

The Esterification of Chlorophyllide a in Greening Bean Leaves

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Z. Naturforsch. **33 c**, 712–714 (1978) ; received July 26, 1978

Phaseolus vulgaris L., Chlorophyll a Biosynthesis, C-20 Diterpene Alcohols

The accumulation of esterified chlorophyll a (Chl a) in etiolated bean leaves was determined during a dark period of 60 minutes after 1 minute irradiation with white light. The separation of the pigments esterified with geranylgeraniol (GG), dihydrogeranylgeraniol (DHGG), tetrahydrogeranylgeraniol (THGG) and phytol (P) was performed by HPLC. The last step for chlorophyll a biosynthesis is confirmed to be an esterification of chlorophyllide a to Chl_{GG} followed by a stepwise hydrogenation of the pigment-bounded alcohols to Chl_{DHGG} and Chl_{THGG} and finally to Chl a.

Introduction

Protochlorophyllid in etiolated leaves is converted by light to Chl. id a* which is then esterified with phytol to Chl a [1]. The detection of an intermediate pigment – discussed to be Chl_{GG} – during the greening of bean leaves, was the first hint, that esterification perhaps is not performed by a direct phytylation [2, 3]. Furthermore, it was also possible to isolate, from other plants, pigments, which are not only esterified with phytol but also with the C-20 diterpene alcohols GG [4, 5], DHGG [6] and THGG [7, 8]. Therefore, a biosynthetic pathway has been deduced for 7 1/2 days old etiolated oat seedling, in which Chl. id a first is esterified to Chl_{GG}, and then hydrogenated via Chl_{DHGG} and Chl_{THGG} to Chl_P [7]. The quantitative analysis of these esterified pigments was based on an indirect method which means saponification of the mixture of pigments and glc-analysis of the alcohol mixture thus obtained [7]. The recently developed analytical method to separate the pigments as pheophytins by HPLC directly [9] prompted us to extend our kinetic experiments to bean seedlings, expecting to get evidence for the generality of the proposed pathway.

* **Abbreviations:** Chl. id: chlorophyllide a; Chl a: chlorophyll a; GG: geranylgeraniol; DHGG: dihydrogeranylgeraniol; THGG: tetrahydrogeranylgeraniol; P: phytol; Chl_P, Chl_{THGG}, Chl_{DHGG} and Chl_{GG}: chlorophyllide a esterified with the respective alcohol; Phe a: pheophytin a; Phe_P, Phe_{THGG}, Phe_{DHGG} and Phe_{GG}: pheophorbide a esterified with the respective alcohols.

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Materials and Methods

Pheophytins: 7 1/2 days old etiolated bean leaves (*Phaseolus vulgaris* L., Bayerische Futter und Saatbau GmbH, München, W. Germany) were harvested and the leaves irradiated with white fluorescent light (2000 lux) for 1 minute. After several dark periods (0.5, 5, 10, 15, 20, 30 and 60 min at 23–24 °C), the mixture of esterified pheophytins was isolated by standard procedures as described earlier [7].

Alcohols: The esterifying alcohols were obtained by the usual method of saponification of the pheophytins [7]. The identification was carried out by glc [9].

Liquid chromatography: HPLC was carried out with a system consisting of two Waters Model 6000 A pumps, an injector model U 6 K and an LDC model 1202 variable-wavelength double-beam detector fitted with a 8 µl flow-through cell (10 mm path-length). For maximum sensitivity, the detector was set at 410 nm (Soret band), but contaminations which absorb at this wavelength were also detected. Therefore, for positive identification in chromatographic analytical separation, the monitor was set to the characteristic wavelength, 667 nm. For preparative separation, the monitor was set to 534 nm, a small maximum for pheophytins, to take a more insensitive system. Runs were made at room temperature.

Analytical separation: stainless-steel column (250 × 5 nm), packed with LiChrosorb RP-8 – 5-10 µm (Knauer, Oberursel, W. Germany); flowrate: 1.5 ml/min; solvents: methanol-water (95:5 v/v).



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Preparative separation: stainless-steel column (250 × 16 nm), packed with LiChrosorb RP-8 – 5-20 μ m (Knauer, Oberursel, W. Germany); flowrate: 4 ml/min; solvents: methanol-acetone (85:15 v/v).

Results and Discussion

A test mixture of esterified pheophytins was separated on HPLC with a preparative column and each fraction then analysed on the analytical column.* The fractions 1, 3 and 4 were completely separated each from another, only fraction 2 was defiled with fraction 1 (20%). To identify the separated fractions they were saponified and the alcohols analysed by glc. The chromatograms show exactly the same pattern as for the separated pheophytins: fractions 1, 3 and 4 contain only the expected alcohols GG, THGG and P, respectively, in fraction 2 the main peak corresponds to DHGG (80%) and the second peak to GG (20%). Based on these results, for the analysis of the mixture of pheophytins, the chromatographic separations by HPLC (see Fig. 1) seems to be the most suitable method.

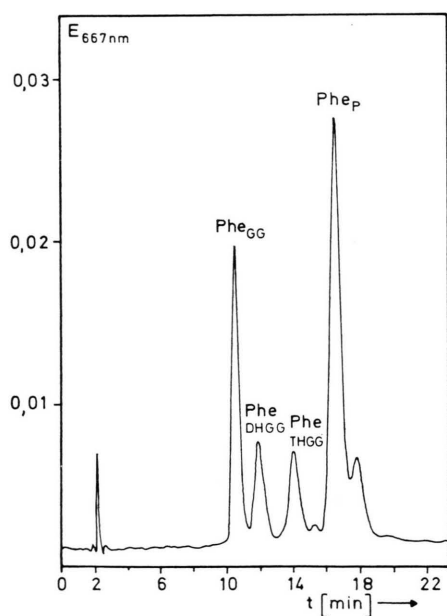


Fig. 1. A typical HPLC-chromatogram derived from an analytical separation of a mixture of PheGG, PheDHGG, PheTHGG and PheP. For details see materials and methods.

* In contrast to former HPLC-separation [9] now a column packed with LiChrosorb RP-8 (Knauer, Oberursel, W. Germany) was used.

The mixture of the esterified pigments from bean leaves was purified by the usual method [7] and then directly applied on HPLC. The relative amounts of the different esterified pheophytins were calculated by the areas of the peaks from the respective chromatogram.

Fig. 2 shows the accumulation of the single pheophytins esterified with GG, DHGG, THGG and P at different times after illumination.

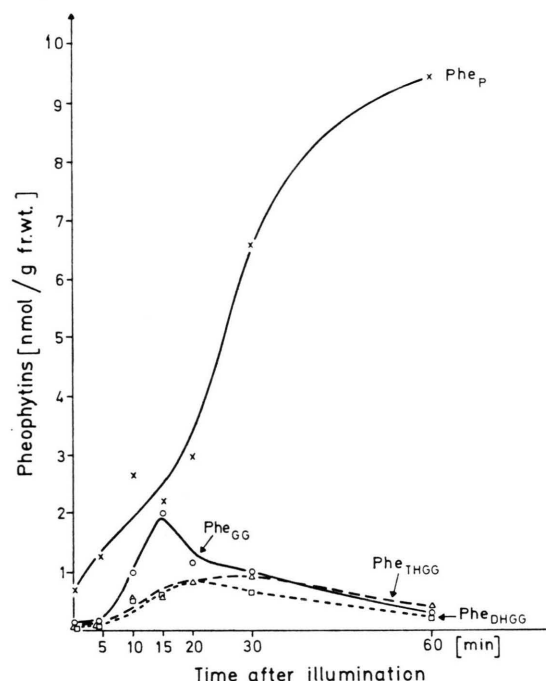


Fig. 2. The accumulation of the esterified pigments during dark period after illumination of etiolated bean leaves. The separation of the pigments – as pheophytins – was performed by HPLC. PheGG (○—○—○), PheDHGG (□—□—□), PheTHGG (△—△—△) and PheP (×—×—×).

The sharp maximum for the accumulation of PheGG after 15 min dark period is remarkable. The rates of accumulation for PheDHGG and PheTHGG reach a broad maximum after 20 and 30 min, respectively. Therefore, between 5 and 15 min after illumination, the esterification with GG seems to be the main process followed by the hydrogenation. After about 60 min of dark period, the esterified pigment is mainly phytylated. At this time, the substrate (Chl. id) for the esterification is almost consumed and therefore the hydrogenation up to the

phytylated pigment seems to be the main process. Besides, a direct phytylation at this time cannot be excluded yet.

This is about the same result — despite of a time shift — what was found for de-etiolated oat seedlings [7].

The compare our results from 7 1/2 days-old etiolated bean leaves with those published for 8 days-old bean leaves [3], the amounts obtained at different incubation times for Phe_{GG}, Phe_{DHGG} and Phe_{THGG} were added up. This resulting curve and those of the accumulation of Phe_P and of the sum of all esterified pigments is represented in Fig. 3. In the first 5 min after illumination, the increase of the amount of esterified pheophytin corresponds only to an increase of Phe_P, since the sum of the three other pheophytins remains constant. In the following 10 min, the rate of accumulation of the summed three esterified intermediate pigments is faster than that for Phe_P. From 15 to 30 min there is only a little change in the accumulation of the summed intermediate pigments whereas in the same time, Phe_P is accumulated very fast. After 30 min, Phe_P reaches a maximum asymptotically, whereas the amounts of the other esterified pheophytins decrease.

Since the pheophytins are the direct derivatives from the chlorophylls, it is possible to correlate these data with those published by Ogawa *et al.* [3]. The authors describe an accumulation of a chlorophyll x which is similar to the accumulation of the mixture of pigments esterified with GG, DHGG, THGG as shown above. Therefore, it is quite probable that this chlorophyll x has been the mixture of Chl_{GG}, Chl_{DHGG} and Chl_{THGG}. These results confirm the generality of the proposed biosynthetic pathway for chlorophyll a for mono- and dicotyledones. After illumination, the first step is the

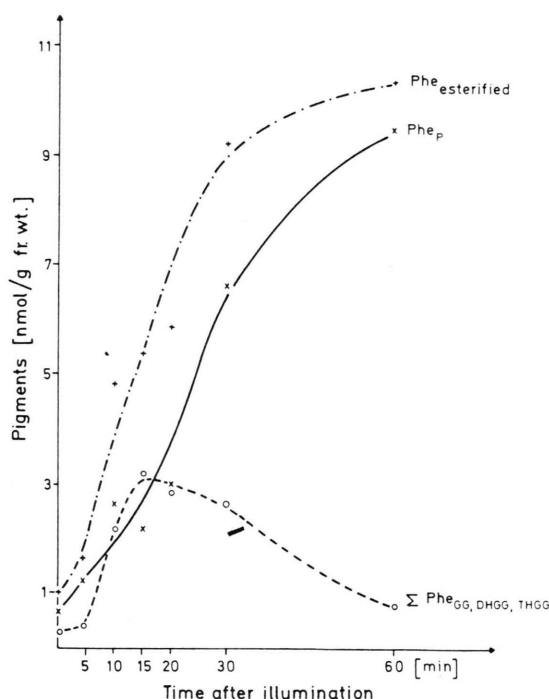


Fig. 3. The amounts in nmol/g fresh weight of total esterified pigment (+ · - · + · - · +), of Phe_P (× - × - ×) and of the sum of Phe_{THGG}, Phe_{DHGG} and Phe_{GG} (○ - - - ○) during dark incubation of 1-min preilluminated etiolated bean leaves.

esterification to Chl_{GG} followed by hydrogenation *via* Chl_{DHGG} and Chl_{THGG} to Chl_P.

This work is supported by a grant from the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg. The author wishes to thank Prof. W. Rüdiger for his helpful discussions and continuing support, to Dr. Ch. Guthoff for critically reading the manuscript, to Miss U. Lempert for skillful technical assistance and to Miss H. Wieschhoff for performing the HPLC-analyses.

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