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Triton X-100 reacts with chlorophyll in the presence of chlorophyllase

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Chlorophyllase (chlorophyll chlorophyllidohydrolase, EC 3.1.1.14) catalyzes the transesterification of chlorophylls with the surfactant Triton X-100, which is widely used in the preparation and study of this enzyme. The preparation and some properties of water-soluble tritonyl chlorophyllide esters are described. A mechanism for the role of Triton X-100 as an inhibitor in chlorophyllase-catalyzed hydrolysis and transesterification of chlorophylls is proposed. Bacteriochlorophyl *a* also has been employed as a substrate for green plant chlorophyllase.

Chlorophyllase; Triton X-100; Transesterification

1. INTRODUCTION

The enzyme chlorophyllase (chlorophyll chlorophyllidohydrolase, EC 3.1.1.14) [1–3] catalyzes esterification, transesterification and hydrolysis at the propionic acid side chain of chlorophyll [4,5]. The nonionic detergent Triton X-100 has been widely used to facilitate the extraction of Chlase from plant material [6,7], and is a frequently used additive in assays of Chlase activity, despite a number of reports that Triton X-100 appears to inhibit Chlase activity [8–10]. We have found that Triton X-100, a primary alcohol, in the presence of Chlase can readily replace the natural esterifying groups (phytyl, farnesyl, geranylgeraniol) to produce chlorophyll derivatives containing transesterified Triton X-100 (fig.1). We have observed the formation of Triton X-100 (tritonyl) esters of several chlorophylls and have character-

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Abbreviations: Chl *a*, chlorophyll *a*; Chlase, chlorophyllase; BChl *a*, bacteriochlorophyll *a*; PChl *a*, pyrochlorophyll *a*; PChlide *a*, pyrochlorophyllide *a*; PbChl *a*, pyrobacteriochlorophyll *a*; PbChlide *a*, pyrobacteriochlorophyllide *a*; PDMS, ^{252}Cf plasma desorption mass spectrometry

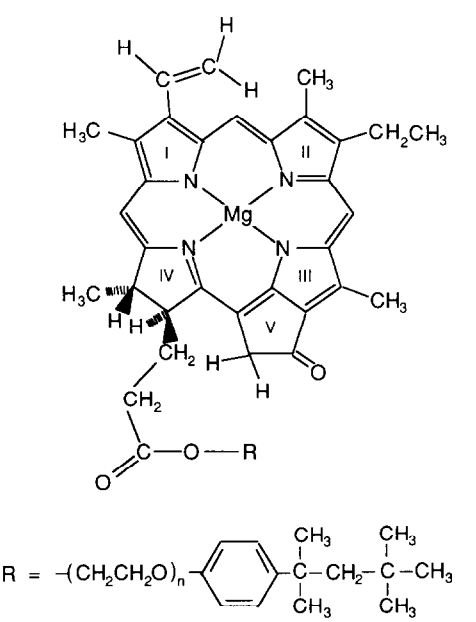

Fig.1. Structure of tritonyl X-100 PChlide *a*.

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ized them by NMR, PDMS [11] and optical spectroscopy. The tritonyl chlorophyllide esters are readily dispersed in water, and offer unique possibilities for the design of aqueous chlorophyll systems.

2. MATERIALS AND METHODS

2.1. *Materials*

Chlase was obtained from chloroplasts of *Ailanthus altissima* leaves by the method of Tanaka et al. [2], modified by the addition of 0.2% pyridine (v/v) during extraction and by several extractions of the product with 95% acetone. The powdered Chlase product can be stored at −24°C for several months without appreciable loss of enzymatic activity. Triton X-100 (Sigma) and reduced Triton X-100 (Aldrich) were used as received. Chl *a*, BChl *a* (from *Rhodobacter sphaeroides*), PChl *a* and PbChl *a* were prepared as described [12].

2.2. *Methods*

2.2.1. Chlase assay

The hydrolytic activity of Chlase preparations was determined by HPLC assay with Chl *a* as substrate. Chlase powder (66 mg), ascorbic acid (20 mg) and 0.4 ml water were gently agitated and sonicated for 30 s; 0.5 ml acetone was then added. Reaction was initiated by addition of 1 mg Chl *a* (1.119 μmol) in 0.5 ml acetone, and the reaction mixture incubated at 22°C in the dark. The progress of the hydrolytic reaction was measured by the change in Chl *a* and/or chlorophyllide *a* concentration. Aliquots of the reaction mixture (5 μl) were taken every 5 min, diluted to 0.130 ml with acetone, and 10 μl samples analyzed by HPLC. Activity of the Chlase, defined here as μmol Chl *a* reacted/min per mg of Chlase powder, was 3.8×10^{-4} (at 30 min). Under these conditions, 95.7% Chl *a* was hydrolyzed to chlorophyllide *a* in 70 min.

2.2.2. HPLC

A dual pump (Beckman 110A) system equipped with a computer controlled diode array detector (HP 8451A) was used for the HPLC analyses. Development of the chromatogram on an Ultrasphere ODS, 5 μm, C-18 (4.6 mm × 25 cm) column was followed simultaneously at 11 different wavelengths. The mobile phase, in all experiments, consisted of acetone/ethanol/water, 72:20:8 (v/v). Preparative scale separations were carried out on a DuPont Zorbax column (21.2 mm × 25 cm) with a refractive index detector.

3. RESULTS

3.1. *Reaction of PChl* a *with Triton X-100*

Small amounts of an unexpected product were detected by HPLC in the Chlase-catalyzed hydrolysis of PChl *a* in the presence of Triton X-100 (0.2%). The spectral properties and chromatographic behavior in reversed-phase column chromatography suggested that this material was the tritonyl ester of PChlide *a*. To test this hypothesis, the concentration of Triton X-100 in the reaction mixture was increased in steps from 0.2% (v/v) to 20% (v/v). The yield of tritonyl PChlide *a* reached its maximum at 22% in a reaction mixture containing Triton X-100/water/acetone in the ratio 15:45:40 (v/v).

PChl *a* is transesterified with reduced Triton X-100 in 18% yield under the same reaction conditions. The reduced surfactant appears to be identical to ordinary Triton X-100 in its behavior with Chlase.

When the concentration of PChl *a* dropped below 10% of the initial concentration during the course of the reaction, the yield of tritonyl ester diminished, presumably due to hydrolysis of the tritonyl ester to PChlide *a*. Support for this hypothesis comes from studies of the hydrolysis of tritonyl PChlide *a*. In a pure sample of tritonyl PChl *a* (0.25 μmol) in 1.5 ml of a 1:1 acetone/water mixture containing 66 mg of a Chlase preparation, 74% of the ester was hydrolyzed in 30 min.

3.2. *Reaction of PbChl* a *with Triton X-100*

The only BChl *a* reaction in the presence of Chlase reported in the literature is the hydrolysis of PbChl *a* [13,14]. BChl *a* is an excellent substrate for the Chlase-catalyzed formation of tritonyl esters of PbChl *a*. Here also the hydrolysis of the tritonyl PbChlide *a* competes with its formation by transesterification. In the Triton X-100 concentration range 0.25% to 0.75% (v/v), yields of only 0.1–6% of tritonyl PbChlide *a* were detected by HPLC. Increasing the Triton X-100 concentration to 18% (0.4% pyridine/10.6% water/71%

acetone, v/v) resulted in a 44% yield of tritonyl PbChlide *a* after 5 h. PbChlide *a* was also formed in 10% yield, and 46% of unreacted PbChl *a* was recovered.

3.3. *Characterization of tritonyl chlorophyllides*

Tritonyl PChlide *a* was prepared on a milligram scale in a reaction mixture containing 2.8 g Chlase, 25 mg PChl *a* (30 μmol), 12% Triton X-100 in 35 ml of a 21% acetone/67% water, v/v, mixture. The progress of the reaction was followed by analytical HPLC (flow rate: 0.7 ml/min). The tritonyl ester (r.t.: 5.8 min) was formed in 32.8% yield after 2 h. The other components of the reaction mixture at that time were PChlide *a* (46.3%, r.t.: 2.9 min) and unreacted PChl *a* (20.9%, r.t.: 16.6 min).

Isolation of pure products was complicated by the large excess of Triton X-100 in the reaction mixture. Chlase was removed by filtration, and the reaction products were extracted from the reaction mixture saturated with NaCl with diethyl ether. The extract was concentrated by evaporation and chromatographed twice on a DEAE Sepharose CL-6B column (2.5 cm × 10 cm) with acetone (1 ml/min). PChlide *a* was retained on the column. The ester fraction was then chromatographed on a Zorbax column (flow rate: 4 ml/min). The pure tritonyl PChlide *a* ester (6 mg, r.t.: 19 min) was obtained after three HPLC separations.

Assignment of the ^{1}H NMR (fig.2A) spectrum confirmed the expected structure of a chlorophyllide ester in which the phytyl chain is replaced by the tritonyl moiety. The ^{1}H NMR spectrum of tritonyl PChl *a* was strongly solvent dependent (fig.2B). It is possible that the principal contributor to solvent effects on the ^{1}H NMR spectrum resulted from differences in aggregation of the methylene groups of (poly)ethylene oxide chains of the tritonyl moiety of the PChlide *a* ester. Integration of the ^{1}H NMR spectrum showed that the average number of ($-CH_2CH_2O-$) units within tritonyl moiety was 10. Triton X-100 is a mixture of polymers containing $(-CH_2CH_2O-)_n$ units with the average number $\bar{n}$ of 10. Additional confirmation of the structure of tritonyl PChlide *a* came

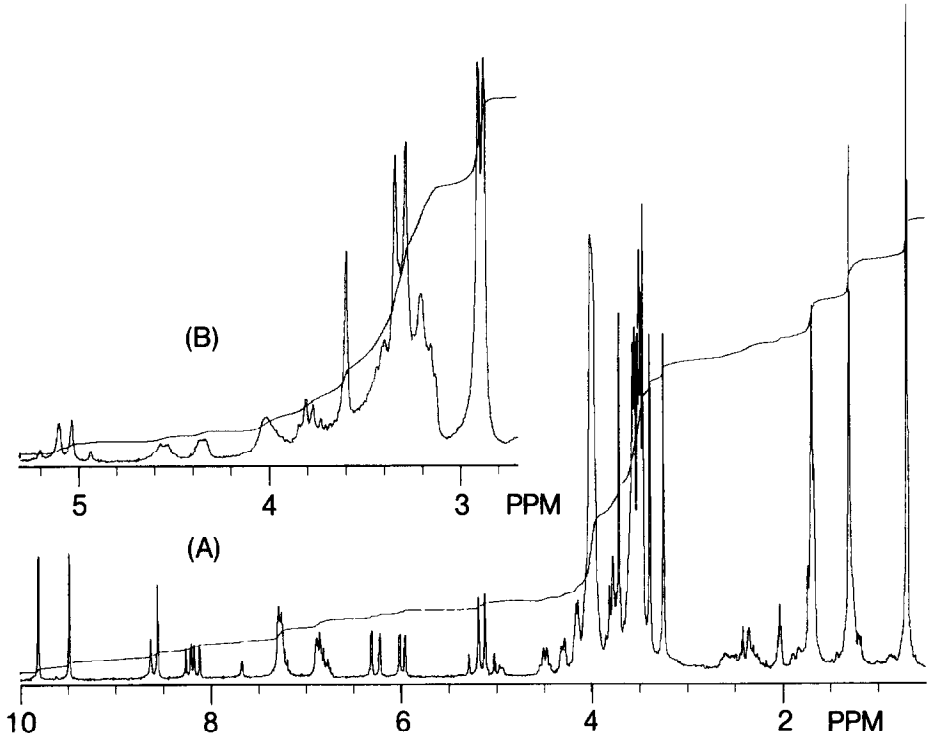

Fig.2. (A) ^{1}H NMR spectrum of tritonyl X-100 PChlide *a* in pyridine-d_5/acetone-d_6 (1:1, v/v); (B) $CDCl_3$/acetone-d_6 (1/4, v/v) (Chl: 3 μl/ml).

from ^{252}Cf-PDMS mass analysis (fig.3). Molecular ions were measured from m/z = 900 (n = 4) to m/z 1296 (n = 13). The weight average molecular mass corresponds to approx. 9 (-CH_2CH_2O-) units. The lower estimate derived from mass spectrometry probably is a consequence of the under representation of higher mass molecular ions of polymers in the mass spectrum.

Tritonyl PChlide *a* is a dark green solid soluble in common organic solvents including chlorocarbons, alcohols and acetone, and in water. Its UV-VIS absorption spectrum in acetone shows a characteristic Chl *a* type spectrum: 412(s), 432(v.s), 618(w) and 662(v.s) nm, with a λ_{max}(blue)/λ_{max}(red) ratio of 1.34. It forms stable solutions (0.12 μmol/ml) in water/acetone (50:1). The optical spectrum of tritonyl PChlide *a* in this solution resembled its spectrum in acetone: 416(s), 436(v.s), 624(w) and 668(v.s) nm with a λ_{max}(blue)/λ_{max}(red) ratio of 1.34. Thus, the macrocycles have a monomer type spectrum.

3.4. *Transesterification of Chl* a *and BChl* a

Formation of tritonyl esters of Chl *a* and BChl *a* in significant concentrations occurred only in the presence of a large excess of Triton X-100. In a reaction mixture containing Triton X-100/water/acetone, 17:60:23 (v/v), and Chl *a* (0.72 μmol/ml), tritonyl Chlide *a* was formed in 1.3% yield. Tritonyl Chlide *a* was obtained in 6.3% yield when the Triton X-100 concentration was increased to 30%. Similar results were obtained with BChl *a*.

4. DISCUSSION

The apparent inhibition [8–10] in Chlase-catalyzed reactions of chlorophylls in the presence of Triton X-100 is likely to be due to competitive transesterification reactions in which the detergent is the substrate. Accumulation of tritonyl chlorophyllide esters occurs only for chlorophylls or chlorophyll derivatives lacking a carbomethoxy group in ring V. Hydrolysis is markedly faster in chlorophyll derivatives that possess a carbomethoxy group in ring V, and this could account for the relative difficulty in obtaining high yields of tritonyl ester from intact chlorophylls. As

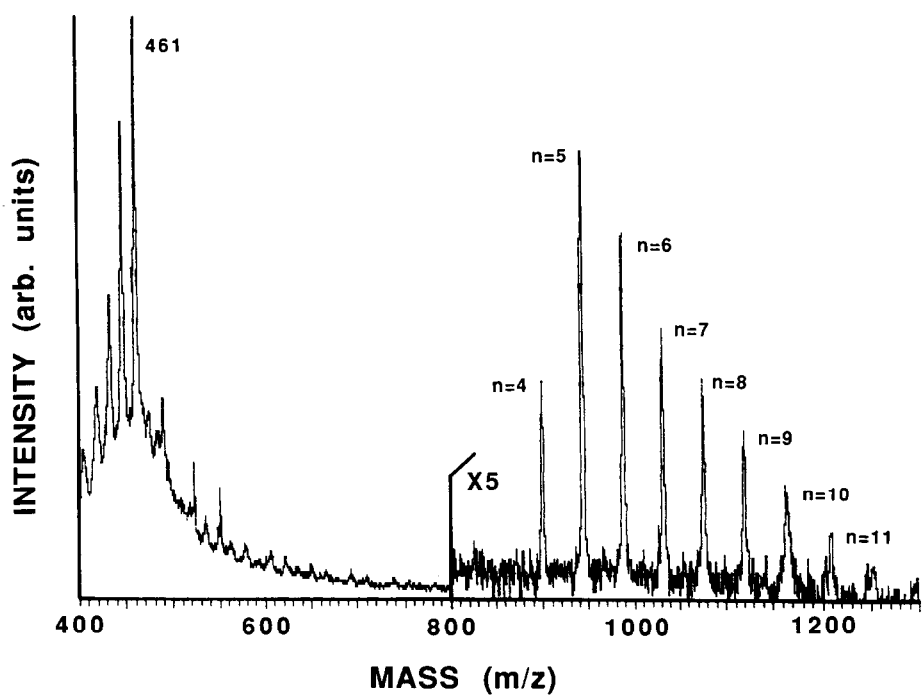

Fig.3. The positive ion ^{252}Cf-PDMS mass spectrum of tritonyl pyropheophorbide *a* ester, containing the molecular ion envelope. *n* corresponds to the number of repeating (-CH_2CH_2O-) units in Triton polyether.

bacteriochlorophyll *c* and *d* are naturally occurring chlorophylls that lack a carbomethoxy group, we expect these chlorophylls to be particularly prone to form hydrolysis-resistant transesterified esters [6].

Triton X-100 has been widely used in the isolation of photosynthetic membrane proteins from plant and bacterial material, usually at concentrations below 0.2%. The extent of Chlase-catalyzed hydrolysis of Chl *a* in chloroplasts and chlorophyll protein complexes was reported to be dependent on the presence of Triton X-100 [18]. There is a growing awareness of the possibility of artifact formation due to Triton X-100 [15–17]. Any tritonyl esters that might be formed in the presence of Chlase would be difficult to detect because of the subsequent rapid hydrolysis of the tritonyl esters of Chl *a* and BChl *a*.

The solubility of the new chlorophyll derivatives in water should make it possible to study aqueous chlorophyll systems containing water-soluble components such as proteins. Since Chlase is rather unevenly distributed in the photosynthetic membrane [1], it may be present as a contaminant during the isolation of cellular components. Therefore, in the presence of Triton X-100 or any other primary alcohol group, transesterification involving chlorophyll species may occur.

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