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FORMATION OF δ-AMINOLEVULINIC ACID FROM GLUTAMIC ACID BY A PARTIALLY PURIFIED ENZYME SYSTEM FROM WHEAT LEAVES

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Summary

A method for partial purification of an enzyme system from greening wheat leaves which converts ^{14}C-labeled glutamate to δ-aminolevulinic acid is described. The purification entails the successive use of anion and cation exchange, followed by molecular sieving. The enzyme system is unstable in crude form, but the stability is markedly increased after column chromatography on CM-cellulose. The pH profile and the cofactor requirements suggest that at least two enzymes are involved.

Introduction

The synthesis of δ-aminolevulinic acid in many chlorophyll-forming organisms has fairly recently been shown to use glutamate and/or α-ketoglutarate as the carbon source [1,2]. Although in vivo investigations of this phenomenon have proliferated [2], only a few very recent in vitro studies have been reported. These have involved extracts and/or plastid preparations from spinach [3], barley [4,5], cucumber [6] and maize [7] leaves. In this paper, we report the preparation, partial purification and partial characterization of an enzyme system from greening wheat leaves which yield ^{14}C-labeled δ-aminolevulinic acid from [^{14}C]glutamate.

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Materials and Methods

Growth conditions

100—125 g wheat seeds (Hard, Winter, 1977, Country Life Natural Foods, Oak Haven, Pullman, MI) were sown/12 × 33 × 5 cm Pyrex dish filled with moist vermiculite. The dishes were covered, and the seeds germinated in the dark at 25° C. Darkness was maintained, but the covers were removed 4 days later so as not to restrain the upward growth of the seedlings.

Harvest conditions

After 7 days, the dishes with the etiolated seedlings were exposed to fluorescent light for 2—3 h before leaves were removed. The leaves were cut into 0.5 cm lengths with scissors and placed on ice in a glass container..

Extraction procedure

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

Diced leaves were ground by hand for 2 min with buffer A (0.05 M Tris—HCl, pH 8.0, 25% glycerol, 20 mM $MgCl_2$, and 10 mM 2-mercaptoethanol) and glass beads (Glasperlen, 0.1—0.11 mm, B. Braun, Melsungen). This mixture was ground in a Sorvall Omnimixer for three times 2 min at 20% maximum. The mixture was centrifuged at 48 000 × *g* for 30 min. The supernatant was respun at this speed and then filtered through three layers of cheese cloth.

Purification of glutamate-δ-aminolevulinic system

DEAE-cellulose chromatography. A 2.5 × 30 cm column of DEAE-cellulose (DE 52, Whatman) equilibrated in buffer A′ (2-mercaptoethanol at 1 mM) was used. The pH of the crude extract from 100 g leaves was adjusted to 7.5—8.0, glycerol was added to 25%, and the mixture was then applied to the column. The enzyme was eluted using a 500 ml linear gradient of 0—1 M KCl in buffer A′ (see Fig. 1).

CM-cellulose chromatography. A 1.0 × 15 cm column of CM-52 (Whatman) equilibrated with buffer B (0.01 M potassium phosphate, pH 6.0, 20 mM Mg^{2+}, 25% glycerol and 1 mM 2-mercaptoethanol) was used. The active fractions from the DE-52 column were concentrated in an Amicon cell (PM 10 membrane), and 25% glycerol was added to give a salt concentration less than 0.01 M. Bound protein was eluted with a 100 ml linear gradient of 0—1 M KCl in buffer B (see Fig. 2).

Molecular sieving. An Ultrogel (LKB, AcA 54) column (1 × 20 cm) equilibrated in buffer C (0.1 M potassium phosphate, pH 6.8, 20 mM $MgCl_2$, 10 mM 2-mercaptoethanol) was prepared, and the active fractions from CM-52 column eluted (flow rate, 0.5 ml/min) with buffer C (Fig. 3).

Assay procedure

Incubations were carried out under argon in sealed vials for 15 min at 30° C using the following reagents and cofactors: buffer concentration, 20 mM (Tris-HCl or potassium phosphate), pH 6.8 or 7.2, 20 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 5 mM glutathione, 1 mM ATP (Sigma), 0.05 mM glutamate (sodium salt, Sigma), 0.05 mM NADPH (Sigma, purified), 0.25—0.3 μCi L-[U-^{14}C]gluta-

mate (New England Nuclear) in 0.01 M HCl, enzyme in buffer to 0.25 ml final volume. In assays of the crude supernatant levulinic acid was added to 0.05 mM final concentration.

The reaction was stopped in each vial by the addition of 20 μmol HCl. This was followed by 2 ml water and 2.5 μmol of δ-aminolevulinic acid as carrier. The pH was adjusted to 2.5 with 1 N NaOH, and the solution was extracted three times with ether to remove interfering radioactive materials. The pH was then raised to 6.8, 20 μl distilled ethyl acetoacetate was added, and the solution was placed in a boiling water bath for 10 min to generate δ-aminolevulinic acid pyrrole (2-methyl-3-carbethoxy-4-(3-propionic acid) pyrrole) [3,8].

δ-Aminolevulinic acid pyrrole was purified as follows: After cooling, the solution was extracted three times with diethyl ether, the ether extracts were discarded, the pH was then lowered to 2.5 with 2 N HCl, and δ-aminolevulinic acid pyrrole was removed via another ether extraction. The combined ether extracts containing δ-aminolevulinic acid pyrrole were washed with water, a drop of NH_4OH was added [3], and the extracts were brought to near dryness under a stream of argon. The residue was subjected to electrophoresis on paper in a 0.01 M potassium phosphate buffer, pH 6.65 (23 V/cm applied gradient). After 90 min, the papers were dried, spliced and counted on a strip counter (Nuclear Chicago Actigraph III). The area corresponding to δ-aminolevulinic acid pyrrole was cut out, and the pyrrole eluted with 0.5 ml NH_4OH/methanol/water (1 : 4 : 5, v/v) [3], and by three 0.5 ml portions of water. The solution was brought to pH 2.5 and the pyrrole extracted into ether to separate it from phosphate salts which had also been eluted from the paper. The combined ether extracts were reduced in volume under a stream of argon and spotted on a 4 × 40 cm Whatman No. 1 paper strip for ascending chromatography in NH_4OH/*n*-butanol/water (5 : 5 : 45, v/v, upper layer) for 16—18 h [8]. The δ-aminolevulinic acid pyrrole was identified by means of the coincidence of radioactivity and the development of pink color upon spraying the dried papers with modified Ehrlich's reagent (1 g dimethylaminobenzene in acetone/conc. HCl, 4 : 1, v/v). All reported δ-aminolevulinic acid pyrrole yields have been corrected for blank values obtained when the same assays were performed using either no enzyme or in some cases acid-killed enzyme.

Results and Discussion

The partial purification of a glutamate-dependent δ-aminolevulinic acid-synthesizing system has been accomplished, and some characteristics of this system have been established.

The purification involves three different column steps starting with the crude supernatant obtained by grinding and centrifugation of greening wheat leaves. The most crucial step in terms of enzyme recovery involved the chromatography, on a large DEAE-cellulose bed, of the crude supernatant immediately after preparation. Failure immediately to perform this chromatography resulted in a 80—100% loss of activity over a 24 h period. Although most of the protein in the crude fraction passed directly through the DEAE-cellulose column, the fraction with δ-aminolevulinic acid-synthesizing activity was bound, and was eluted just after the main peak when a salt gradient was applied

to the column (Fig. 1). Further purification of this activity included chromatography on CM-cellulose (Fig. 2), followed by molecular sieving chromatography (Fig. 3). Fractions from the two last purification steps were used to investigate the properties of the enzyme system. The enzyme fractions obtained from Ultrogel chromatography were found to be stable in buffer with 25% glycerol for several days at −20° C.

Assays of the glutamate-dependent δ-aminolevulinic acid-synthesizing system included, after incubation, the preparation, isolation and purification of δ-aminolevulinic acid pyrrole in a series of tedious but reproducible steps similar to those reported by others [1,3]. Blank runs with known quantities of δ-aminolevulinic acid gave a yield after the final chromatography step of about 10% (raw cpm from strip counting of paper). All data reported here have been corrected for losses in the assay procedure.

The time course of the reaction (Fig. 4) shows an initial rapid rise at 1 min, followed by a nearly linear increase to 30 min. The pH dependence of the reaction consistently showed a bimodal form, even when different enzyme preparations were used to assay pH dependence. Fig. 5 illustrates not only this point, but also the fact that the more purified (Ultrogel) enzyme fraction shows more activity at pH 7.5 than at 5.5, whereas the less purified (CM-cellulose) enzyme fraction shows just the reverse pH optimum. This difference may indicate the presence of two different enzymes contributing to the final product, each with it own pH optimum. Unfortunately, instability of the enzyme fractions did not allow reproducible assay results at pH 8 or above. We assume, however, that activity decreases above pH 7.5. The behavior of the purified enzyme preparations as pH was varied appears to corroborate the work of others which has indicated at least two enzymes (or at least one stable intermediate) in the pathway from glutamate (or α-ketoglutarate) to δ-aminolevulinic acid [5,9].

The dependence of the conversion on added cofactors is summarized in Table I. There was a consistent dependence on reduced pyridine nucleotide,

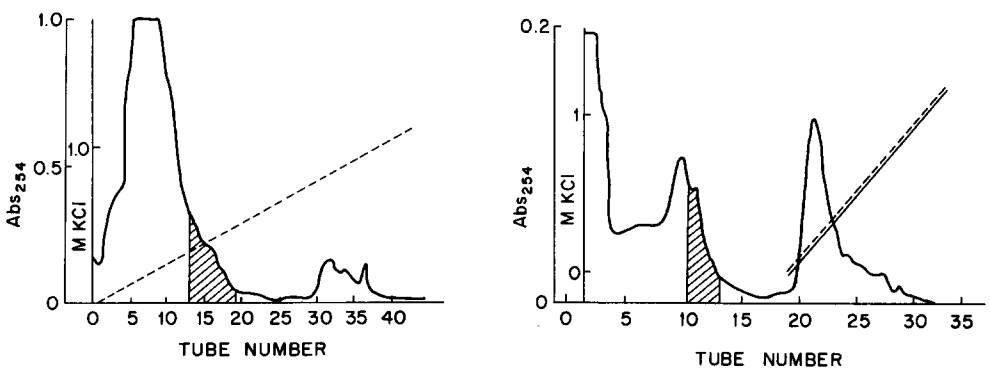
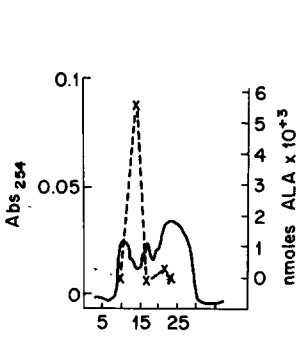


Fig. 1. DEAE-cellulose chromatography of crude supernatant on a 2.5 × 30 cm column. Eluted with 0—1 M KCl in buffer A'. ———, absorbance at 254 nm; - - - - - -, concentration of KCl; shaded area indicates fractions having enzyme activity.

Fig. 2. CM-cellulose column chromatography of pooled, concentrated eluates from DEAE-cellulose chromatography. Column dimensions, 1.5 × 13 cm. ———, absorbance at 254 nm; - - - - - -, KCl concentration; shaded area indicates fractions having enzyme activity.



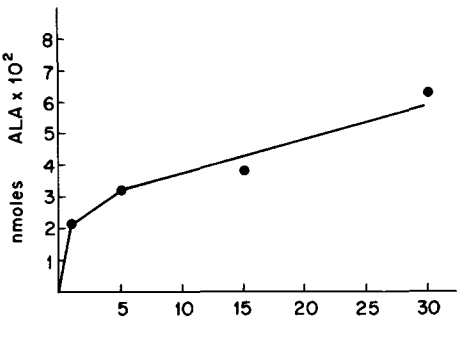


Fig. 3. Ultrogel column chromatography of pooled, concentrated eluates from CM-cellulose column chromatography. ———, absorbance at 254 nm; - - - - - -, nmol of δ-aminolevulinic acid $\times 10^3$.

Fig. 4. Time course of ^{14}C-labeled δ-aminolevulinic acid from L-[U-^{14}C]glutamate using enzyme purified through CM-cellulose column chromatography.

with NADPH being more productive than NADH. In contrast, the results for ATP and glutathione varied from preparation to preparation, often by as much as 30—50%, although some dependence on both was always found, in agreement with Gough and Kannangara [4]. We have no clear explanation for these variations, but is is likely that they are due to the differential stability and cofactor requirements of two (or more) different enzymes.

Recent publications have shown that synthetically prepared glutamate-1-semialdehyde can be converted to δ-aminolevulinic acid by enzyme preparations from barley leaf chloroplasts [5] without the participation of an externally added amino group donor, while in cell-free preparations from greening maize leaves α-ketoglutarate was converted to δ-aminolevulinic acid in the presence of an amino donor and of pyridoxal phosphate as well as of NADH [7]. In the latter system NADPH was less effective. It is possible that the earlier observations from this laboratory using α-ketoglutarate as a precursor of δ-aminolevulinic acid [9] are due to the presence of glutamate dehydrogenase

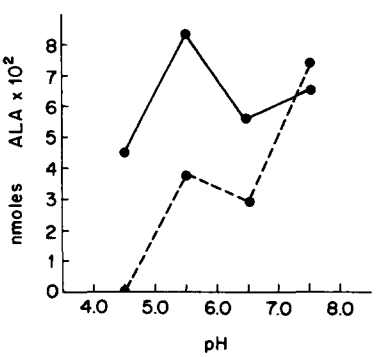


Fig. 5. pH Dependence of ^{14}C-labeled δ-aminolevulinic acid formation from L-[U-^{14}C]glutamate using purified enzyme. ———, enzyme from CM-cellulose column chromatography; - - - - - -, enzyme from Ultrogel column chromatography.

TABLE I

DEPENDENCE ON ADDED COFACTORS OF THE FORMATION OF ^{14}C-LABELED δ-AMINOLEVULINIC ACID FROM L-[U-^{14}C]GLUTAMATE IN GREENING WHEAT LEAVES

Assays were performed at pH 7.2. Enzyme fractions used were from CM-cellulose chromatography or from Ultrogel chromatography.

Conditions	Relative activity
+ All cofactors	100
— ATP	61
— Glutathione	81
— NADPH	31
— NADPH; + NADH	39.5
No cofactors	10
— Levulinic acid *	100

* Levulinic acid was required for δ-aminolevulinic acid accumulation from glutamate only in assays of crude extract supernatant.

in the preparations used. Hedley and Stoddart [10] reported the discovery of a light-dependent alanine aminotransferase in *Lolium temulentum* L. leaves whose activity could be correlated with chlorophyll synthesis, and they suggested that this enzyme might be involved in the conversion of dioxovalerate to δ-aminolevulinic acid.

Further progress on this problem awaits separation and characterization of the two or more enzymes apparently involved in this process. Preparation of probable intermediates as substrates is also necessary. This approach is now being taken in this laboratory.

Acknowledgement

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