Biochimica et Biophysica Acta, 1019 (1990) 137–141
Elsevier

BBABIO 43261

A simple method for extracting chlorophylls from the recalcitrant alga, *Nannochloris atomus*, without formation of spectroscopically-different magnesium-rhodochlorin derivatives

Robert J. Porra

Botanisches Institut der Universität, München (F.R.G.), and Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Canberra (Australia)

(Received 3 April 1990)

Key words: Chlorophyll extraction; Chlorophyll assay; Recalcitrant alga; Dithionite; Ascorbic acid; (*N. atomus*)

A spectrophotometric assay is described for chlorophylls *a* and *b* extracted by a simple procedure from the recalcitrant alga, *Nannochloris atomus* (strain CS 183). In this assay method, the extraction is performed at 60 °C with 85% aqueous methanol containing 1.5 mM sodium dithionite or sodium ascorbate. The success of this extraction method is consistent with the view that the reductant cleaves disulphide bridges of structural proteins of the cell wall to render the cell permeable to aqueous methanol. In this assay, chlorophylls *a* and *b* are extracted without conversion to magnesium-3^1,3^2-didehydrorhodochlorin-15-acetic acid 13,15,17-trimethyl ester and its 7^1-oxo derivative (also known as magnesium-rhodochlorins *a* and *b*) as occurs in an alternative assay for recalcitrant algae where the structural cell-wall proteins are hydrolysed by an aqueous alkaline methanol reagent which is used as extractant (see Porra, R.J. (1990) Biochim. Biophys. Acta 1015, 493–502). The new assay procedure described in this paper is suitable for biochemists and physiologists who wish to express the activity of photosynthesis-related reactions and processes in recalcitrant algal cells on a chlorophyll basis.

Introduction

Recently, Porra et al. [1] obtained corrected extinction coefficients for chlorophylls (Chls) *a* and *b* in buffered 80% aqueous acetone from which they they derived simultaneous equations for the determination of Chls *a* and *b* extracted from higher-plant tissues with this solvent system. These equations are intended to replace the equations of Arnon [2], which were derived from the inaccurate coefficients of Mackinney [3]. Similar corrections were also made for the estimation of Chls *a* and *b* extracted with methanol or DMF so that compatible results could be obtained using all three of the above-mentioned solvent systems. None of these solvent systems, however, could totally remove Chls *a* and *b* from the recalcitrant alga, *Nannochloris atomus* (strain CS 183).

To overcome this problem with recalcitrant algae, Porra [4,5] developed a new extraction technique using 2% KOH in 85% aqueous methanol containing 1.5 mM sodium dithionite. Preliminary data were presented which showed that the aqueous alkaline component of this solvent system was hydrolysing structural proteins of the cell wall, so rendering the cell permeable to the alkaline-methanol component [4]. The extraction with alkaline methanol, however, converted chlorophylls *a* and *b* (Fig. 1, structure I) to magnesium-3^1,3^2-didehydrorhodochlorin-15-acetic acid 13,15,17-trimethyl ester and its 7^1-oxo derivative. These rhodochlorin derivatives, previously referred to as magnesium-rhodochlorins (Mg-Rchlns) *a* and *b* [5] and represented by structure III (see Fig. 1), are formed either by methanolysis of the labile $C13^1$-13^2 double bond of the enolate anion (Fig. 1, structure II), which is produced when chlorophyll is exposed to alkaline conditions [6], or by nucleophilic attack by methoxyl ions at the $C\text{-}13^1$ carbonyl [7]. Sodium dithionite (1.5 mM) prevented allomerization, the oxidative cleavage of ring E, which produces an unassayable mixture of allomerization products includ-

Abbreviations: Chl, chlorophyll; DMF, N,N^1-dimethylformamide, DTT, dithiothreitol; Mg-Rchln *a*, magnesium-3^1,3^2-didehydrorhodochlorin-15-acetic acid 13,15,17-trimethyl ester; Mg-Rchln *b*, magnesium-3^1,3^2-didehydro-7^1-oxorhodochlorin-15-acetic acid 13,15,17-trimethyl ester.

Correspondence: (permanent address): R.J. Porra, CSIRO-Division of Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia.

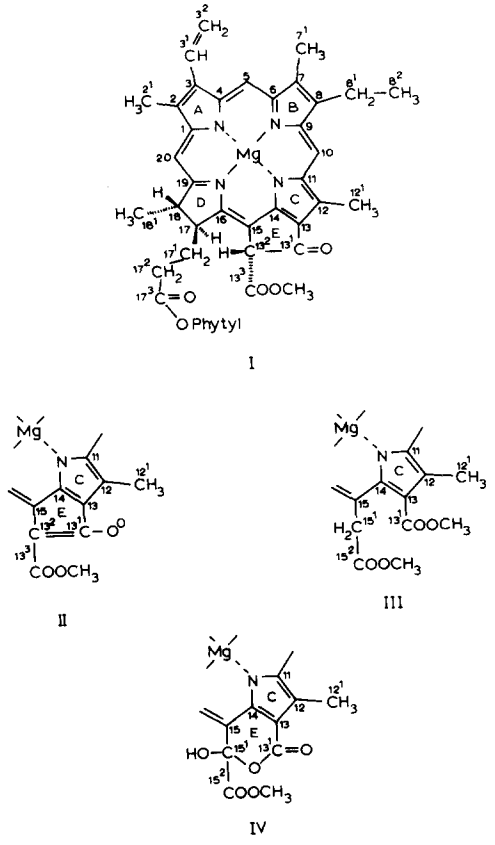

Fig. 1. The structures of chlorophylls and their derivatives. Chl *a* is represented by structure I. Chl *b* differs only in the presence of a formyl group (-CHO) at C-7 rather than a methyl group. The structural differences of the enolate anions, which occur only in rings C and E, are shown in structure II. The structural changes, also occurring only in rings C and E, are shown for Mg-Rchlns *a* and *b* (III) as well as the magnesium-hydroxylactones (IV). C-15[1] and C-15[2] of structures III and IV are derived from C-13[2] and C-13[3] of structure I, respectively. The tetrapyrrole numbering system used is that currently approved by the international Union of Biochemistry [13].

ing magnesium-hydroxylactones (Fig. 1, structure IV) in addition to the magnesium-rhodochlorin derivatives produced by methanolysis.

In this paper, another method is described for the total extraction of Chls *a* and *b* from *N. atomus* (strain CS 183). In this method, the structural proteins are again perturbed to render the cell wall permeable to aqueous methanol, but not by alkaline hydrolysis, rather by reductive cleavage of disulphide bonds (cf. Ref. 8) using 1.5 mM sodium dithionite or sodium ascorbate in 85% aqueous methanol. This method has several advantages over the aqueous alkaline methanol procedure [4,5]: the chlorophylls are not exposed to alkaline conditions which render them liable to allomerization and they are extracted as Chls *a* and *b* with stronger absorption peaks at 664 and 650 nm than those of Mg-Rchlns *a* and *b* at 642 and 623 nm [5]. No spectral assay distinguishes Chl *a* or *b* from their spectrally identical allomers and epimers: but, spectral identity ensures no assay error if such artefacts arise during extraction.

Experimental

Chemical and reagents

Acetone, diethylether and methanol were all Analar grade reagents supplied by BDH Chemicals (Australia), Port Fairy, Australia. Buffered aqueous acetone is 80% aqueous acetone containing either 2.5 mM sodium phosphate buffer (pH 7.8) or 50 mM Hepes-KOH buffer (pH 7.5) to minimize conversion of chlorophylls to phaeophytins. Sodium dithionite was obtained from E. Merck, Darmstadt, dithiothreitol from Boehringer-Mannheim and sodium ascorbate from Sigma.

Purification of chlorophylls a and b

Chls *a* and *b* were purified by the method of Porra [5].

Extinction coefficients of chlorophylls a and b in 85% aqueous methanol

These coefficients were obtained as previously described by Porra et al. [1] for chlorophylls dissolved in 100% methanol, DMF and 80% buffered aqueous acetone.

Extraction of chlorophylls from N. atomus (strain CS 183)

Cultures of *N. atomus* (strain CS 183) were provided by Dr. Shirley Jeffrey, CSIRO Division of Fisheries Research, Hobart, Australia. Aliquots (12–40 ml) of cultures (36–250 μg dry wt./ml) were harvested by centrifugation and suspended in 5 ml of 85% aqueous methanol containing 1.5 mM sodium dithionite or sodium ascorbate using a Potter-Elvehjem homogenizer. The suspension was transferred to a glass screw-capped centrifuge tube and closed. After wrapping in foil to exclude light, the contents were heated to 60 °C for 20 min. After centrifugation, the pellet was extracted in the same way with a further 5 ml of the same reagent until the pellet was no longer green or exhibiting red fluorescence under ultraviolet light. Normally, two or three extractions were sufficient for total extraction. The Chl *a* and *b* concentrations in *N. atomus* cells from identical aliquots of the same culture were also determined as Mg-Rchlns *a* and *b* by extractions carried out with 2% KOH in 85% aqueous methanol containing 1.5 mM sodium dithionite as previously described [5].

Results

It is well known that it is difficult to totally extract chlorophylls from many fresh-water and marine algae using methanol, DMF or aqueous acetone. In an earlier paper it was shown that less than 10 and 75% of Chls *a* and *b* were extracted from *N. atomus* (strain CS 183)

cells with buffered 80% aqueous acetone and methanol, respectively [5]; further, repeated (four-fold) extraction with 33% (v/v) of petroleum ether (b.p. 40–60 °C) in methanol, a solvent used by Strain [9], showed that the presence of the petroleum ether reduced the efficiency of chlorophyll extraction of methanol (approx. 75%) to that of aqueous acetone (< 10%). The difficulty appears to be associated with the impermeability of the cell walls to these solvents [4,5]. Supporting this view, algal cells which are recalcitrant to normal chlorophyll extraction procedures include the armoured dinoflagellates with their cellulose walls, heavily silicified berthic diatoms, blue-green algae with multi-layered walls and heavily walled green algae. Strong solvents such as dimethylsulphoxide, freezing and thawing procedures and mechanical techniques of cell disruption including grinding have frequently been employed but without achieving total extraction.

In 1968, Thompson and Preston [8] reported that dithiothreitol (DTT) caused a considerable weakening of the cell walls of the green alga, *Cladophora rupestris*. This phenomenon was ascribed by these authors to the breaking of disulphide bonds in structural proteins of the cell wall. Working on the premise that DTT might weaken the cell walls of *N. atomus* and render the cells permeable to solvents, it was found that the chlorophylls were indeed totally extracted from this alga with 12.5% DTT in 85% aqueous methanol using the procedure described in the Experimental section. However, it was found that the results, including the Chl *a*/*b* ratios, were variable and this variability may be due to a side-reaction between the thiol compound and the formaldehyde group of Chl *b* to form a cyclic dithioether. Support for this side-reaction was previously obtained [5] when purified Chl *b* was treated at room-temperature with 2% KOH in 85% aqueous methanol containing 12.5 mM DTT: the isosbestic points, observed under identical conditions when Chl *a* is converted to Mg-Rchln *a*, were lost (cf. Ref. 5). The side-reaction does not occur at room-temperature, as judged by the presence of isosbestic points, when using 1.25 mM DTT, 1.5 mM sodium dithionite or 1.5 mM sodium ascorbate; however, at the higher temperatures (60 °C) used in the extraction procedure (see Experimental section) the side-reaction with DTT is again present (cf. Ref. 5). For this reason, the preferred reductant used with both 85% aqueous methanol and with alkaline 85% aqueous methanol containing 2% KOH is always dithionite or ascorbate (1.5 mM). The oxidized ascorbate is not very soluble in methanolic solvents but the gelatinous precipitate, which interferes with spectroscopy, was removed simultaneously with cell debris by centrifugation.

Determination of extinction coefficients of Chls a and b in 85% aqueous methanol and derivation of appropriate simultaneous equations

To develop this extraction and assay process, the extinction coefficients of Chls *a* and *b* were determined in 85% aqueous methanol using the method of Porra et al. [1]. The extinction coefficients are given in Table I both as millimolar extinction coefficients (ε_{mM}; $l \cdot mmol^{-1} \cdot cm^{-1}$) and as specific extinction coefficients (α; $l \cdot g^{-1} \cdot cm^{-1}$). The simultaneous equations derived from these coefficients for solving Chl *a*, Chl *b* and Chl *a* + *b* concentrations are given below in nmol/ml (Eqns. 1–3):

$$\text{Chl } a \text{ (nmol/ml)} = 18.36\, A^{664} - 9.06\, A^{650} \quad (1)$$

$$\text{Chl } b \text{ (nmol/ml)} = 39.94\, A^{650} - 13.85\, A^{664} \quad (2)$$

$$\text{Chl } a + b \text{ (nmol/ml)} = 30.88\, A^{650} + 4.51\, A^{664} \quad (3)$$

and in μg/ml (Eqns. 4–6):

$$\text{Chl } a \text{ (μg/ml)} = 16.41\, A^{664} - 8.09\, A^{650} \quad (4)$$

$$\text{Chl } b \text{ (μg/ml)} = 30.82\, A^{650} - 12.57\, A^{664} \quad (5)$$

$$\text{Chl } a + b \text{ (μg/ml)} = 22.73\, A^{650} + 3.84\, A^{664} \quad (6)$$

TABLE I

Millimolar and specific difference extinction coefficients for chlorophylls a and b in 85% aqueous methanol

Because the spectrophotometer was zeroed at 750 nm, the extinction coefficients are difference coefficients as indicated in column 1. Each coefficient is the mean of three separate determinations. The percentage variation about the mean (m) is presented as $100 \cdot \sigma/m$ where σ represents standard deviation. The percentage variation is presented (in brackets) beside the millimolar coefficients (ε_{mM}) but would be identical for the specific coefficients (α) which are derived from the same data.

Wavelengths appropriate to the difference coefficients	Difference extinction coefficients			
	Chl *a*		Chl *b*	
	(ε_{mM}) ($l \cdot mmol^{-1} \cdot cm^{-1}$)	α ($l \cdot g^{-1} \cdot cm^{-1}$)	(ε_{mM}) ($l \cdot mmol^{-1} \cdot cm^{-1}$)	α ($l \cdot g^{-1} \cdot cm^{-1}$)
664 minus 750	68.18(±0.36%)	76.26	18.19(±0.44%)	20.03
650 minus 750	27.81(±0.39%)	31.11	36.88(±0.34%)	40.62

TABLE II

Extraction and assay of chlorophylls from Nannochloris atomus (strain CS183) cells

Samples (15–40 ml) of freshly-grown cells (Culture 1) and of cells maintained at room temperature under low light conditions for the various indicated periods (Cultures 2 and 3) were harvested by centrifugation and extracted with 85% aqueous methanol containing either 1.5 mM sodium dithionite or sodium ascorbate (as indicated) or with alkaline aqueous methanol in the presence or absence of 1.5 mM dithionite (as indicated). Cultures 1, 2 and 3 contained 36, 16 and 250 μg dry wt. of cells/ml, respectively.

Culture No.	Exposure to low light (days)	Extraction No.	Extraction procedure	Reductant used	Chlorophyll content of cells (nmol/mg dry wt. of cells)			Chl *a*/*b* ratio
					Chl *a*	Chl *b*	Chl *a* + *b*	
1	0	1	aqueous methanol	ascorbate	16.76	7.03	23.79	2.38
		2	aqueous methanol	dithionite	16.64	6.60	22.24	2.52
		3	alkaline aqueous methanol	dithionite	16.43	6.02	22.45	2.73
		4	alkaline aqueous methanol	–	16.45	6.75	23.20	2.44
2	4	5	aqueous methanol	dithionite	12.88	5.03	17.91	2.56
		6	alkaline aqueous methanol	dithionite	12.41	4.88	17.29	2.54
2	8	7	aqueous methanol	ascorbate	12.01	5.86	17.87	2.04
		8	aqueous methanol	dithionite	10.66	5.37	16.03	1.98
		9	alkaline aqueous methanol	dithionite	10.53	5.02	15.55	2.09
3	10	10	aqueous methanol	dithionite	16.01	8.48	24.49	1.69
		11	alkaline aqueous methanol	dithionite	17.05	9.34	26.39	1.82
		12	alkaline aqueous methanol	–	17.05	10.24	27.29	1.66

Assaying the chlorophyll content of N. atomus cells

The assays of the chlorophylls extracted with 85% aqueous methanol containing dithionite or ascorbate from cells harvested from aliquots of three different cultures of *N. atomus* are shown in Table II (see extractions 1, 2, 5, 7, 8 and 10). For comparison, Chls *a* and *b* in identical aliquots of these cultures were assayed by the method of Porra [4,5] as Mg-Rchlns *a* and *b* formed during extraction with aqueous alkaline methanol containing dithionite (see Table II, extractions 3, 6, 9 and 11). The comparison clearly shows that both methods give reliable and consistent results. Also it was shown that extraction at 60 °C with alkaline aqueous methanol containing no reducing agent (see Table II, extractions No. 4 and 12) gave consistent results.

Another interesting feature of the results is the decrease in the Chl *a*/*b* ratio when cells are maintained for long periods in low light conditions (see Table II, extractions 5–12). This is consistent with results obtained with higher plants [10].

Discussion

Earlier work [5], in which *N. atomus* cells were broken in a high-pressure cell of Milner et al. [11], clearly showed that the resistance of these cells to extraction of their chlorophylls was due to the impermeability of the cell walls to acetone or methanol or methanol containing 33% (v/v) petroleum ether (b.p. 40–60 °C); unfortunately, the cells of many other recalcitrant species are not readily broken to afford such an easy method of extraction.

This work shows that reductants such as dithionite and ascorbate (see Table II, extractions 1, 2, 5, 7, 8 and 10) render *N. atomus* cells permeable to aqueous methanol allowing chlorophyll extraction. This is consistent with the view that the reducing agents disrupt the cell wall by cleaving disulphide bridges of cell-wall structural proteins as previously observed with DTT by Thompson and Preston [8]; another view appears later.

Perusal of the results in Table II also shows that treatment of *N. atomus* cells at 60 °C with alkaline aqueous methanol with dithionite (extractions 3, 6, 9 and 11) or without dithionite (extractions 4 and 12) produces nearly identical data. This observation shows that at 60 °C, where oxygen is insoluble in organic solvents, the reducing agent is not essential to prevent allomerization reactions in the alkaline extractant. This confirms not only that aqueous alkaline methanol can make cell walls permeable to solvents in the absence of reductants but also that reductants are only needed with the alkaline extractant when manipulations prior to

heating at 60 °C (i.e., grinding in a mortar) occur at room temperature (cf. Ref. 5).

These results are consistent with a single, simple explanation for the successful extraction of chlorophylls from recalcitrant algae by the aqueous alkaline conditions of aqueous alkaline methanol reagents [5] or of aqueous alkaline pyridine [12] on the one hand and the reducing conditions of aqueous methanol containing dithionite or ascorbate on the other because both conditions are capable of causing structural alterations of proteins. The aqueous alkaline solvents [5,12], while replacing the phytyl group with a methyl group and altering the *iso*cyclic ring to form chlorophyll derivatives, would also be capable of hydrolysing peptide bonds of structural proteins of the cell wall, possibly the same proteins susceptible to modification by reductive cleavage of their disulphide bonds in aqueous methanol containing reductants. Alternatively, the reductants may remove oxygen and prevent oxidations such as disulphide bridge formation in cell-wall proteins during denaturation in aqueous methanol which might produce a tighter-meshed, less-permeable wall structure. The much older assay procedure of Porra and Grimme [12] employing alkaline aqueous pyridine to extract Chls *a* and *b* from recalcitrant *Chlorella fusca (pyrenoidosa)* 211-15 cells does not hydrolyse Chls *a* and *b* to stable Mg-Rchlns *a* and *b* (see Fig. 1, structure III), as occurs with aqueous alkaline methanol, but oxidizes them to their respective less stable magnesium hydroxylactones (see Fig. 1, structure IV). Preliminary experiments (Lorenz, Porra and Grimme, unpublished results) indicate that chlorophylls can also be extracted unaltered from recalcitrant *C. fusca* cells using aqueous methanol with dithionite or as magnesium rhodochlorins with the aqueous alkaline methanol procedure [4,5].

Finally, this and other [5,12] chlorophyll assays for recalcitrant algae may be useful in marine biology where marine-productivity estimates are based on the chlorophyll content of marine algae which include many recalcitrant forms.

Acknowledgements

This work, started at the laboratories of the CSIRO-Division of Plant Industry, Canberra, was completed and the manuscript prepared during the author's visit to the laboratories of Professor Hugo Scheer, Botanisches Institut der Universität, München, which was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 143, Projekt A1. The author thanks Professor Hugo Scheer, Munich, and Drs. Jan Anderson and W.S. Chow, CSIRO-Division of Plant Industry, Canberra, for helpful discussions.

References

1 Porra, R.J., Thompson, W.A. and Kriedemann, P.E. (1989) Biochim. Biophys. Acta 975, 384–394.
2 Arnon, D.I. (1949) Plant Physiol. 24, 1–15.
3 Mackinney, G. (1941) J. Biol. Chem. 140, 315–322.
4 Porra, R.J. (1990) in Current Research in Photosynthesis (Baltscheffsky, M., ed.), Vol. 2, pp. 237–240, Kluwer, Dordrecht.
5 Porra, R.J. (1990) Biochim. Biophys. Acta 1015, 493–502.
6 Hynninen, P.H. (1973) Acta Chem. Scand. 27, 1771–1780.
7 Lötjönen, S. and Hynninen, P.H. (1980) Synthesis 541–543.
8 Thompson, E.W. and Preston, R.D. (1968) J. Exp. Bot. 61, 690–697.
9 Strain, H.H. (1958) in Chloroplast Pigments and Chromatographic Analysis, Thirty-second Annual Priestley Lecture, pp. 26–27, The Pennsylvania State University, University Park, PA.
10 Anderson, J.M. (1968) Annu. Rev. Plant Physiol. 37, 93–136.
11 Milner, H.W., Lawrence, N.S. and French, C.S. (1950) Science 111, 633–634.
12 Porra, R.J. and Grimme, L.H. (1974) Anal. Biochem. 57, 255–267.
13 Merritt, J.E. and Loening, K.L. (1979) Pure Appl. Chem. 51, 2251–2304.