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RESEARCH NOTE

AN IMPROVED ANALYSIS FOR DETERMINATION OF MONOVINYL AND DIVINYL PROTOPORPHYRIN IX

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Abstract — For studying chlorophyll biosynthetic heterogeneity of plants, it is necessary to establish a technique for microassay of a putative monovinyl and divinyl protoporphyrin IX. Precise determination of the amounts of monovinyl and divinyl protoporphyrin IX is difficult with optical electronic spectroscopy even at 77°K. Such a problem could be solved by conversion of protoporphyrin IX to protoporphyrin IX dimethylester with diazomethane and subsequent Mg insertion into protoporphyrin IX dimethylester by reaction with a Grignard solution. The proportion of monovinyl and divinyl Mg-protoporphyrin IX dimethylester formed was measured instead of direct measuring that of protoporphyrin IX by low-temperature spectrofluorometry. The relative proportions of monovinyl and divinyl of protoporphyrin IX, Mg-protoporphyrin IX, and Mg-protoporphyrin IX dimethylester have not changed during the chemical conversion steps. This analysis system could be useful for the study of the monovinyl and divinyl chlorophyll biosynthetic routes in plants.

INTRODUCTION

In green plants Chl *a* is formed via two parallel biosynthetic routes; the DV and MV carboxylic Chl *a* routes.[1,2] The intermediates of the DV carboxylic route have two vinyl groups, one at position 2 and the other at position 4 of the macrocycle (Fig. 1). In contrast, the intermediates of the MV carboxylic route have one vinyl and one ethyl group at positions 2 and 4 of the macrocycle, respectively (Figure 1). The two routes are assumed to be linked by 4-vinyl reductases at several levels of Chl *a* biosynthetic pathway.[3,4,5,6] Fluorescence spectroscopy has successfully been used for the detection and quantitation of very small amount of tetrapyrroles in unpurified mixtures.[7,8,9,10] The determination of DV and MV tetrapyrroles is usually done by using room temperature[8,9,10] and 77°K spectrofluorometry for quantifying tetrapyrroles and for measuring proportion of DV and MV tetrapyrroles,[7,10] respectively. MV tetrapyrroles beyond Proto in the porphyrin pathway are known to be easily detected. However, those up to Proto in the pathway have not been determined precisely, since proper techniques for determining them have not been established. Determination of the precise proportion of MV and DV Proto with optical electronic spectroscopy has not been successful even under 77°K condition. This is because the fluorescence emission and excitation maxima of MV and DV Proto are not sufficiently separated, and their Soret excitation bands are rather broad.[11,12] In this work, we report microassay technique for determining putative MV and DV Proto. In this technique, Proto was converted to MPE for determining the proportion MV and DV of MPE by

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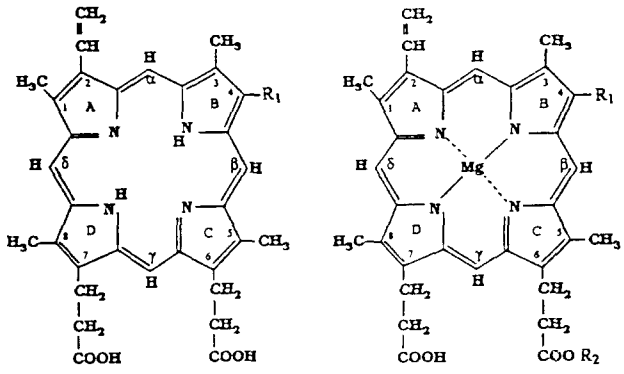
† *Abbreviations*: Chl, Chlorophyll; Chlide, Chlorophyllide; DV, Divinyl; Mg-Proto, Mg-Protoporphyrin IX; MPE, Mg-Protoporphyrin IX methyl ester; MP(E), a mixture of Mg-Proto and MPE; MV, Monovinyl; Proto, Protoporphyrin IX; Protogen, Protoporphyrinogen IX.
‡ Unless preceded by MV or DV, tetrapyrrole names are used generically to designate metabolic pools that may consist of MV and/or DV components.



Protoporphyrin IX Mg-Protoporphyrins

R_1	R_2	Compounds
CH_2 - CH_3	-	MV Proto
CH_2=CH_3	-	DV Proto
CH_2 - CH_3	H	MV Mg-Proto
CH_2=CH_3	H	DV Mg-Proto
CH_2 - CH_3	CH_3	MV Mg-Proto momomethyl ester
CH_2=CH_3	CH_3	DV Mg-Proto momomethyl ester

Figure 1. Chemical structures of monovinyl and divinyl protoporphyrin IX and Mg-protopophyrins.

using typical 77°K spectrofluorometry. The proportion of MV and DV Proto was then indirectly calculated. This technique could widely be used in the study of the MV and DV Chl *a* biosynthetic routes in plants.

MATERIALS AND METHODS

Chemicals. DV Mg-Proto and DV MPE were from Porphyrin Products, Inc. (Logan, UT, USA). DV Proto was from Sigma Chemical Co. (St. Louis, MO, USA). Protogen was prepared by the reduction of Proto with sodium amalgam as previously described by Jacobs and Jacobs.[13] Organic solvents (GR or HPLC grade) were purchased from Fisher Scientific (Norcross, GA, USA).

Catalytic hydrogenation of tetrapyrroles. Catalytic hydrogenation of tetrapyrroles was conducted by gentle bubbling hydrogen through a solution of tetrapyrrole in methanol:methylene chloride (3:1, v/v). The catalyst was 10% palladium in asbestos. The hydrogenation of the authentic DV Mg-Proto and DV MPE was carried out on ice for 25 s and 10 min, respectively. After the hydrogenation, the reacted mixture was briefly centrifuged until the centrifugal force reached at 1500 g to remove the debris. The resulting supernatant was dried under nitrogen gas and resolved in ether for further experiments.

Methylation of acidic tetrapyrroles. An excess of ethereal solution of diazomethane was added to an ethereal solution of the extracted Proto pool (see below). The mixture was briefly swirled on ice and then completely dried under nitrogen gas. The ethereal solution of diazomethane was prepared from diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) (Aldrich Chemical Co., Milwaukee, WI, USA) as described in Aldrich Technical Information Bulletin Number AL-180.

Insertion of Mg into Proto. Insertion of magnesium into the Proto pool was performed by a modification of the method of Tripathy and Rebeiz.[12] The dried Proto dimethyl ester was dissolved in methylene chloride prior to reaction with a Grignard solution.[12] The latter was prepared as follows: In a round bottom flask, 20 mL of diethyl ether were added to 1 g of crushed magnesium turnings and a few milligrams of iodine crystals. The reaction mixture was gently heated (heating mantle rheostat at position 40). When the ether started to boil, 3 mL of methyl iodide were added dropwise. Following the most of the magnesium had reacted, the heater was turned off and the Grignard reagent was stored at -84°C under nitrogen gas. Under these conditions it was stable for several months. The Proto dimethyl ester was dissolved in 1.0 mL of methylene chloride containing 27μM of 2,6-di-*t*-butyl-4-methyl phenol which was prepared freshly. At this stage, 0.2-0.4 mL of the 2 *M* Grignard solution was added to the reaction mixture and the latter was immediately mixed with 1.0 mL of diethyl ether and washed with 10-13 mL of 0.1 N NH_4OH. An additional 1.0 mL of diethyl ether was added and the ether phase containing Mg-Proto dimethyl ester was collected with a Pasteur pipette. The ether extract was briefly centrifuged until the centrifugal force reached at 1500 g to remove suspended particles. The resulting supernatant was used for spectrofluorometric determinations at 77°K. Loss of Mg inserted-Proto dimethyl ester during the wash with 0.1 N NH_4OH was found to be negligible (data not shown).

Determination of MV and DV proportion of Mg-Proto pools. The proportion of MV and DV Proto was determined by spectrofluorometry at 77°K in ether and caculated with the equation represented by Belanger and Rebeiz.[10]

Spectrofluorometry. Fluorescence spectra were recorded on a fully corrected photon-counting, high-resolution SLM spectrofluorometer Model 8000C interfaced with an IBM Value Line microcomputer. Determination at room temperature were performed on an aliquot of the hexane-extracted acetone fraction in cylindrical microcells (3 mm in diameter) at emission and excitation bandwidths of 4 nm.[8,9,10] Fluorescence spectra at 77°K were recorded at emission and excitation bandwidths that varied from 0.5 to 4 nm depending on signal intensity.[7,10] The photon count was integrated for 0.5 s at each 1 nm increment.

RESULTS AND DISCUSSION

To investigate a putative MV Proto in plants and to characterize the related enzymes *in vitro*, an appropriate analytical method should be established for determination of MV and DV forms from a small amounts of Proto. No such method has been

published, since small amount of Proto having two carboxyl groups (Fig. 1) hinders its analysis. Furthermore, there are technical difficulties to distinguish between MV and DV Proto by spectrofluorometry even at 77°K.[11,12] As an attempt to overcome these experimental difficulties, Tripathy and Rebeiz[12] tried to determine MV and DV Proto indirectly after converting all Proto to Mg-Proto by chemical Mg insertion, since the quantitative determination of MV and DV Mg-Proto can routinely be accomplished with precision.[10] However, their attempt was found to be not efficient for the analysis of a small amount of MV and DV Proto. When Mg is inserted to Proto, the Mg-inserted Proto becomes more hydrophilic and thus is easily run out during the extraction and purification procedures, especially in alkali washing solution which is generally used for preventing Mg deletion during the purification. Furthermore, since it is sticky on glass wall in the presence of ether, extraction of small amounts of Mg-Proto with ether has not been complete.

In this work, we convert Proto to Proto dimethylester with diazomethane and then insert Mg into the Proto dimethylester by reaction with a Grignard solution, rather than the direct insertion of Mg into Proto as before.[12] Our MPE converted from Proto exhibited sufficiently seperated spectra of MV and DV MPE, which allowed the determination of them (Fig. 2). To ascertain whether this is appropriate for the determination of MV and DV Proto, any changes at side chains of Proto and Mg-Proto pools were examined during methylation and Mg-insertion reaction. First, authentic DV Mg-Proto and DV MPE were partially reduced by the chemical hydrogenation as described in Materials and Methods. Then, MV and DV proportion of the heterogeneous compounds, which probably consist of mixture of 2,4-divinyl, 2,4-diethyl, and 2-vinyl and 4-ethyl, was examined before and after the chemical reaction of methylation and/or Mg-insertion. It would be expected that if the methylation and/or Mg-insertion reaction affect at the side chains of the tetrapyrroles, a difference in the MV/DV ratio will occur before and after reactions. However, such differences were not observed (Table 1). Second, DV Proto was converted to MPE by the reaction of methylation and Mg-insertion as above and the MV and DV proportion of MPE was examined. The converted MPE was found to be all DV form, indicating that the reactions did not affect at the side chains of Proto (Table 1). However, Protogen which was prepared by the reduction of DV Proto with sodium amalgam had MV proportion of 32.4 %. It is speculated that a change at position 2 and 4 of macrocycle might occur during the reduction of DV Proto. Therefore, prepared Protogen seems not to be a proper substrate source for a research of Chl *a* heterogeneity. In addition, effects of volume of the Grignard reagent (0.05-0.4 ml), pH of extraction solution (pH 2-6), and the sample concentrations in

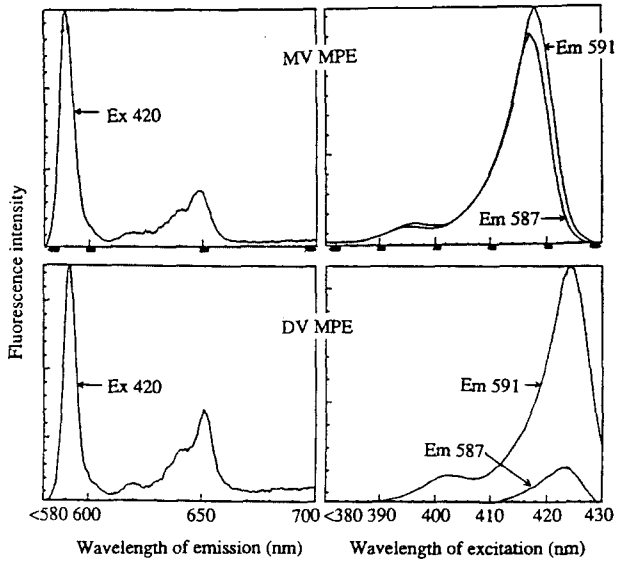


Figure 2. Fluorescence emission and excitation spectra of monovinyl and divinyl Mg-protopophyrin IX methylester (MPE) in ether at 77°K.

Table 1. Proportion of MV and DV components of standard tetrapyrroles in ether, before and after reaction, at 77°K.

Reaction step	Protogen (MV:DV%)	Proto (MV:DV%)	Mg-Proto (MV:DV%)	MPE (MV:DV%)
Initial[a]	-	0 : 100	0 : 100	0 : 100
After hydrogenation[b]	-	-	26.8 : 73.2	57.9 : 42.1
After methylation[c]	-	-	-	52.0 : 48.0
After Mg insertion[d]	32.4 : 67.6	0 : 100	26.0 : 74.0	56.1 : 43.9

[a] Protogen was prepared by the reduction of DV Proto with sodium amalgam.[13]
[b] Hydrgenation of DV Mg-Proto and DV MPE was carried out on ice for 25 s and 10 min, respectively, with the catalyst of 10 % palladium in asbestos.
[c] An excess of ethereal solution of diazomethane was added to an ethereal solution of the tetrapyrroles.
[d] Grignard's reagent was added to reaction mixture as described in Materials and Methods.

ether on the MV and DV proportion of Proto were not found (data not shown). With our method, MV and DV proportion of Proto could be measured even at the level of 50 pmol/mL. The analysis system would efficiently be used for the detection of MV Proto from higher plants.

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