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Porphyrin biosynthesis in soybean callus tissue system. Isolation, purification and general properties of δ-aminolaevulinate dehydratase

δ-Aminolaevulinate dehydratase (5-aminolaevulinate hydro-lyase (adding 5-aminolaevulinate and cyclizing), EC 4.2.1.24) catalyses the porphobilinogen biosynthesis from two molecules of δ-aminolaevulinate[1,2]. This enzyme was partially purified, mainly from animal and microorganism sources[3] and recently NANDI AND WAYGOOD[4] reported a partial purification of the "dehydratase" from wheat leaves.

Soybean callus tissue system, known as a vegetable tissue with an active cell division was selected as the source material of the "dehydratase" in our work.

Wound callus from soybean seeds were obtained following the procedure described by MILLER[5]. Porphobilinogen was estimated according to an assay used by MOORE AND LABBE[6]. Protein content was determined by the Folin–Ciocalteu micromethod[7]. Enzyme activity was estimated *in vacuo* in Thunberg tubes at 38° with 200 μmoles of Tris buffer (pH 8.8); 5 μmoles of δ-aminolaevulinate (pH 9.0) in a final volume of 2.5 ml, for 1 h. After incubation the reaction was terminated with 0.5 ml of 25% trichloroacetic acid.

The 'dehydratase' was isolated and purified as follows. All operations were carried out in the cold room at 4°. Homogenate (60% wet wt./vol.) of wound callus was prepared in a Potter–Elvehjem type homogeniser with 0.1 M glycine–NaOH buffer (pH 9.0). After centrifugation at 800 × *g* for 10 min, the sediment was washed 3 times. All the supernatants were collected together, constituting the crude extract. The crude extract was centrifuged at 70 000 × *g* with the activity remaining in the supernatant. The supernatant was fractionated with ammonium sulphate (up to 50% satn.), using conc. NaOH to maintain an alkaline pH. The fraction precipitating at 50% satd. ammonium sulphate was dissolved in 0.1 M glycine–NaOH buffer (pH 9.0) and chromatographed on a Sephadex G-100 column (1.8 cm × 30 cm). Protein was eluted with the same buffer and its content and specific activity were determined in each fraction eluted from the column. On the "pooled" fraction containing high activity a second fractionation using up to 50% satd. ammonium sulphate was performed. The sediment was stored at −15° but it was not convenient to prolong storage.

This preparation migrated as a single band in starch-gel electrophoresis[8] at pH 7.4 and 8.8. According to these results, the dehydratase, purified 7-fold, yielded a solution behaving as a homogeneous protein which we considered pure enough to study.

During gel filtration a coloured, low molecular weight component was detected which was difficult to eluate completely from the column. A lyophilised extract of it partially inhibited the enzyme. Although Fe^{3+} was qualitatively detected in the eluate by the *o*-phenanthroline test[9], Fe^{3+} itself did not inhibit the reaction. The absorption spectrum of the eluate was measured and a shoulder between 254 and 259 mμ was found.

A broad optimum pH between 8.4 and 8.8 for the purified preparation was observed; this is different from the values reported for the dehydratase obtained from other sources. The K_m at pH 8.8 and 38° was $3.5 \cdot 10^{-4}$ M. Maximal enzyme

TABLE I

PURIFICATION OF δ-AMINOLAEVULINATE DEHYDRATASE FROM SOYBEAN CALLUS TISSUE SYSTEM

Incubation conditions are described in the text. A dehydratase unit is defined as the amount of enzyme that catalyses the formation of 1 mμmole of porphobilinogen in 1 h under standard conditions; the specific activity being the units of dehydratase per mg of protein.

Fraction	*Total protein (mg)*	*Total units*	*Yield (%)*	*Specific activity*	*Purification (-fold)*
1. Crude extract	360.0	2860	100	7.9	1.00
2. 70 000 × *g* supernatant	280.0	2260	79	8.1	1.01
3. 50% satd. ammonium sulphate fraction	82.5	1165	41	14.2	1.78
4. Combined fractions from Sephadex G-100 column	29.6	1262	44	42.7*	5.60
5. Second 50% satd. ammonium sulphate fraction	15.0	834	29	56.0	7.00

* Specific activity in the main tube was 46.6.

activity was observed at 55°. 0.2 M Tris buffer enhanced enzyme activity showing an effect opposite to that obtained with mammalian liver dehydratase.

Various compounds have been examined for their effects on dehydratase activity. A 30-min preincubation at 38° was performed in these cases before adding the δ-aminolaevulinate. Depending on the compound being studied, a saturated solution of $CuSO_4$ *plus* trichloroacetic acid was used for terminating the reaction. Of the thiol activators studied (cysteine, sodium thioglycollate and dithioerytritol in a final concentration of 10 mM), none increased enzyme activity; on the contrary, they all produced partial inactivation. 1 mM cysteine had practically no effect on enzyme activity. This is a significant finding because this preparation does not require GSH and cysteine for maximum activity, as is the general case for the dehydratase from mammalian and other sources. Nevertheless, of the thiol inhibitors studied (*p*-chloromercuribenzoate, iodoacetamide, *N*-ethylmaleimide and iodosobenzoate at a final concentration of 1 mM), all produced marked inhibition of activity confirming the presence of –SH groups related to enzyme activity. GSSG, a reagent capable of oxidizing –SH groups, did not produce inactivation of the dehydratase. EDTA up to 10 mM inhibited enzyme activity by 40%.

Results reported here show that some of the properties of the soybean enzyme are similar to those of the dehydratase arising from other sources, mainly those referring to the essential thiol reacting groups. However it is possible to speculate that the position or distribution of the –SH and the probable disulphide groups present in this enzyme is not the same in the tertiary structure of the protein. This hypothesis seems reasonable considering the different behaviour of the enzyme with GSH and cysteine, particularly compared with that of the mammalian liver enzyme, but further studies would be necessary to ellucidate this problem. On the other hand, the presence of a low molecular weight compound which inhibited enzyme activity is noted for the first time.

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PRELIMINARY NOTES

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Spécificité "hydrophobique" d'une endopeptidase isolée de *Bacillus megaterium*

Un enzyme protéolytique exocellulaire provenant d'une culture en phase exponentielle de *Bacillus megaterium* (souche MA) en milieu minimal glucosé à 30° (réf. 1), a été purifié par précipitation fractionnée au moyen de l'alcool et du sulfate d'ammonium, puis par passage sur Sephadex G-200 (réf. 2). L'enzyme est stabilisé par le calcium et présente un optimum d'action sur la caséine à pH 7.2 (tampon Tris) en présence de $CaCl_2$ $2 \cdot 10^{-3}$ M. Le matériel s'est révélé homogène au cours de l'électrophorèse sur Cellogel réalisée à différents pH entre 4 et 10.3. D'autre part il ne fournit qu'un seul pic lorsqu'il est soumis à une filtration sur colonne de Sephadex G-75. Le produit paraissant correspondre à une seule espèce moléculaire, une étude de la spécificité d'action a été entreprise.

Activité exopeptidasique. L'enzyme est totalement dépourvu d'activité exopeptidasique. Il est sans action sur les substrats habituels des carboxypeptidases: Bz-Gly–Phe* (Yeda) et Bz-Gly–Leu (Fluka) pour la carboxypeptidase A, Bz-Gly–Arg

* Tous les acides aminés des substrats synthétiques contenant un carbone α-asymétrique sont de configuration L. Les abréviations suivantes sont utilisées: Bz, benzoyl; Z, carbobenzoxyl; Ac, acétyl; BAEE, benzoyl-arginyl ethyl ester; TAME, tosyl-arginyl methyl ester; BANA, benzoyl-arginyl *p*-nitranilide; ATEE, acetyl-tyrosyl ethyl ester; BTEE, benzoyl-tyrosyl ethyl ester.