I. D. Jones, L. S. Butler, E. Gibbs and R. C. White, *J. Chromatogr.*, 70 (1972) 87.
15 R. J. Daley, C. B. J. Gray and S. R. Brown, *J. Chromatogr.*, 76 (1973) 175.
16 P. H. Hynninen, *Acta Chem. Scand.*, submitted for publication.
17 P. H. Hynninen and N. Ellfolk, *Acta Chem. Scand.*, 27 (1973) 1463.
18 P. H. Hynninen, *Acta Chem. Scand.*, 27 (1973) 1771.
19 F. H. Verhoek, *J. Amer. Chem. Soc.*, 58 (1936) 2577.
20 P. H. Hynninen, *Acta Chem. Scand.*, 27 (1973) 1487.
21 J. J. Katz, G. D. Norman, W. A. Svec and H. H. Strain, *J. Amer. Chem. Soc.*, 90 (1968) 6841.