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PURIFICATION BY AFFINITY CHROMATOGRAPHY AND PROPERTIES OF UROPORPHYRINOGEN I SYNTHETASE FROM *CHLORELLA REGULARIS*

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Summary

Uroporphyrinogen I synthetase (porphobilinogen ammonia-lyase (polymerizing), EC 4.3.1.8) from *Chlorella regularis* was purified to homogeneity by affinity chromatography on porphobilinogen-AH-Sepharose 4B, which was prepared by reacting carbodiimide with substrate, porphobilinogen. The enzyme was purified 232-fold from the initial crude extract and specific activity was 348 nmol porphyrinogen I formed (mg protein)$^{-1} \cdot h^{-1}$ at pH 7.4. The molecular weight of the enzyme was 35 000—36 000 as determined by Sephadex G-100 gel filtration. This enzyme was acidic protein having an isoelectric point of 4.2. The enzyme exhibited a single pH optimum at a pH value of 7.4 both in phosphate and Tris-HCl buffer. The K_m value for porphobilinogen was 89 μM as measured by its consumption and 85 μM when uroporphyrin formation was used. The Arrhenius plot obtained from the enzyme activity measurements appeared triphasic with breaks occurring at 35 and 46°C and activation energy was calculated to be 21 700 (10—35°C), 12 700 (35—46°C) and 1800 cal · mol^{-1} (46—65°C). This enzyme was heat stable and the enzyme still retained 87% of activity, even after 1 h incubation at 75°C.

Introduction

The enzyme, uroporphyrinogen I synthetase catalyzes condensation of four molecules of porphobilinogen to form the cyclic tetrapyrrole, uroporphyrinogen I. In the presence of additional enzyme, uroporphyrinogen III cosynthetase, porphobilinogen is converted into the uroporphyrinogen III, which is the precursor of heme, chlorophyll and cobyrinic acid [1]. The cosynthetase by itself is inactive toward porphobilinogen and uroporphyrinogen I. There is not

yet enough information concerning the functional role of cosynthetase and the mechanism of uroporphyrinogen III formation as a result of the action of these two enzymes.

Although uroporphyrinogen I synthetase has been studied from a number of different sources and preparations of varying degrees of purity have been obtained [2—9], the homogeneous preparation of algal enzyme has not been reported. To provide a better understanding of the manner in which these proteins associate to form uroporphyrinogen III from porphobilinogen, comprehension of the various properties existent in each enzyme is necessary. Apparently, it is geatly dependent on the availability of the highly purified enzyme preparations.

We here report a simple affinity chromatographic procedure for the preparation of *Chlorella regularis* uroporphyrinogen I synthetase and its characterization.

Materials and Methods

Materials

Chemicals. δ-Aminolevulinic acid, serum albumin (bovine), ovalbumin (chicken), chymotrypsinogen A (bovine), cytochrome *c* (horse), and Tris (Trizma base) were obtained from Sigma Chemical Co. (U.S.A.). Sephadex G-100, Sephacryl S-200, DEAE-Sephacel, AH-Sepharose 4B and Pharmalyte (pH 2.5—5.0) were products of Pharmacia Fine Chemicals (Sweden). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) from Fluka (Switzerland). All other chemicals were reagent grade.

Algal strain and culture conditions. The green alga, *C. regularis* (S-50) was obtained from the Yakult Institute for Microbiological Research, Tokyo, Japan.

The culture conditions were the same as those reported previously [10]. The cells were harvested by centrifugation with a yield of about 30 g wet weight per l medium. After washing twice with distilled water, the cells were suspended in 20 mM Tris-HCl buffer (pH 7.4) at a concentration of 200 g wet weight per l and stored in the dark at −15°C until used.

Preparation of porphobilinogen. Porphobilinogen was prepared enzymatically from δ-aminolevulinic acid in the presence of δ-aminolevulinic acid dehydratase, which was purified from the same *Chlorella* strain as described previously [10]. Porphobilinogen was purified by chromatography through a Dowex 1-X8 acetate column according to the method of Urata and Granick [11]. The lyophylized porphobilinogen was identified by paper chromatography in a *n*-butanol/acetic acid/water mixture (12 : 3 : 5, v/v) which gave an Ehrlich-positive spot with R_F 0.59 [12]. The presence of δ-aminolevulinic acid was checked by incubating a sample with purified δ-aminolevulinic acid dehydratase to see if any further porphobilinogen was formed [13].

Preparation of porphobilinogen-AH-Sepharose 4B. Porphobilinogen-AH-Sepharose 4B was prepared by coupling with substrate, porphobilinogen and AH-Sepharose 4B in the presence of carbodiimide using the following method. AH-Sepharose 4B (3 g) was swollen and washed with 0.5 M NaCl and further with distilled water (pH 4.5 with acetic acid). To the washed gel, 15 ml of 1.6

mM porphobilinogen solution (pH 4.5) and 12 ml of 10 mg/ml EDC solution (pH 4.5) were added and the mixture was incubated with gentle shaking in the dark for 20 h. After incubation, the gel was packed into the column and washed with about 30 ml of 0.05 M Tris-HCl (pH 7.4) containing 1 M NaCl, and with 0.05 M acetic acid (pH 3.1) containing 1 M NaCl, to remove unreacting porphobilinogen and carbodiimide. In this preparation, about 92% of porphobilinogen was coupled to the gel. The resulting gel, equilibrated with 20 mM Tris-HCl (pH 7.4) was used as porphobilinogen-AH-Sepharose 4B for affinity chromatography.

Analytical methods

Assay of enzyme. Uroporphyrinogen I synthetase was assayed in a reaction mixture containing 40 μmol Tris-HCl (pH 7.4), 88 nmol of porphobilinogen and enzyme, in a total volume of 1.0 ml. Incubation was carried out at 37°C. Uroporphyrinogen was determined spectrophotometrically after oxidizing the uroporphyrinogen to uroporphyrin according to the method of Jordan and Shemin [7]. The amount of uroporphyrinogen I in 1 N HCl was calculated from millimolar extinction coefficient of $5.48 \cdot 10^3$ at 406 nm [14]. 1 unit of enzyme activity was defined as the amount of enzyme producing 1.0 nmol urogen I/h. Specific activity was expressed as units/mg protein. Porphobilinogen was determined with modified Ehrlich assay [15] and 1 unit of enzyme activity was taken to be the amount of enzyme required to catalyze the consumption of 1 nmol/h.

Uroporphyrin isomers were analyzed on paper chromatography by the method of Cornford and Benson [16] after isolation from the mixture and esterification of uroporphyrin according to the procedure of Bogorad [14].

Protein content. Protein content was determined by the method of Lowry et al. [17], with bovine serum albumin as a standard.

Determination of molecular weight. Molecular weight was determined by the gel filtration through a Sephadex G-100 column (1.6 × 92 cm) at 4°C. The marker proteins used were bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), chymotrypsinogen A (M_r 22 000), and horse heart cytochrome *c* (M_r 12 400). The moving phase consisted of 20 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl. The void volume was determined by Blue Dextran 2000.

Determination of isoelectric point. The isoelectric point, p*I*, was determined using an Ampholine column (110 ml) and Pharmalyte (pH range 2.5—5.0) at 4°C for 45 h as described previously [10].

Gel electrophoresis. Polyacrylamide gel electrophoresis of the purified enzyme was carried out by the use of 9% gel in Tris-glycine buffer (pH 9.0) according to the procedure of Davis [18].

Isolation and purification of uroporphyrinogen I synthetase

All of the following procedures were carried out at 0—4°C.

Extraction, heat treatment and $(NH_4)_2SO_4$ fractionation. The algal cells, suspended with 20 mM Tris-HCl buffer (pH 7.4) were disrupted with a 20 kHz ultrasonic oscillator for 30 min in 3-min periods. The resulting cell homogenate was centrifuged at 22 000 × *g* for 1 h. The clear green supernatant was incubated at 65°C for 10 min and was centrifuged at 17 000 × *g* for 15 min to

remove denatured proteins. Then the precipitate forming at 40—75% saturation of $(NH_4)_2SO_4$ was prepared and dissolved in a small volume of 20 mM Tris-HCl buffer (pH 7.4).

Sephacryl S-200 gel filtration. After clarification by centrifugation at 12 000 × *g* for 15 min, the solution was applied on to a column of Sephacryl S-200 (2.6 × 65 cm), previously equilibrated with 20 mM Tris-HCl buffer (pH 7.4) and eluted with the same buffer. The active fractions were pooled.

DEAE-Sephacel column chromatography. The resulting active solution was loaded on to a column of DEAE-Sephacel (2.6 × 15 cm), previously equilibrated with 20 mM Tris-HCl buffer (pH 7.4). After washing the column with 120 ml of the same buffer, the enzyme was eluted with 500 ml of the same buffer with a linear gradient of 0—0.4 M NaCl. The uroporphyrinogen I synthetase fractions (elution peak 0.075 M NaCl) were concentrated with Minicon B15 (Amicon Co., U.S.A.).

Porphobilinogen-AH-Sepharose 4B affinity chromatography. The concentrated enzyme (approx. 800 units) was loaded on to a column of porphobilino-

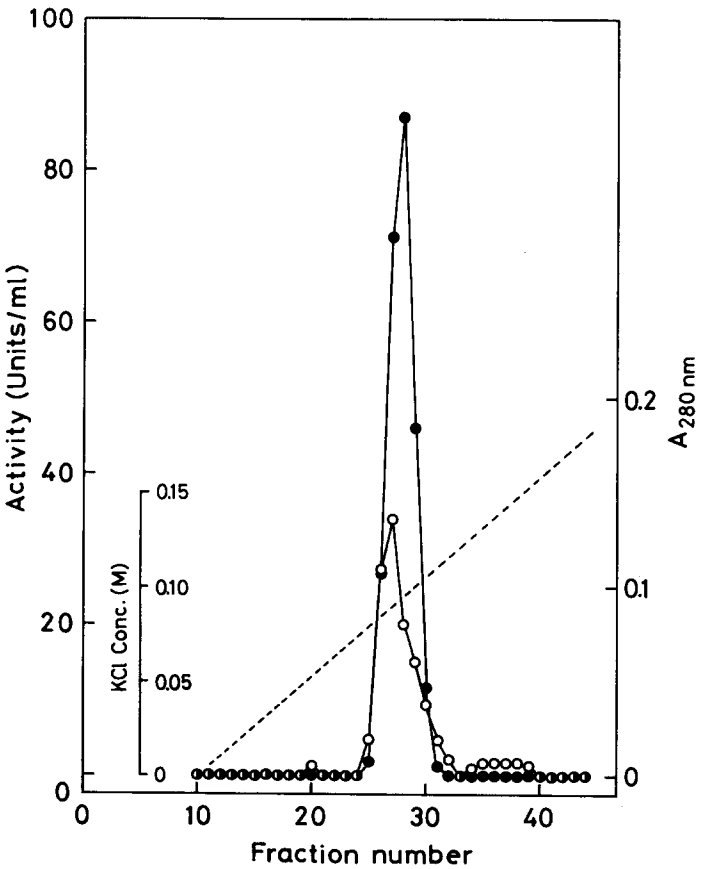

Fig. 1. Porphobilinogen-AH-Sepharose 4B affinity chromatography elution profile of the uroporphyrinogen I synthetase. The column was equilibrated with 20 mM Tris-HCl buffer (pH 7.4) and eluted with the same buffer with linear gradient of 0—0.3 M KCl. 3-ml fractions were collected. ○———○, absorbance at 280 nm; ●———●, uroporphyrinogen I synthetase activity. - - - - - -, KCl concentration.

gen-AH-Sepharose 4B (1 × 15 cm), which was previously equilibrated with 20 mM Tris-HCl buffer (pH 7.4). After washing with 40 ml of the same buffer, the enzyme was eluted with the same buffer with a linear gradient of 0—0.3 M KCl. A single symmetrical peak eluted with 0.095 M KCl was found (Fig. 1). The active fractions were concentrated with Minicon B15 and stored in the dark at —15°C. The enzyme preparation was stable under this condition, with a little loss of the activity (about 14% decrease) for at least 1 month.

Results

Purification and purity

Results of enzyme purification are summarized in Table I. The purified enzyme preparation obtained after porphobilinogen-AH-Sepharose 4B affinity chromatography (Fig. 1) was purified 232-fold from the initial crude extract, with a recovery of 29%.

The purified enzyme exhibited a single protein band after polyacrylamide gel electrophoresis; no minor bands could be detected either visually or by densitometric tracing of the gels (Fig. 2).

Physicochemical properties

Molecular weight. Molecular weight was estimated to be 35 000—36 000 by Sephadex G-100 gel filtration. Higuchi and Bogorad [9] determined the molecular weight of spinach enzyme to be 40 000 by Sephadex G-100 gel filtration and 38 000 by sucrose density gradient centrifugation.

Isoelectric point. The isoelectric point was determined on the homogeneous enzyme preparation, and a p*I* value of approx. 4.2 was obtained. This value was similar to those reported for the spinach enzyme (p*I* 4.2—4.5) [9] and *Rhodopseudomonas spheroides* enzyme (p*I* 4.46) [6].

Amino acid analysis. The results of all amino acid analyses, after 24 h hydrolysis, are given in Table II. An excess of acidic amino acid groups was observed over basic groups, thus accounting for the acidic isoelectric point of uroporphyrinogen I synthetase. From the amino acid residues, the molecular weight of the enzyme was calculated to be 33 300.

TABLE I

PURIFICATION OF *CHLORELLA* UROPORPHYRINOGEN I SYNTHETASE

PBG, porphobilinogen.

Purification step	Protein (mg)	Total activity ** (units)	Specific activity (units/mg protein)	Purification (-fold)	Yield (%)
Crude extract *	2566	3838	1.5	1	100
Heat treatment	801	3843	4.8	3	100
$(NH_2)_4SO_4$ (40—75% saturation)	213	3453	16.2	11	90
Sephacryl S-200	61.4	3166	51.6	34	83
DEAE-Sephacel	6.21	1649	266	177	43
PBG-AH-Sepharose 4B	3.21	1116	348	232	29

* Uroporphyrinogen I synthetase was extracted from 30 g (wet weight) of cells.

** Enzyme was assayed by uroporphyrinogen I formation.

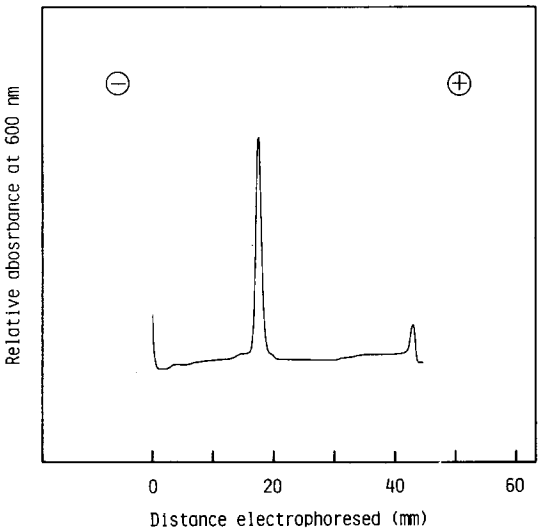

Fig. 2. Scanning profile of polyacrylamide gel electrophoresis of the purified uroporphyrinogen I synthetase. Electrophoresis was carried out in a Tris-glycine buffer (pH 9.0), using 9% acrylamide gel. The protein (30 μg) was applied to gel and stained with Amido black 10B for 1 h at 30°C and electrophoretically destained.

TABLE II

AMINO ACID COMPOSITION OF UROPORPHYRINOGEN I SYNTHETASE

Amino acid analysis was carried out with a Hitachi amino acid analyzer, model 835 F. The enzyme protein was hydrolyzed at 110°C for 24 h in 6 N HCl. Tryptophan content was determined after hydrolysis with 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole. Threonine and serine were corrected for degradation during hydrolysis: Thr, 5%, Ser, 10% [19].

Amino acid	Average number	Integral number
Asp	25.3	26
Thr	16.7	17
Ser	52.6	53
Glu	51.1	51
Pro	12.7	13
Gly	47.6	48
Ala	36.1	36
Cys	0.21	0
Val	16.4	16
Met	2.28	2
Ile	9.72	10
Leu	18.6	19
Tyr	5.54	6
Phe	6.36	6
Lys	12.4	12
His	6.80	7
Try	0.52	1
Arg	7.58	8
Total		331

Catalytic properties

Stoichiometry and K_m value. The stoichiometry of the reaction was estimated spectrophotometrically by measuring the ratio of substrate, porphobilinogen consumption and the tetrapyrrole formation [14]. We obtained the ratio of 4.0—4.5, indicating that about 4 mol substrate was consumed for 1 mol product formation. This stoichiometry matches the theoretical production of 1 mol porphyrin from 4 mol porphobilinogen.

The isomer analysis of the reaction product by paper chromatography [16] gave uroporphyrinogen I; no type III isomer could be detected.

The K_m value for substrate porphobilinogen was 89 μM as measured by its consumption and 85 μM when uroporphyrin formation was used. Our value for porphobilinogen consumption is similar to those reported for plant enzymes; spinach (72 μM) [9] and wheat germ (50 μM) [8].

pH profile. This enzyme shows a single, but slightly broad pH optimum at a pH value of 7.4 in Tris-HCl buffer. A similar profile was obtained using phosphate buffer; in this case the enzyme was more active at pH optimum (127%) than with Tris-HCl buffer. The enzyme was stable in the pH range from 6 to 9. pH optimum at a pH value of 7.8—8.2 has been reported for *R. spheroides* [6,7]. Frydman and Feinstein found a value of pH 8.2 for wheat germ [8].

Temperature profile and thermal stability. The enzyme showed a maximum activity at a temperature of 65°C (Fig. 3) 240% over the activity shown at 37°C. Arrhenius plots obtained from the activity measurements were triphasic with breaks occurring at 35°C and 46°C, indicating the conformational change

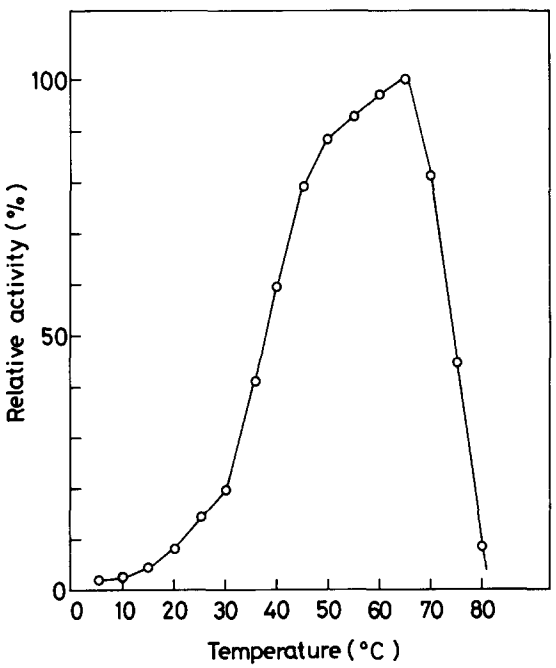

Fig. 3. Effect of temperature on the activity of purified uroporphyrinogen I synthetase. Control rate: 153.4 units/ml at 65°C (100%).

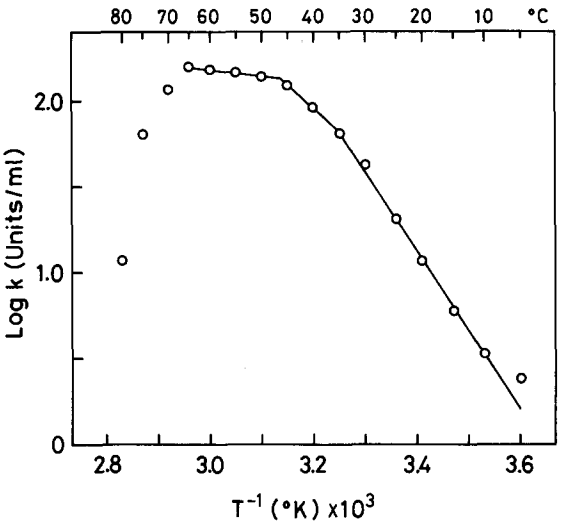

Fig. 4. Arrhenius plot of log rate constant (k) against reciprocal of absolute temperature (T) for uroporphyrinogen I synthetase. 1 unit of enzyme activity was expressed as nmol uroporphyrinogen I formed/h.

in the enzyme molecule (Fig. 4). Activation energy, calculated from the slopes in Fig. 4, was 21 700 (10—35°C), 12 700 (35—46°C), and 1800 cal · mol^{-1} (46—65°C).

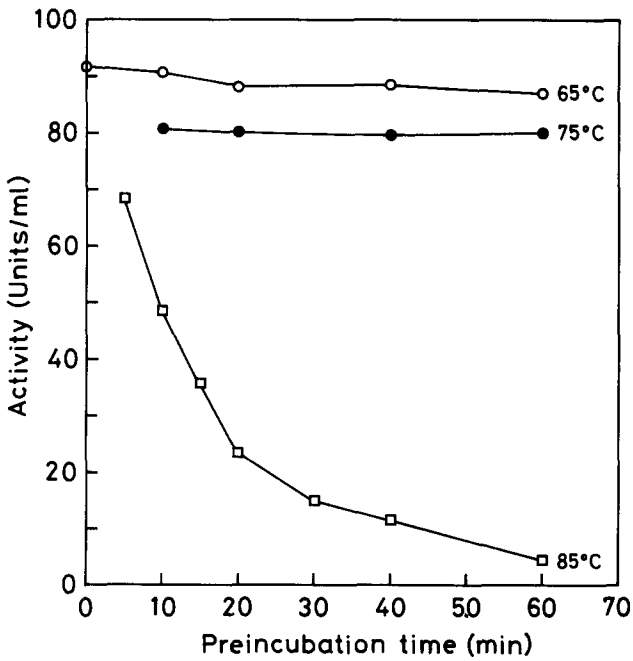

Fig. 5. Thermostability of uroporphyrinogen I synthetase. The enzyme was incubated prior to assay in the standard reaction mixutre. At the end of each preincubation period, rapidly cooled and the remaining activity of the enzyme was measured. 1 unit of enzyme activity was expressed as nmol uroporphyrinogen I formed/h.

Uroporphyrinogen I synthetase is known to be relatively stable to heat treatment compared with uroporphyrinogen III cosynthetase [2,4]. The thermal stability of the enzyme was examined by incubation of the homogeneous preparation in a normal reaction mixture, prior to start of the reaction with porphobilinogen. The results showed little loss of activity after 1 h incubation at a high temperature range (Fig. 5). Increasing the temperature results in a progressive loss of activity with time, but even after 1 h at 75°C, the enzyme still retained 87% of the activity.

Inhibitors. The enzyme was strongly inhibited by sulfhydryl reagents such as 25 μM *p*-chloromercuribenzoate (62% inhibition) and 1 mM *N*-ethylmaleimide (59% inhibition). Metal-chelating reagents, 1 mM EDTA and 1 mM *o*-phenanthroline had no effect on the enzyme activity. A similar situation has been reported for wheat germ [4], avain erythrocyte [5] and *R. spheroides* enzyme [7].

Discussion

Uroporphyrinogen I synthetase was purified to homogeneity from *C. regularis* by affinity chromatography and physical and enzymic properties were characterized. The procedure described here is simple and useful to purify the homogeneous preparation of uroporphyrinogen I synthetase, although uroporphyrinogen I synthetase binding characteristics were decreased after repeated use of the same affinity column material. It is probably better to use the affinity chromatography with partial purified preparations, because the Sepharose-bound porphobilinogen can be reduced to oxidized form by pyrooxygenase, which is present in the crude extract preparation [20]. Besides this affinity chromatography method, homogeneous preparation could be obtained with repeated molecular sieving through Sephacryl S-200 after DEAE-Sephacel column chromatography, in this case recovery was about 13%.

Chlorella uroporphyrinogen I synthetase is a simple and heat-stable, acidic protein with a molecular weight of 35 000—36 000. In most respects, *Chlorella* enzyme resembles those that have been reported from other higher plants [8,9] and bacterial sources [6,7], all of which are acidic proteins (p*I* 4—4.6) having relatively low molecular weights under 40 000, neutral to slightly alkaline pH optimum (pH 7.0—8.2) and K_m values of 30—90 μM for porphobilinogen consumption.

The unique nature of the enzyme is its inherent thermal stability as previously pointed out by several investigators [2,4]. Up to date, however, its nature has not been sufficiently studied. Our enzyme showed maximum activity at a temperature of 65°C (Fig. 3) and was reasonably stable at 75°C for 1 h in the absence of co-factors or stabilizing ions (Fig. 5). Interestingly, these characteristics are compared to the thermal stability of various enzymes isolated from thermophylic organisms.

We are now studying the mechanism of the thermophilic nature of uroporphyrinogen I synthetase and purification and characterization of uroporphyrinogen III cosynthetase from *C. regularis*.

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