

The chlorinated chlorophyll RC I, a preparation artefact

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A special chlorophyll, designated chlorophyll RC I, has been reported by our and other laboratories to be a stable chlorophyll, extractable from organisms with oxygenic photosynthesis. Its structure was revealed as 13²-hydroxy-20-chloro-chlorophyll *a*. We revise the finding that chlorophyll RC I is a naturally occurring chlorophyll, and show that it is an artificial chlorination product created during thin-layer chromatography on silica gel. Simultaneous hydroxylation prevents further chlorination and stabilizes the already chlorinated chlorophylls. Avoiding TLC by applying HPLC we were unable to find chlorinated chlorophylls in pigment extracts and, therefore conclude that chlorophyll RC I is an artefact generated during thin-layer chromatography.

Chlorophyll RC I; Chlorophyll chlorination; Chlorophyll hydroxylation; TLC

1. INTRODUCTION

Seven years ago an unknown chlorophyll was found in extracts from a green alga, separated by TLC. It was designated as chlorophyll RC I [1]. Subsequently it was extracted from many other photosynthetic organisms [2–4]. The structure of this chlorophyll was identified as 13²-hydroxy-20-chloro-chlorophyll *a* [4,5]. Because it was known that chlorophylls can easily be halogenated in the C-20 position [6], considerable precautions were taken to rule out such a possibility. Cells were extracted in chloride free medium [4] or extracted in the presence of ³⁶chloride [5], the chlorophyll was rechromatographed and transferred to several other solvents [7], but no changes in structure or amount of chlorophyll RC I could be observed. All these control experiments revealed chlorophyll RC I as a stable compound extractable from photosynthetic organisms.

Except for a general disbelief, the only substantial criticism was raised by Watanabe et al. [8]. In contrast to the generally used thin-layer separation and purification they applied HPLC and could not

confirm the existence of chlorophyll RC I, but later detected a component similar to chlorophyll RC I under certain circumstances [9]. Applying reversed-phase HPLC [10] for separation we were unable to find chlorophyll RC I with this method.

In this paper we demonstrate that chlorophyll RC I is most probably an artefact generated during thin-layer chromatography.

2. MATERIALS AND METHODS

Scenedesmus obliquus pigment mutant C-6E, having no chlorophyll *b* and carotenoids, was grown heterotrophically in the dark at 30°C as described by Bishop and Senger [11]. Pigments were extracted with boiling methanol and cell debris was then removed by centrifugation for 5 min at 3000 × *g*. The methanolic solution was cooled and immediately extracted with petroleum ether (40–60°C fraction) and diethylether. The solvent was evaporated under reduced pressure and the residue was taken up in acetone.

Thin-layer chromatography (TLC) was performed on Merck silica-gel thin-layer plates (Merck, Darmstadt, FRG) according to the method of Dörnemann and Senger [7].

For HPLC analysis an RP-18 based system was employed. The elution solvent consisted of CH₃CN/CH₃OH = 75:25 (v/v) superimposed by a multilinear gradient of water. The water content was diminished within 40 min from 10% down to 0%, then held for 10 min at 0%. Finally the water content was increased again up to 10% within 10 min. For separation a Spherisorb ODS column (Kontron, München, FRG) with 5 µm material (250 mm × 4 mm i.d.) was used. For details see Humbeck et al. [10]. Absorption was detected at 430 nm.

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Pigments were identified either by analytical HPLC, using known compounds as internal or external standards, or by spectroscopy. Quantitative HPLC analysis was achieved by a number of calibration runs injecting known amounts of pigments and obtaining calibration curves by plotting the amount of pigment versus arbitrary units given by the integrating unit of the HPLC apparatus. HPLC analysis of samples after TLC was performed as follows: the TLC plate was developed [5], dried and the whole plate was scraped off. Pigments were extracted exhaustively with acetone and diethylether and the silica gel was removed by centrifugation. The organic solution was dried over MgSO_4 and evaporated under reduced pressure at 40°C . The residue was taken up in a defined volume of acetone and then injected into the HPLC apparatus.

3. RESULTS AND DISCUSSION

The chlorinated chlorophyll RC I was first isolated by TLC from extracts of a pigment mutant C-6E of the unicellular green alga *Sc. obliquus* [1]. To test whether this chlorophyll RC I was an artefact of TLC separation or is present in the extract of the green alga, reversed-phase HPLC was applied (fig.1). The HPLC elution profile (fig.1A) shows only chlorophyll *a* (retention time 32 min) and its stereoisomer chlorophyll *a'* (34 min) as major constituents. No chlorinated chlorophylls could be detected. To exclude a possible masking of chlorophyll RC I by chlorophyll *a* or other components, chlorophyll RC I, purified by TLC, was added to the C-6E extract (1% of total Chl). Fig.1B shows that the chlorinated chlorophyll is clearly separated and distinguished from chlorophyll *a*. Hence, chlorinated chlorophylls are not

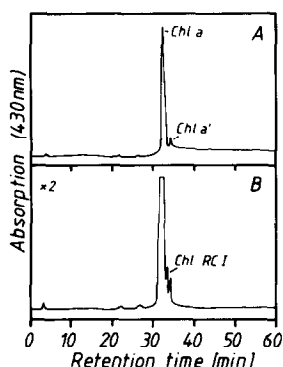


Fig.1. (A) HPLC elution profile of pigment extract from *Scenedesmus* mutant C-6E. (B) Elution profile of C-6E pigment extract with added chlorophyll RC I. The amount of the chlorinated chlorophyll was 1% of total chlorophyll. The attenuation of (B) is two times that of (A).

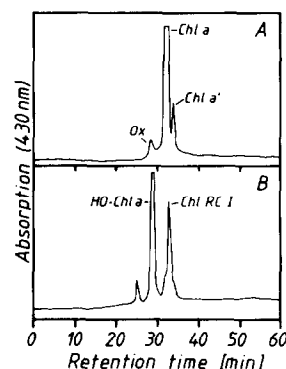


Fig.2. HPLC elution profiles of the separation of a sample of chlorophyll *a* before (A) and after TLC (B). The sample before TLC contained 97% chlorophyll *a* and oxidized chlorophyll (Ox) and chlorophyll *a'* as minor impurities. HO-Chl *a* = 13^2 -hydroxy-chlorophyll *a*.

natural constituents of this green alga. Tests with extracts of other organisms yielded the same results (not shown).

Because hydroxylation of chlorophylls occurs easily during TLC on silica gel materials [12] and preparation of pigment extracts in the presence of ^{36}Cl yielded no chlorinated chlorophyll [5] the only step where introduction of the chlorine atom could have occurred was TLC. Therefore we prepared pure chlorophyll *a* by HPLC (fig.2A) and then subjected this sample to the TLC system which was used in the isolation of chlorophyll RC I [7]. After 2.5 h of separation the plates were scraped off, pigments were extracted with acetone

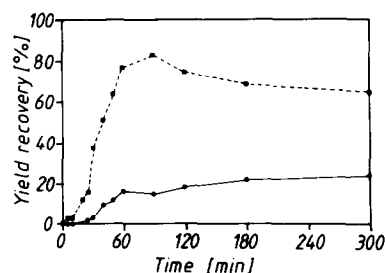


Fig.3. Quantitative composition of chlorophyll *a* samples after different times of development on silica gel TLC plates. Equal amounts ($200\ \mu\text{g}$ each) were applied to the plates, developed, scraped off, extracted and analyzed by HPLC. The data were calculated from the arbitrary units produced by the integrating unit during HPLC. The yield of pigments is calculated in relation to the molar amount of chlorophylls recovered after TLC separation. (●—●) Chlorophyll RC I; (■---■) 13^2 -hydroxy-chlorophyll *a*.

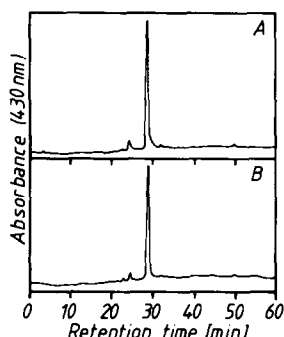


Fig.4. HPLC elution profiles of a sample of 13^2 -hydroxy-chlorophyll *a* before (A) and after TLC (B).

and diethylether and subjected again to an HPLC analysis. Fig.2B shows that the major portion of chlorophyll *a* has been converted to a compound now eluting with a retention time of 29 min. This compound could be identified as 13^2 -hydroxy-chlorophyll *a* by mass spectrometry (not shown). In addition a new compound, eluting with a retention time of 33 min had been formed. This compound showed UV/vis and fluorescence spectra identical to chlorophyll RC I and in fluorescence emission the height of this peak in the elution profile was only one third when compared to that of chlorophyll *a* in accordance with the lower fluorescence yield of chlorophyll RC I [7]. This proves that chlorophyll RC I had been formed during the chromatographic procedure.

To quantitate these reactions, similar amounts of chlorophyll *a* were applied to silica gel TLC plates and separated. After different times the plates were scraped off and the pigment composition was determined by HPLC. The formation of hydroxylated chlorophyll *a* and of chlorophyll RC I, occurring during TLC, is shown in fig.3. Both curves of chlorophyll RC I and 13^2 -hydroxy-chlorophyll *a* formation reach saturation. When all chlorophyll had been completely hydroxylated no further increase in chlorophyll RC I content could be observed.

Therefore, we applied purified 13^2 -hydroxy-chlorophyll *a* to TLC plates and analyzed its purity

before (fig.4A) and after (fig.4B) TLC. In both cases similar elution profiles were obtained, showing that hydroxylated chlorophyll is not altered during TLC.

These experiments indicate that chlorophyll RC I is not a naturally occurring chlorophyll, but a product of TLC with siliceous materials. Extensive reports about chlorination and hydroxylation of chlorophylls and their relation to Chl RC I are in preparation.

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