

# Hydroxylation of Chlorinated and Unchlorinated Chlorophylls *in vitro*

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Chlorophyll *a* is hydroxylated quantitatively in the 13<sup>2</sup>-position, when chromatographed on silica gel thin-layer plates. This was shown by using HPLC as a non-destructive method for analyzing photosynthetic pigments. The hydroxy-group of Chl RC I, a 13<sup>2</sup>-hydroxy-20-chloro-chl *a*, described by Dörnemann and Senger (1986) and Scheer *et al.* (1986), is shown to be artificially introduced during the purification procedure by TLC.

## Introduction

During the experiments revealing the structure of an unknown chlorophyll, designated Chl RC I, isolated by thin-layer chromatography (TLC) from bulk chlorophylls, a hydroxy-group was detected at the 13<sup>2</sup>-position [1, 2]. It was already discussed whether this hydroxylation took place *in vivo* or was an artefact, induced during the isolation procedure [1, 2]. Such an assumption was suggested by divergent results with HPLC separation [3], and by preceding investigations of chlorophyll alterations on silica gel layers [4, 5]. The reinvestigation of this question was possible by the application of a non-destructive HPLC method for separation and identification of chlorophylls, together with the use of synthetic pigments.

## Materials and Methods

Chlorophyll *a* (Chl *a*) was prepared from *Scenedesmus* mutant C-6E and *Scenedesmus obliquus* WT-D3 and prepurified according to Omata and Murata [6].

20-Chloro-pheophytin *a* and 13<sup>2</sup>-hydroxy-20-chloro-pheophytin *a* were prepared by a modification of the method of Woodward and Skaric [7] from the respective 20-unsubstituted pigments [2]. Metalation with Mg [8] yielded the respective chlorophylls (2 isomers each). The structures were confirmed by NMR and absorption spectroscopy, interconversions and by demetalation to the pheophytins (A. Struck and H. Scheer, to be published).

For enzymatic chlorination, Chl *a* was incubated with KCl and H<sub>2</sub>O<sub>2</sub> in KH<sub>2</sub>PO<sub>4</sub>-buffer (0.3 M, pH 5.0) in the presence of chloroperoxidase (EC 1.11.1.10, Sigma, Munich, C-0278) for 60 min. The reaction products were extracted with diethylether, dried over MgSO<sub>4</sub> and subjected to TLC or HPLC analysis.

The pigments were identified by absorption spectroscopy, using an Uvikon 820 (Kontron) spectrophotometer. Molecular weights were determined by plasma desorption mass spectrometry (PDMS) [Senge and Senger in preparation, 9–11].

TLC was performed on Merck silica gel plates (#5553, Merck, Darmstadt, F.R.G.), according to Dörnemann and Senger [1].

HPLC analysis was carried out using a Spherisorb S5 RP-18 column with 5 μm material (250 mm × 4 mm ID, Kontron). The elution system consisted of CH<sub>3</sub>CN/CH<sub>3</sub>OH (75/25; v/v) superimposed with a multilinear gradient of water content, starting with 10% water. The water content was diminished to 0% within 40 min, followed by a 10 min phase of regeneration at 0% water. Finally the water content was increased again up to 10% within 10 min [12]. The detection wavelength was 430 nm, the flow rate 1 ml/min.

## Results and Discussion

The reaction product of an enzymatic chlorination of Chl *a* (comp. I in Fig. 1) was shown to be 13<sup>2</sup>-hydroxy-20-chloro-chl *a* (comp. IV) after purification by TLC [9]. This compound is identical to the Chl RC I described by Dörnemann and Senger [1]. The purified compound IV was eluted in the above

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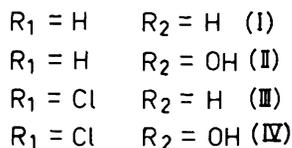
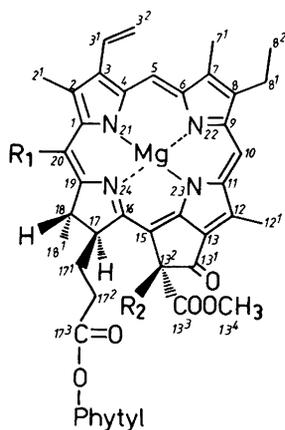


Fig. 1. Structural formula of Chl *a* (I), 13<sup>2</sup>-hydroxy-chl *a* (II), 20-chloro-chl *a* (III), 13<sup>2</sup>-hydroxy-20-chloro-chl *a* (IV). All compounds exist as epimers at C-13<sup>2</sup>.

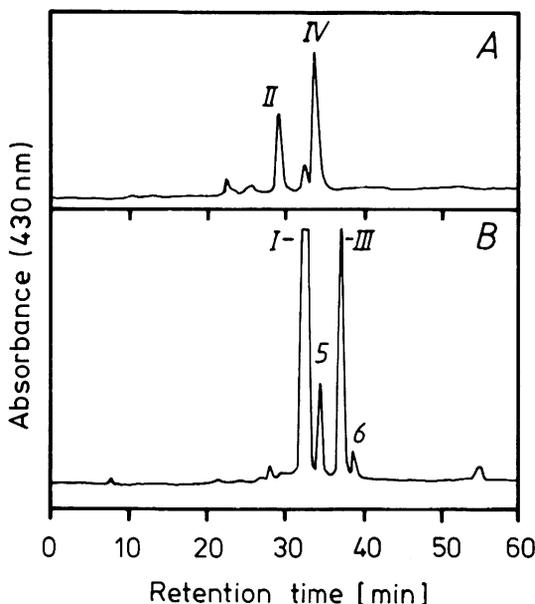


Fig. 2. HPLC elution profiles for the reaction products of an enzymatic chlorination of 13<sup>2</sup>-hydroxy-chl (A) and of Chl *a* (B). I – Chl *a*, II – 13<sup>2</sup>-HO-chl *a*, III – 20-Cl-chl *a*, IV – 13<sup>2</sup>-HO-20-Cl-chl *a*, 5 – chl *a*', 6 – 20-Cl-chl *a*'.

described HPLC system after a retention time of 33 min.

In order to circumvent the laborious TLC separation, the reaction products of the enzymatic chlorination of Chl *a* were extracted from the reaction mixture and directly subjected to HPLC analysis. The elution profile (Fig. 2B) showed major peaks at retention times of 32 and 37 min. Minor peaks derived from epimerization of chlorophylls at C-13<sup>2</sup>, generally occurring in polar solvents and during extraction in chlorophylls containing an enolizable β-ketoester. By using Chl *a*, purified by HPLC as internal and external standard, the peak at 32 min was identified as Chl *a* (I). It showed the typical absorption spectrum with maxima at 661 nm and 428 nm in acetone, and PDMS revealed its molecular weight to be 893 (Table I). The compound eluting after 37 min exhibited absorption maxima at 668 nm and 432 nm in acetone. The absorption spectrum was identical to the one obtained for Chl RC I (IV), but while Chl RC I has a molecular weight of 943 [1], this compound gave a M<sup>+</sup>-peak at 926 ± 1 in the PDMS-spectrum. It follows that different products are obtained, when either TLC or HPLC are used for separation of the reaction products of an enzymatic chlorination of Chl *a*.

Similar discrepancies were observed, when Chl *a* was purified by HPLC or TLC. When Chl *a* was analyzed by HPLC before and after being subjected to TLC it showed different retention times. Chl *a* purified by HPLC was eluted after 32 min, while Chl *a* purified by TLC eluted in the HPLC system after 29 min. A spectral analysis showed that both compounds exhibited the same absorption spectrum, with maxima at 661 nm and 428 nm in acetone. PDMS confirmed that only the HPLC purified Chl *a* had a molecular weight of 893, in conformity with the

Table I. Absorption maxima in acetone, retention times in HPLC, and plasma desorption mass spectrometry data of Chl *a* (I), 13<sup>2</sup>-hydroxy-chl *a* (II), 20-chloro-chl *a* (III), and 13<sup>2</sup>-hydroxy-20-chloro-chl *a* (IV).

Compound	Retention time [min]	Absorption maxima [nm]	Mass No. of M <sup>+</sup> -peak
I	32	661 428	893
II	29	661 428	908
III	37	668 432	927
IV	33	668 432	943

weight expected from the formula. The Chl *a* subjected to TLC gave a PDMS-spectrum with the  $M^+$ -peak at 908 mass units. Hence, Chl *a* before and after TLC showed the same mass difference of 16 mass units as enzymatically chlorinated Chl *a*, when purified either by TLC or HPLC.

When Chl *a*, purified by TLC, *i.e.* the compound eluting at 29 min, was incubated with chloroperoxidase and the extracted reaction products were analyzed by HPLC, an elution profile as given in Fig. 2A was obtained. It shows major peaks at 29 min, *i.e.* not converted starting material and at 33 min, *i.e.* compound IV.

Since hydroxylation of chlorophyll is a very common allomerization reaction of porphyrins [13–16], and the mass difference of 16 mass units before and after TLC would be in good accordance with the introduction of a hydroxyl-group, chemically synthesized compounds of known structure were used for comparison.  $13^2$ -hydroxy-20-chloro-chl *a* (IV) was

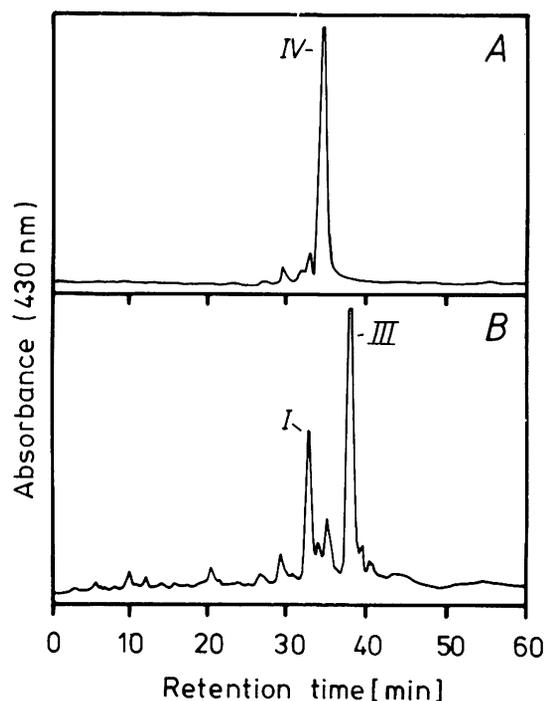


Fig. 3. HPLC elution profiles for the reaction products of a chemical synthesis of  $13^2$ -hydroxy-20-chloro-chl *a* (A) and a mixture of chl *a* (I) and 20-chloro-chl *a* (III) (B). For identification of the peaks see Fig. 2. The epimer mixture of IV is not separated under the conditions of the separation.

eluted after a retention time of 33 min (Fig. 3A), and 20-chloro-chl *a* (III) was eluted after 37 min (Fig. 3B). Hence, the reaction product of the enzymatic chlorination of Chl *a* is 20-chloro-chl *a* (III) and the hydroxylgroup of Chl RC I is introduced artificially during thin layer chromatography.

Further support for this conclusion was obtained by the following experiments. Pure Chl *a* (I) was converted with chloroperoxidase to chlorinated chlorophyll (III) [9], and then subjected to TLC separation. The whole TLC plate was scraped off, and the pigments were extracted exhaustively with acetone. When this sample was analyzed by HPLC an elution profile similar to Fig. 2A was obtained (*cf.* Fig. 5).

The hydroxylation of 20-chloro-chl *a* (III) to compound (IV) could be followed during separation on the TLC plate. When a sample of the chemically synthesized compounds (III) and (IV) were applied to a TLC plate and developed after the method of Dörnemann and Senger [1], the band of compound (III) migrated initially ahead of the band of compound (IV). During the development the spatial difference between both bands became smaller, and at the end of the TLC procedure the band of compound (III) comigrated with the band of (IV). The compound (IV) deriving from compound (III), was scraped off, extracted and subjected to HPLC analysis. The elution profile is given in Fig. 4. The major peak has a retention time of 33 min, hence compound (III) has been converted to compound (IV) during the TLC procedure.

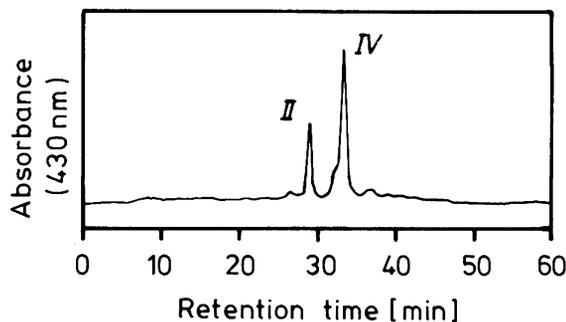


Fig. 4. HPLC elution profile of chemically synthesized 20-chloro-chl *a* (see Fig. 2B) after development on Merck TLC plates.

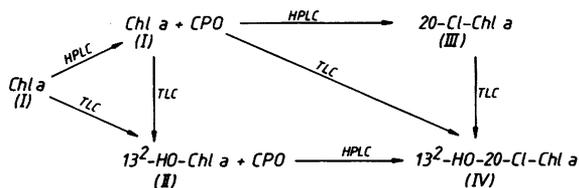


Fig. 5. Scheme of the connections between pure Chl *a* and the products obtained in enzymatic chlorination reactions, using either TLC or HPLC for purification of the reaction products. CPO = chloroperoxidase and cosubstrates, see [9].

The scheme of Fig. 5 summarizes the different experiments. The results clearly demonstrate that hy-

droxylation of chlorophylls on silica gel plates is a nearly quantitative reaction. This supports the warning of Strain *et al.* [4] not to use silica gel materials in chlorophyll chromatography. The results reported here have to be considered during further investigations on the nature of Chl RC I.

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