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# ACTIVITY AND PROPERTIES OF (−)-*S*-ADENOSYL-L-METHIONINE: MAGNESIUM-PROTOPORPHYRIN IX METHYLTRANSFERASE IN CRUDE HOMOGENATES FROM WHEAT SEEDLINGS

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SUMMARY

A direct, rapid assay for the enzyme *S*-adenosylmethionine:magnesium-protoporphyrin IX methyltransferase (EC 2.1.1.11) is presented. Using the assay developed, the effect of temperature and pH on the activity of the enzyme in a crude homogenate of etiolated wheat seedlings was studied. The pH optimum was found to be 7.7 using phosphate buffer; the enzyme exhibited maximum activity at 37 °C although activity was found to be nearly optimal at 25 °C. The $K_m$ (pH 7.7, 25 °C) values determined for Mg-protoporphyrin IX and *S*-adenosylmethionine were 36 and 48 $\mu$M, respectively. Treatment of the crude preparation from etiolated wheat with *n*-butanol to remove endogenous pigments did not result in a decrease in specific activity of the preparation. A crude preparation from green wheat seedlings when treated with butanol exhibited an activity comparable with that of the etiolated wheat preparation so treated thereby demonstrating that this assay could be used in green plant systems.

## INTRODUCTION

The enzyme (−)-*S*-adenosyl-L-methionine:magnesium-protoporphyrin IX methyltransferase (EC 2.1.1.11), first observed by Tait and Gibson[1] in *Rhodopseudomonas spheroides* and later demonstrated in a chloroplast suspension derived from corn seedlings by Radmer and Bogorad[2], is apparently solubilized to some degree by extraction of higher plant tissues with a buffered sucrose solution[3]. This enzyme is strongly associated with subcellular particles and/or membranes[1-3] but the proportion of the enzyme solubilized is not known. Previous assays of this enzyme have been limited to the use of $^{14}C$-labeled substrates due to the presence of high concentrations of endogenous porphyrins and to low yields of the Mg-protoporphyrin IX monomethyl ester product. This report describes a reliable, rapid and direct procedure, not requiring labeled substrates, suitable to assay this enzyme in a crude homogenate

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of etiolated wheat seedlings. In addition, it was found that the assay may be used on preparations from green plants after extraction of endogenous pigments from the crude homogenate with *n*-butanol.

## METHODS

### *Preparation of substrates*

Protoporphyrin IX was prepared from commercially available hemin (Sigma Chemical Co., St. Louis, Mo. U.S.A.)[4]. The protoporphyrin IX thus obtained was partitioned between 1.0 and 2.5% (w/v) aqueous HCl prior to use. $Mg^{2+}$-protoporphyrin IX was prepared by the insertion of Mg into protoporphyrin IX using the method of Baum *et al.*[5]. The *S*-adenosyl-L-methionine used was obtained from Sigma Chemical Co.

The *S*-adenosylmethionine was added to reaction mixtures dissolved in 0.2 M phosphate buffer (pH 7.7). Porphyrins were added to reaction mixtures in a solution of 1% (w/v) Triton X-100 in the same 0.2 M phosphate buffer.

### *Preparation of crude homogenates*

40 g of 7-day-old etiolated wheat (*Tricticum aestivum*, L. emend. Thell. Kharkov 22 M.C.) seedlings were gently ground in a mortar in 40 ml of chilled 0.2 M phosphate buffer (pH 7.7) containing 0.02 mole sucrose. The resulting homogenate was then filtered through Whatman No. 40 filter paper; the filtrate was the crude homogenate used. The preparation was freshly prepared for each experiment. All operations were carried out at room temperature under subdued light unless otherwise noted.

### *Preparation of butanol-treated homogenates*

For assay of the enzyme in 8-day-old green plants, 1.5 vol. of *n*-butanol were added to the crude preparation and shaken briefly in a separatory funnel and then filtered through Whatman No. 40 filter paper. The aqueous layer, containing 2.5 mg protein per ml, was removed for assay. That no fluorescence was observed in the aqueous layer or subsequent ethereal or acidified ethyl acetate extracts of the aqueous layer suggested that the *n*-butanol treatment had removed all endogenous porphyrins. Crude homogenates from etiolated seedlings were also treated in the same way where specified in the text.

Proteins were determined in these preparations using the biuret reaction[6].

### *Preparation of reaction mixtures*

To 5 ml of the crude preparation (containing 6–9 mg proteins per ml) were added *S*-adenosylmethionine (0.03–1.1 $\mu$moles) in 0.5 ml of 0.2 M phosphate (pH 7.7) buffer and Mg-protoporphyrin IX (0.04–1.1 $\mu$moles) in 0.01–0.05 ml 1% Triton X-100 solution; Triton X-100 concentration was maintained at 0.01% in all assays. Final volume was 5.55 ml. Reaction mixtures were then covered with foil and shaken for 3 h at room temperature unless otherwise specified; reasonable product yields of 10–20% were obtainable within 1 h, but reactions run 3 h provided more reproducible results. The effect of pH was studied in phosphate buffer of appropriate pH.

*Analysis of reaction mixtures*

Reactions were stopped by adding 50 ml of a solution of ethyl acetate–acetic acid (3:1, v/v). The mixture was filtered to remove protein and shaken with 10% (w/v) sodium acetate. The ethyl acetate layer resulting, containing more than 90% of pigment, was washed several times with 10% (w/v) aqueous sodium acetate. In some experiments the acidified ethyl acetate extract was treated with solid $NaHCO_3$ to obtain an ethyl acetate layer containing pigment: nearly identical results were obtained and therefore the more rapid sodium acetate method was routinely used. Pigments were then extracted from the ethyl acetate layer with 6% (w/v) HCl. The pigments (free base porphyrins) were then re-extracted into fresh ethyl acetate by raising the pH with $NaHCO_3$. The ethyl acetate layer was then washed thrice carefully (avoiding shaking) with 0.1 M aqueous ammonia and thrice with distilled water. The ethyl acetate solution containing metallo-free protoporphyrins (as evidenced by absorption spectrophotometry) was then evaporated to dryness. The residue was redissolved in 0.5 ml pyridine and then applied to silica gel thin layers (Sil-HR silica gel polygrams, Brinkman Instruments, Westbury, N.Y., U.S.A.) which were developed in 2,6-lutidine to facilitate separation of protoporphyrin IX and its monomethyl ester as described earlier[7]. Carotenoids present in the pigments extracted from reaction mixtures ran ahead of the protoporphyrin IX monomethyl ester band and, therefore, did not interfere with the evaluation of the chromotograms. Evaluation of chromatograms was done densitometrically using a Gelman Integrating Scanner (Gelman Instruments, Ann Arbor, Mch., U.S.A.). Duplicate experiments rarely varied from each other by more than 5%. From the ratio of the absorbance of protoporphyrin IX monomethyl ester band to that of protoporphyrin IX, the percent yield of product was determined. Since the amount of Mg-protoporphyrin IX added

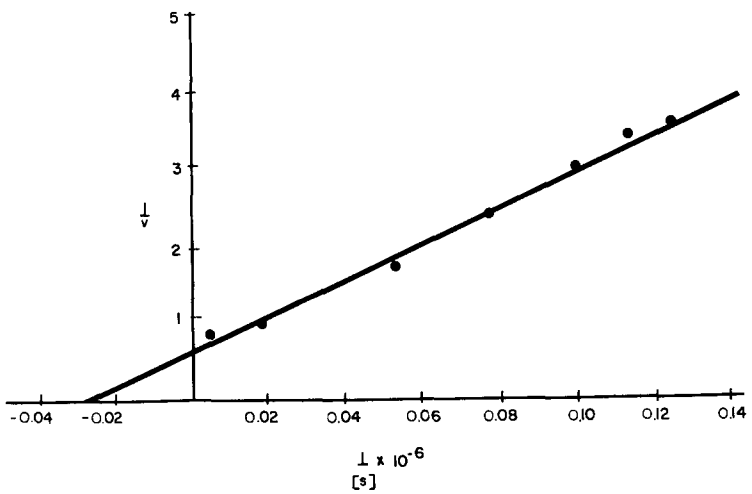


Fig. 1. Lineweaver–Burk plot of the effect of Mg-protoporphyrin IX concentration on Mg-protoporphyrin IX monomethyl ester synthesis by the crude homogenate from etiolated wheat seedlings. Conditions for assay were as described in Methods except that Mg-protoporphyrin IX concentration was varied as indicated. The $K_m$ value estimated from the data in the figure was 36 $\mu$M for Mg-protoporphyrin IX. The concentration of *S*-adenosylmethionine was 200 $\mu$M. $v$, $\mu$moles ($\times 10^6$) monoester formed per min per mg protein; [*S*], concn of Mg-protoporphyrin IX (M).

to reaction mixtures was known, the number of $\mu$moles of product formed could be calculated. If a densitometer is not available, a less rapid but almost equally reliable procedure is available by scraping off chromatographic bands, eluting porphyrins into ethyl acetate[7], and determining porphyrins in solution by absorption spectrophotometry.

## RESULTS AND DISCUSSION

The crude homogenate was found to be capable of esterifying nearly 50 pmoles of Mg-protoporphyrin IX in 1 min per mg protein. Fig. 1 represents the results of a typical experiment performed at non-limiting concentration of *S*-adenosylmethionine and at increasing concentrations of Mg-protoporphyrin IX (pH 7.7, 25 °C); the $K_m$ value was found to be 36 $\mu$M for Mg-protoporphyrin IX. The $K_m$ for *S*-adenosylmethionine was found to be 48 $\mu$M (see Fig. 2). Similar $K_m$ values for Mg-protopor-

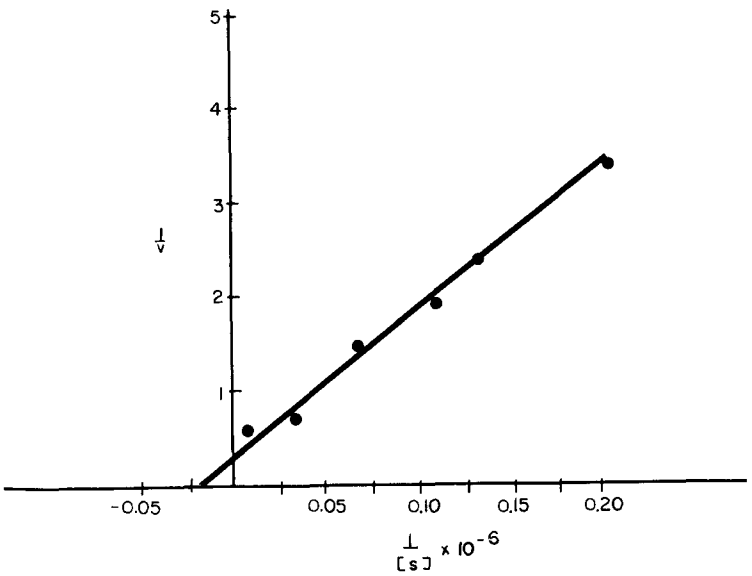


Fig. 2. Lineweaver–Burk plot of the effect of *S*-adenosyl-L-methionine concentration on Mg-protoporphyrin IX monomethyl ester synthesis by the crude homogenate from etiolated wheat seedlings. Conditions for assay were as described in Methods except that *S*-adenosylmethionine concentration was varied as indicated. The $K_m$ value estimated from the data in the figure was 48 $\mu$M for *S*-adenosylmethionine. The concentration of Mg-protoporphyrin IX was 200 $\mu$M. $v$, $\mu$moles ($\times$ $10^5$) monoester formed per min per mg protein; [*S*], concn of *S*-adenosylmethionine (M).

phyrin IX and *S*-adenosylmethionine of 40 and 55 $\mu$M, respectively, measured at pH 7.5 and 37 °C, were reported by Gibson *et al.*[8] for the *Rhodopseudomonas* enzyme. The reaction appeared to be first order with respect to enzyme and both substrates.

The enzyme exhibited a maximum activity near pH 7.7 using phosphate buffer (Table I); Gibson *et al.*[8] observed a pH maximum of 8.4 in their preparation from *R. spheroides* using Tris–HCl buffer. The optimum temperature was 37 °C (Table II), although activity in this study was almost maximal at room temperature (25 °C).

TABLE I

THE EFFECT OF pH ON THE ACTIVITY OF *S*-ADENOSYL-L-METHIONINE: MAGNESIUM-PROTOPORPHYRIN IX METHYLTRANSFERASE IN A CRUDE HOMOGENATE FROM ETIOLATED WHEAT SEEDLINGS

The homogenate was assayed for 3 h at 25 °C in the presence of 128 $\mu$M Mg-protoporphyrin IX and 160 $\mu$M *S*-adenosylmethionine (see Methods) at the pH indicated. The specific activity is expressed as $\mu$moles ($\times 10^5$) of Mg-protoporphyrin IX monomethyl ester formed per min per mg of protein.

| *Expt No.* | *pH* | *Specific activity* |
|---|---|---|
| 1 | 6.7 | 1.0 |
| 2 | 6.8 | 1.2 |
| 3 | 7.0 | 1.6 |
| 4 | 7.7 | 4.3 |
| 5 | 7.9 | 3.2 |

After heating a portion of the enzyme preparation at 60 °C for 1 h prior to incubation, no activity remained.

Because the detergent used to solubilize Mg-protoporphyrin may inhibit the enzyme, a study of the effect of Triton X-100 on the activity of *S*-adenosylmethionine: magnesium-protoporphyrin IX methyltransferase was made. It can be seen from the results (Fig. 3) that concentrations greater than 0.5 mg/ml of Triton X-100 depressed the activity of this enzyme; since the concentration of Triton X-100 in the assay was only 0.01% it had no effect upon the enzyme.

That the product was Mg-protoporphyrin monomethyl ester was proven after extracting this pigment as described by Granick[9]: it was identical to an authentic

TABLE II

THE EFFECT OF TEMPERATURE ON THE ACTIVITY OF *S*-ADENOSYL-L-METHIONINE: MAGNESIUM-PROTOPORPHYRIN IX METHYLTRANSFERASE IN A CRUDE HOMOGENATE FROM ETIOLATED WHEAT SEEDLINGS

The enzyme was assayed as described in Table I at the temperatures indicated. Specific activity units are described in Table I.

| *Expt No.* | *Temperature (°C)* | *Specific activity* |
|---|---|---|
| 1 | 0–4 | 1.0 |
| 2 | 23–26 | 4.2 |
| 3 | 37 | 4.4 |

sample of this compound when subjected to thin-layer chromatography[7] and spectrophotometry in ether solution.

Activities determined following extraction of Mg-protoporphyrins were in close agreement with those obtained by extracting metallo-free porphyrins.

Protoporphyrin IX was a poor substrate; methylation of protoporphyrin occurred at less than 10% of the rate observed when Mg-protoporphyrin IX was used.

That the assay may be applied to green plants was also demonstrated. Crude homogenates and butanol-treated homogenates from etiolated seedlings gave almost

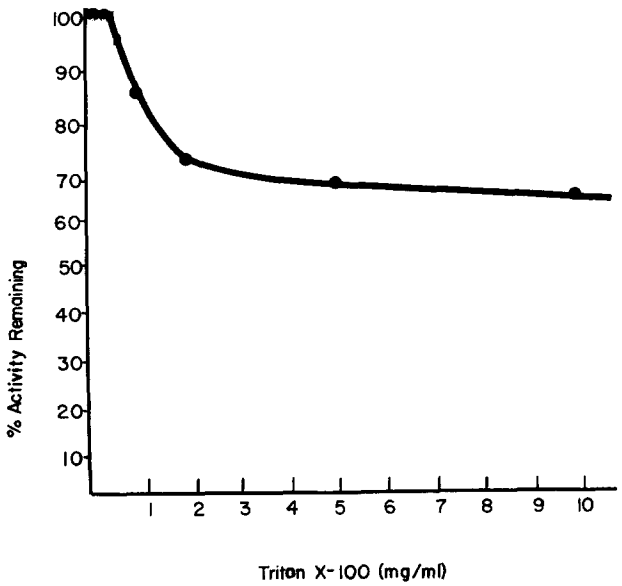


Fig. 3. The effect of Triton X-100 concentration on Mg-protoporphyrin IX monomethyl ester synthesis in a crude preparation from etiolated wheat seedlings. Conditions for assay were as described in Methods. Concentration of Mg-protoporphyrin IX and *S*-adenosylmethionine were 128 and 160 $\mu$M, respectively. In the other studies reported Triton X-100 never exceeded 0.5 mg/ml.

identical specific activities (Table III) suggesting that *n*-butanol treatment of crude homogenates from green plants might provide a protein system free from endogenous chlorophyll pigments that would allow the use of this method on green plants. The specific activity of the *n*-butanol treated preparation from green plants (Table III) was about half of that observed with the etiolated leaf preparation. The lower activity may be due to lower efficiency of extraction and solubilization of the enzyme from leaves with mature chloroplasts or, alternatively (but less likely), these green leaves may contain less enzyme than etiolated leaves.

TABLE III

THE ACTIVITY OF *S*-ADENOSYL-L-METHIONINE: MAGNESIUM-PROTOPORPHYRIN IX METHYLTRANSFERASE IN VARIOUS PREPARATIONS FROM GREEN AND ETIOLATED WHEAT SEEDLINGS

A crude homogenate of etiolated seedlings was prepared and a portion treated with butanol; in addition, a butanol-treated homogenate was prepared from green leaves (see Methods). All three preparations were assayed and results presented as in Table I.

| *Preparation* | *Specific activity* |
|---|---|
| (I) Crude protein homogenate from etiolated wheat | 4.2 |
| (II) *n*-Butanol extracted crude protein homogenate from etiolated wheat | 4.5 |
| (III) *n*-Butanol extracted crude protein homogenate (obtained from 8-day-old green wheat) | 2.0 |

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