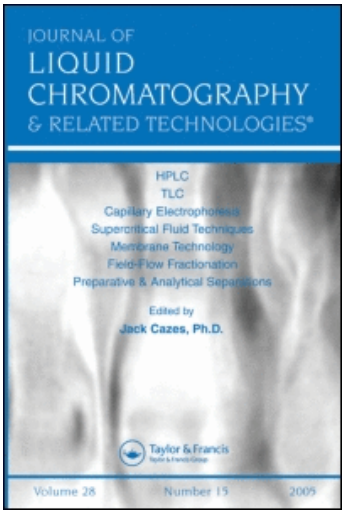

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Preparation and Purification of Chlorophylls, Bacterio-Chlorophylls and of Their Derivatives

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PREPARATION AND PURIFICATION OF CHLOROPHYLLS, BACTERIOCHLOROPHYLLS AND OF THEIR DERIVATIVES

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ABSTRACT

Rapid purification of chlorophylls and bacteriochlorophylls (2–4 hours), at the level of 20–100 mg, is described, combining preseparation on reversed phase cartridges and purification on C18 preparative HPLC columns.

Several derivatives (pyro, oxidized or reduced compounds, pheophytins) are prepared from these chlorophylls and purified by HPLC.

U.V. visible, infrared and NMR spectroscopy are used for identification of these derivatives and analytical HPLC for the evaluation of their purity.

INTRODUCTION

Pure derivatives of chlorophylls and bacteriochlorophylls, such as pyroderivatives, pheophytins, reduced or oxidized derivatives, are needed for spectroscopic and physicochemical studies in photosynthesis (1) or as standards in chromatographic analysis. Different methods for HPLC

separation of chlorophylls and their derivatives have been described, only a few of them at the preparative scale (2–6), sometimes requiring a long prepurification step with dioxan (6).

We have previously developed a purification method for Bacteriochlorophyll a (BChl a) and Bacteriochlorophyll b (BChl b) (7), using preseparation on C18 cartridges, already described for analytical purposes, followed by HPLC on reversed phase column. This procedure allows to purify 20–100 mg in 2–4 hours, avoiding contact with active material, so that allomerization and epimerization products, difficult to separate from chlorophylls, are reduced to minimum. In this work, preparation and purification of fifteen chlorophylls and derivatives are described.

MATERIAL AND METHODS

All derivatives were prepared from various chlorophylls extracted from plants or bacteria.

1) Chlorophyll extraction

Chlorophyll a and b were extracted from spinach leaves by grinding in a Waring blendor with methanol (4 volumes per g. of wet weight). The same operation was repeated twice. The resulting solution was filtrated on glass filter and water was added up to the concentration of 20 %.

Pigments were separated from polar soluble compounds by rapid passage under pressure through Sep Pak C18 cartridges (Millipore–Waters), for small quantities (≈ 10 mg) or by suction through a small column (3–5 cm high, 2 cm diameter) of reversed phase powder (particle size 40 nm, J.F. Baker Co), for larger amounts of chlorophylls (≈ 100 mg). Polar impurities were eliminated by washing with methanol containing 20 % H_2O, then chlorophyll a and b and several carotenoids were eluted by pure ethanol.

Bacteriochlorophyll a was extracted from *Rhodospirillum rubrum* and Bacteriochlorophyll b from *Rhodopseudomonas viridis*, by vigorous stirring with methanol (4 volumes per gram of wet weight of centrifuged cells). Three repetitions were necessary for complete extraction. Then, prepurification was performed on Sep Pak cartridges or reversed phase column as above. In the case of BChl a, for a better fixation on the resin, the water content of methanol was brought to 30 %.

The ethanolic solutions of pigments were evaporated under vacuum and stored under nitrogen at – 20°C when they were not used immediately. All operations were performed in dim light. The Sep Pak cartridges and the reversed phase powder are reusable several times after extensive washing with acetone.

2) Chlorophyll purification and analysis

These procedure were performed by HPLC with a Waters liquid chromatograph (M 510 pump, U_6K injector, M481 or 490E detectors) on Partisil 10 ODS2 columns (20 mm x 50 cm or 9 mm x 50 cm) (Whatman) for preparative runs and on μ Bondapak C18 columns (3.9 mm x 30 cm) (Waters) for analytical runs. Ethanol–water mixtures were used for elution. Respective percentages were choosen as to have sufficient resolution in a reasonable time (10 – 20 min). They depend on the size of the column and the nature of the chlorophyll (table 1).

3) Pheophytin preparation and purification

Pheophytinization was performed either on a mixture of chlorophylls a and b, obtained after prepurification on Sep Pak cartridges, or on separated chlorophylls or bacteriochlorophylls. It was carried out as in (6) by a stream of nitrogen passing through a solution of concentrated HCl at the rate of 100 ml/mn, bubbling into the solution of chlorophylls in ethyl ether (2–5 ml) at 0°C during one to five minutes. The spectrum was recorded as a function of time and the reaction was stopped by flushing the solvent by nitrogen, when the spectrum corresponded to that of pheophytin or mixtures of pheophytins. Dithiothreitol (5 mg/ml) was added in the case of the preparation of B Pheophytin b, to prevent oxidation which occurs very rapidly (increase of the 680 nm peak).

Purification was then achieved with the same columns as for the chlorophylls, with pure ethanol as solvent, except for B Pheophytin b, which was purified on Partisil 10 (Whatman) with chloroform containing 1 % ethanol as eluent.

4) Pyroderivatives

Pyroderivatives were obtained by keeping pyridin solutions of chlorophyll or pheophytin in an evacuated sealed vial for 15 h at 100°C (8). The solution was then evaporated under vacuum and purification was carried out as for the corresponding chlorophyll or pheophytin.

5) Oxidation product

10–0H BChl a was prepared by action of light (≈ 100 watts), air and LiCl (10 mg/ml) for 2 hours on BChl a in solution in ethanol, at room temperature (9). The extent of the reaction was measured by analytical chromatography (μ Bondapak C18, with ethanol containing 15 % water as eluent). The oxidation product was then separated from residual BChl a and other impurities on a preparative column.

6) Reduction products

Reduction of the 2a acetyl group of BChl a (or B Pheophytin a) dissolved in ethanol was achieved by action of potassium borohydride (10) : 0.4 ml of 10 % KBH4 in 0.1 M Tris buffer pH 8 for 2 ml BChl a ethanolic solution. The reaction was followed by analytical chromatography (same conditions as for 10 OH BChl a). After 15 minutes, the solution was evaporated and purification was performed as above.

7) NMR spectra

Samples for NMR experiments were dissolved in deuterated tetrahydrofuran (S.M.M, CEA, France) at a concentration of pigments of about 0.5 mM. 500 MHz proton spectra were recorded on a Bruker WM 500 spectrometer at room temperature and referenced relative to internal tetramethylsilane (TMS).

8) Infrared spectroscopy

Infrared spectra of pigments in tetrahydrofuran d_8 were recorded on a Nicolet 60 X Fourier transform infrared spectrometer (FTIR) equipped with a MCTA detector.

RESULTS

Chlorophylls

Using the combination of techniques described in Material and Methods, 100 mg of chlorophyll or bacteriochlorophyll a and b can be purified per day. The overall yield, as measured by the optical density at the maximum of absorption, ranged between 50 and 75 %.

The percentages of impurities (epimers, allomerization products, etc.), estimated by the area of the peaks separated by analytical HPLC (conditions Table 1) and recorded at the same wavelength as the corresponding chlorophyll, were comprised between 0.25 and 1 % (Table 2). In particular, the percentage of BChl a' (isomer of BChl a on carbon 10) was estimated to be lower that 0,04 %. In the case of BChl b, the very low absorption at 680 nm indicated the absence of oxidation products (11).

Contamination with phospholipids (measured by gas liquid chromatography of the methyl esters of fatty acids) were found smaller in BChl a and BChl b (respectively 6 and 20 %), than in samples prepared with conventional methods (54 %) (7).

Table 1

CHROMATOGRAPHIC CONDITIONS FOR PIGMENT PURIFICATION AND ANALYSIS : PERCENTAGE OF WATER IN ETHANOL

CHLOROPHYLL OR DERIVATIVE	PREPARATIVE RUN ON MAGNUM 20 COLUMN	PREPARATIVE RUN ON MAGNUM 9 COLUMN	ANALYTICAL RUN ON μ BONDAPAK C18 COLUMN
Chl a	0 to 2	6	8 to 10
Pyrochl a	2	/	10
Pheo a	0	/	0 to 1
Pyropheo a	0	/	1
Chl b	0 to 2	6	8 to 10
Pyrochl b	2	/	10
Pheo b	0	/	0 to 1
Bchl a	4 to 6	12	15 to 20
PyroBchl a	6	12	10 to 15
Bpheo a	0	/	6 to 8
PyroBpheo a	0	/	5 to 6.5
10 OH Bchl a	9	12	15
2a deoxo 2a hydroxy Bchl a	8	/	20
Bchl b	2	8	12

Table 2

PERCENTAGE IMPURITIES RANGE OF CHLOROPHYLLS AND DERIVATIVES

CHLOROPHYLL OR DERIVATIVE	(1)	(2)
Chl a	0.24 to 0.85	
Pyrochl a	< 0.1	
Pheo a	1.75	7.2
Pyropheo a	7	12.4
Pyrochl b		5.7
BChl a	0.45 to 0.63	
PyroBchl a	< 0.1	
Bpheo a	1.2	9
PyroBpheo a	1.2	3.5
10 OH BChl a	0.1 to 2	
2 a deoxo 2a hydroxy BChl a	2.5	
BChl b	0.72 to 0.95	
Bpheo b	2.4	

No protein could be detected in the pigments prepared, after sodium dodecylsulfate polyacrylamide gel electrophoresis and staining with Coomassie Brilliant Blue G, even when the gels were extremely overloaded (7).

Pheophytins

Pheophytin derivatives were less pure than chlorophyll derivatives : 1,75 % impurities for Pheo a, 1,2 % for B Pheo a, 2,4 % for B Pheo b. The chromatographic peaks in preparative runs were often wide because of poor solubility of pheophytins in ethanol.

When HCl treatment was performed directly on crude extracts, from Sep Pak cartridges, and not on purified chlorophylls, one step was omitted and time was saved, but the percentage of impurities was higher (7,2 % for Pheo a, 9 % for B Pheo a).

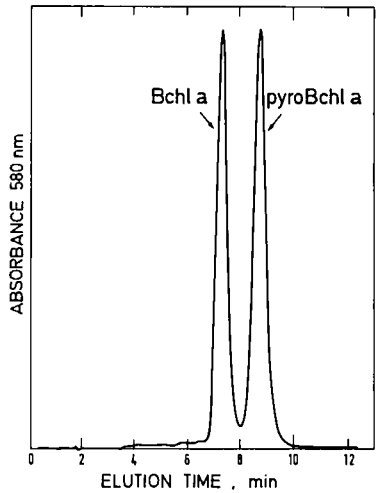

Fig.1 Separation of BChl a – pyro BChl a. Column, μ Bondapak C18. Eluent, Ethanol 15 % H_2O. Flow rate, 1 ml/m

In the case of B Pheo b, a normal phase column, with chloroform +1 % ethanol as eluent was used. The presence of dithiothreitol was necessary to avoid oxidation of BChl b during HCl treatment.

Pyroderivatives (Fig 1)

Pyrochlorophylls were obtained particularly pure, without any detectable impurity absorbing at the maximum absorption wavelength of the corresponding chlorophyll, as judged by analytical chromatography on μ Bondapak C18 (<0.1 % for pyroChl a and pyroBChl a)

The three highest frequency bands at 1737 cm^{-1}, 1684 cm^{-1} and 1658 cm^{-1} in the infrared absorbance spectrum of BChl a (Fig. 2) have been assigned to the C = 0 vibrations of the ester (in tetrahydrofuran, the two ester C = 0 at 7c and 10a positions cannot be distinguished), the 9 keto and the 2a acetyl groups, respectively (1). In the pyroBChl a infrared spectrum, the absorption at 1735 cm^{-1} is about 40 % that of the 1737 cm^{-1} band in the BChl a spectrum, as expected from the loss of the 10a ester group in the pyroderivative.

Pyropheophytins were less pure (1.2 % impurities for pyro B Pheo a, 7 % for pyro Pheo a), especially when they were prepared directly from crude extracts (3.5 % for pyro B Pheo A, 12.4 % for pyro Pheo A).

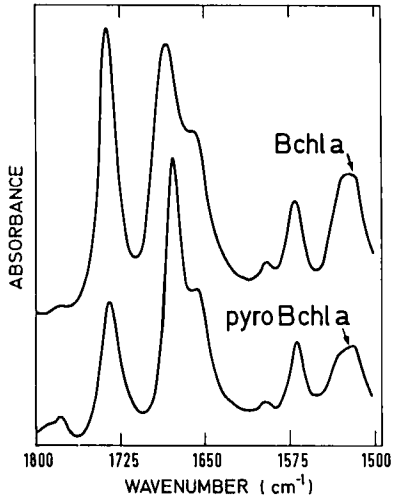

Fig.2 Infrared absorbance spectra of BChl a and PyroBChl a in tetrahydrofuran d8. The bands of the solvent have been substracted. T = 20°C. 64 interferograms co-added. 4 cm^{-1} resolution.

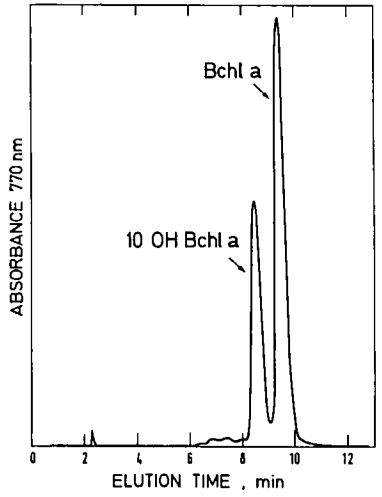

Fig.3 Separation of BChl a – 10 OH BChl a. Same conditions as in Fig.1

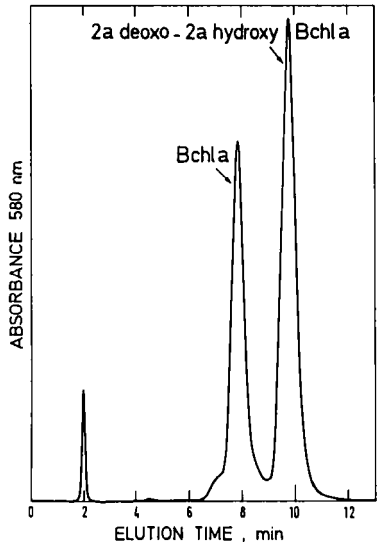

Fig.4 Separation of BCHl a – 2a deoxo 2a hydroxy BChl a. Column, μ Bondapak C18. Eluent, Ethanol 20 % H_2O. Flow rate, 1.5 ml/m.

Table 3

NMR data on Bchl a and 10 OH Bchl a , Chemical shift, ppm (in tetrahydrofuran d8, TSM = 0 ppm)

PROTON	Bchl a	10 OH Bchl a
β	9.08	9.20
α	8.50	8.58
δ	8.40	8.50
10 H	6.04	
10 OH		6.45

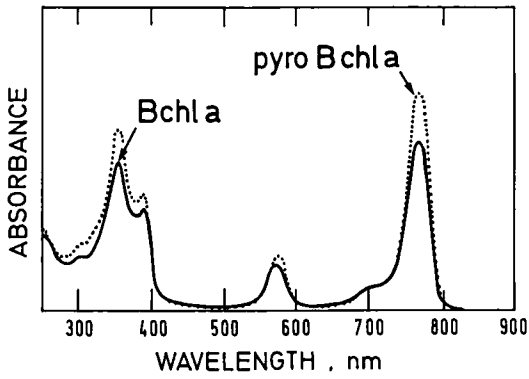

Fig. 5

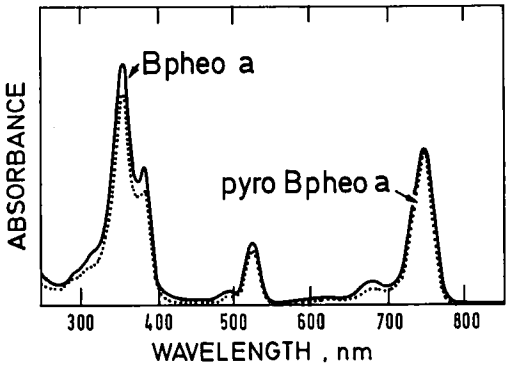

Fig. 6

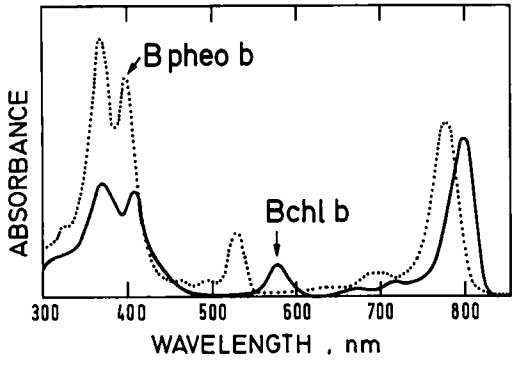

Fig. 7

Fig.5, 6, 7 : UV–visible absorption spectra of different chlorophylls and derivatives in ethyl ether.

10 OH Bacteriochlorophyll a (Fig.3)

10 OH BChl a was prepared from previously purified BChl a. It was separated from several degradation products and from BChl a. The final yield was around 30 %.

Identification was achieved by comparison of the relative retention times to those of BChl a and of NMR data, to those of reference (9) (Table 3). The peak of 6.45 ppm chemical shift disappears completely upon addition of D_2O and has been attributed to the presence of an OH group.

2a deoxo 2a hydroxy BChl a and B Pheo a (Fig.4)

Absorption spectra of the products separated here are identical to those corresponding to 2a deoxo 2a hydroxy BChl a and B Pheo a. The structure of these compounds has been initially determined by NMR by Ditson and coll (10).

CONCLUSION

The methods described here combine prepurification on reversed phase cartridges and purification by HPLC on C18 preparative columns. Owing to rapidity of chromatography and to inertness of the materials involved, epimerization and allomerization reactions are minimized. The purity of the chlorophylls and of their derivatives is comparable, or in some cases, better than that obtained by other methods (6).

Quantities on the range of 20–100 mg are easily obtained, which are sufficient for optical and electrochemical experiments, and they can be prepared before use, avoiding long storage degradation.

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