Journal of Chromatography, 166 (1978) 632–636
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CHROM. 11,386

Note

Application of affinity chromatography to the purification of wheat germ porphobilinogen deaminase

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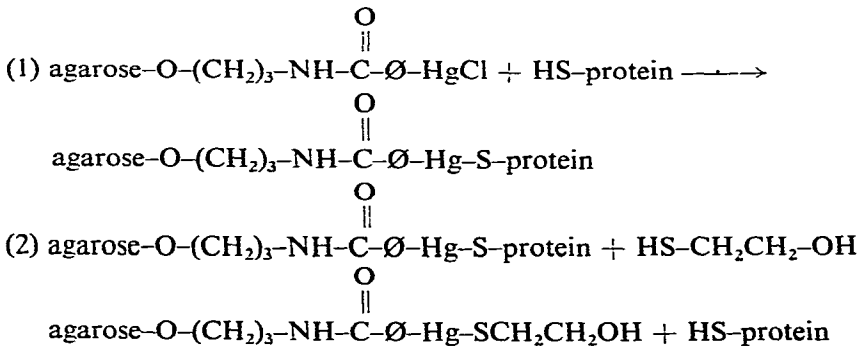
(First received June 13th, 1978; revised manuscript received August 14th, 1978)

Porphobilinogen deaminase (uroporphyrinogen I synthetase) was first isolated from spinach[1] and wheat germ[2] by Bogorad. This enzyme catalyzes the head-to-tail condensation and cyclization of four moles of porphobilinogen (PBG) to form uroporphyrinogen I. Deaminase has been prepared from a variety of sources including avian[3] and mammalian erythrocytes[4], *Euglena gracilis*[5] and *Rhodopseudomonas spheroides*[6]. The latter was purified by Jordan and Shemin[7] by ammonium sulfate precipitation, column chromatography and preparative acrylamide gel electrophoresis. Frydman and Frydman[8] have reported on the purification and properties of wheat germ deaminase. Deaminase from various sources appears to be a single protein of molecular weight about $3.6 \cdot 10^4$ with a pH optimum near 8.0 and K_m values in the range of 20 μM.

Wheat germ deaminase is inhibited by sulfhydryl reagents: Ag^+, Hg^{++} and *p*-chloromercuribenzoate (PCMB)[2]. Inhibition by PCMB is reversed by cysteine. It was concluded that a cysteine residue is essential for enzymatic activity.

In connection with other studies on wheat germ deaminase, the need arose for a large-scale, homogeneous preparation of the enzyme. A form of affinity chromatography has been successful in removing many impurities from deaminase in one rapid step. Chromatography on organomercurial agarose has been used to purify plasma factor XIII, fraction 4 (ref. 9). The reaction of a sulfhydryl protein with organomercurial agarose removes it from a crude mixture [reaction (1)]. After washing the column with buffer, a concentration gradient of mercaptoethanol in the same buffer is used to elute the sulfhydryl protein from the organomercurial bond (2).



The success of this approach depends on the removal of deaminase from a crude preparation because the sulfhydryl groups of the former will bind to mercury on the agarose support. Elution with a mercaptoethanol concentration gradient should release the enzyme from the solid support.

MATERIALS AND METHODS

PBG was purchased from Porphyrin Products (Logan, Utah, U.S.A.) and from Sigma (St. Louis, Mo., U.S.A.). Affi-gel 501 was purchased from Bio-Rad Labs. (Richmond, Calif., U.S.A.). All other chemicals were reagent grade or better. Wheat germ was obtained from Sigma.

Spectrophotometric measurements were made with the Cary 15 spectrophotometer. Fluorimetric measurements were made with the Perkin-Elmer MPF-2A spectrofluorometer.

Protein was determined by measuring absorbance at 280 nm or according to Lowry *et al.*[10] with bovine serum albumin as standard. PBG was determined using the modified Ehrlich reagent of Mauzerall and Granick[11]. Uroporphyrinogen I was oxidized by the method of Jordan and Shemin[7] and determined as uroporphyrin spectrophotometrically or fluorimetrically. Polyacrylamide disc gel electrophoresis was performed using the method of Davis[12]. Wheat germ deaminase was prepared according to Bogorad[2] and carried through the heat-treatment step.

Enzyme assay

The fractions eluted from agarose columns were assayed in the following way. In a 400-μl tube were placed 100 μl of the contents of the fraction and 25 μl of a PBG solution (0.2 mg/ml). The tube was stoppered and incubated at 37°. The assay tubes were evaluated when fluorescence appeared under ultraviolet (UV) light in the control prepared from crude sample. The elution profile was obtained by adding one drop of the iodine reagent of Jordan and Shemin[7] to 50 μl from each tube and 350 μl of buffer and reading the intensity of fluorescence at 598 nm in the spectrofluorimeter with excitation at 410 nm.

Specific activities were determined by assaying for PBG consumption and uroporphyrinogen formation [incubation mixtures containing 1.0 ml of enzyme preparation, 4.0 ml of 0.1 *M* Tris buffer, pH 8.2, 0.50 ml 0.001 *M* EDTA and 0.20 ml of PBG (1.0 mg/10 ml)] at zero time and at 4 to 5 h.

Enzyme purification with Affi-gel 501

In a typical run, a preparation of wheat germ deaminase prepared by the method of Bogorad[2] and carried through heat-treatment contained 9 mg protein/ml and had a specific activity of 0.322 nm uroporphyrinogen/h/mg. A 10-ml volume was mixed with 15 ml of Affi-gel 501 (mercuriphenylagarose) which had been washed three times with three volumes each of 0.075 *M* Tris buffer, pH 8.0. The mixture was stirred at room temperature for 10 min with a glass rod and then poured into a small chromatography column and washed with the same buffer until the UV monitor (254 nm) indicated that no more protein was being eluted from the column (fraction 9). A large amount of protein and the yellow–brown color of the crude mixture was removed in this step. A linear gradient of Tris buffer 0.075 *M*, pH 8.0, containing 0.001

M EDTA and the same buffer with 0.02 *M* mercaptoethanol was started and fractions of 3.3 ml collected.

Affi-gel 501 can be regenerated by washing first with 10 m*M* $HgCl_2$, 20 m*M* EDTA in 50 m*M* sodium acetate pH 4.8. Excess $HgCl_2$ is removed by washing the gel with 0.2 *M* NaCl, 1 m*M* EDTA in 50 m*M* sodium acetate at pH 5.0. It is important to avoid introducing phosphate ions during regeneration of the gel.

Chromatography on DEAE-cellulose

In a typical run, a sample of enzyme (6 mg protein/5 ml) was dialyzed against 25% glycerol, 10 m*M* mercaptoethanol in 0.02 *M* phosphate buffer pH 7.9 at 4° overnight. The sample was put on a column 1 × 7 cm and eluted with a linear gradient of 0–0.4 *M* KCl in the same buffer. Activity eluted in one peak at 0.13 *M* KCl.

Analytical polyacrylamide disc gels of the active tubes from the chromatography runs were cut into 0.5-cm slices, and each slice incubated with 25 μl PBG (0.1 mg/ml 0.75 *M* Tris, pH 8.0) at 37° and evaluated for fluorescence under UV light. Parallel gels were stained whole for protein with Coomassie Blue.

RESULTS AND DISCUSSION

Because deaminase activity is vulnerable to sulfhydryl reagents, it was reasonable to expect rapid immobilization by mercuriphenylagarose if the support arm was long enough to reach an accessible sulfhydryl group. It was hoped that the extraneous proteins in the crude preparations would be far less susceptible to this inhibitor. A yellow–brown enzyme preparation which was the result of carrying Bogorad's wheat germ preparation "B" through the heat treatment step, and the mercurated chromatography support were mixed batch-wise and loaded onto a column. A yellow-fluorescent colored impurity and a large amount of protein were removed by simple elution with buffer (Table I). Elution with a mercaptoethanol gradient showed two protein peaks, one associated with enzymatic activity as determined by spectrophotometric and fluorimetric assays of aliquots of the individual fractions which had been incubated with PBG. The activity and elution profiles appear in Fig. 1. Table I presents the results of enzyme assays. A large amount of non-enzyme protein is removed in one step by this method. Recovery of activity was over 50%. The method is fool-proof, rapid and reproducible. Frydman and Frydman[8] have noted that purification of the enzyme lowers its stability considerably. We found the purified enzyme was fairly stable in 0.1 *M* Tris buffer and 20 m*M* mercaptoethanol, but lost activity with freezing and thawing and by removal or oxidation of mercaptoethanol.

TABLE I

PURIFICATION OF WHEAT GERM DEAMINASE BY CHROMATOGRAPHY ON MERCURIPHENYLAGAROSE

	Total protein (mg)	*Units*	*Specific activity**
Heat-treated enzyme	89.6	28.0	0.311
Fractions 21–30	2.12	9.79	4.62
Fractions 31–40	1.72	4.21	2.45

* Specific activity is expressed as nmoles of uroporphyrinogen produced per h per mg protein.

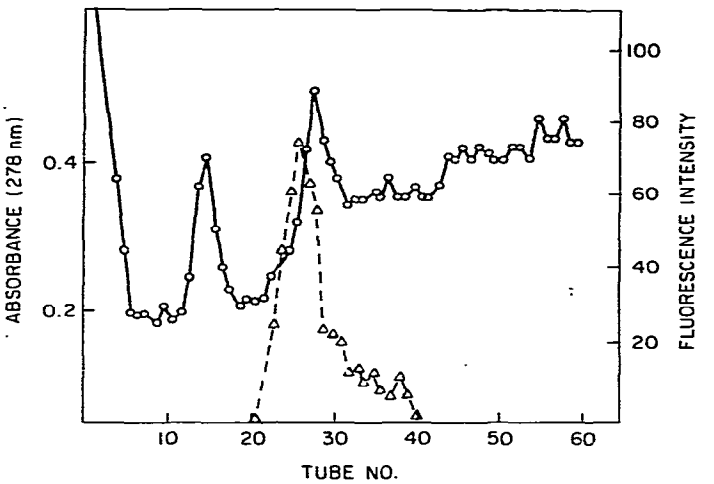


Fig. 1. Elution profile of PBG deaminase from mercuriphenylagarose column. Conditions of absorption and elution are described in the text. Enzyme activity is described in experimental methods. ○, Absorbance at 278 nm; △, uroporphyrinogen-forming activity. Fractions of 3.3 ml were collected after tube 9 when gradient was started.

Analytical polyacrylamide gels of the active fractions off mercuriphenylagarose chromatography showed a pronounced protein band at R_F 0.43 (using mobility of bromphenol blue as reference) and a faint band at R_F 0.87. The latter band was the only one with enzymatic activity. DEAE-cellulose chromatography in 25% glycerol and 10 mM mercaptoethanol of the active fractions off mercuriphenylagarose, removed most but not all of the band with R_F 0.43. While much protein was removed by DEAE-cellulose chromatography the specific activity of the enzyme did not increase, purification probably being offset by destabilization of the enzyme[8]. Preparative gel electrophoresis[7] preferably in buffer containing mercaptoethanol after mercuriphenylagarose chromatography would be the method of choice for preparing homogeneous wheat germ deaminase.

ACKNOWLEDGEMENTS

This investigation was supported by grants from the General Research Support Branch, Division of Research Resources, Bureau of Health Professions Education and Manpower Training, National Institutes of Health, (5S07RR07132), the City University of New York PSC-BHE Research Award Program, and the National Institute of Arthritis, Metabolism and Digestive Diseases (5R01AM17890).

The invaluable assistance of Ms. Irene Winocov and Ms. Eugenia Jacobs is gratefully acknowledged.

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