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Effect of Growth Regulators and Group-Modifiers on NADH-Glutamate Synthase of Marrow Cotyledons

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Abstract: NADH-Glutamate synthase (GOGAT, E.C. 1.4.1.14) from *Cucurbita pepo L.* (marrow) cotyledons was inhibited by abscisic acid (ABA) at 10^{-6} M. Addition of either gibberellic acid (GA_3) or dichlorophenoxyacetic acid (2,4-D) at 10^{-6} M to ABA counteracted its inhibitory effect. Kinetin at the same concentration increased inhibition of the enzyme by ABA. Cycloheximide, rifampicin, cordycepin and chloramphenicol at 0.3 M reduced mediated-increase in the enzyme activity by 2,4-D particularly *in vivo*. The enzyme was purified to homogeneity as determined by sodium dodecyl sulfate-polyacrylamide gels. The subunit Mr of GOGAT as determined by SDS-PAGE was 200 KDa. The specific activity was 131.2 U mg^{-1} protein. Group-specific modifiers like N-bromosuccinimide (NBS), Densyl chloride (DC), Tetranitromethane (TNM) and 2-ethoxy-1-ethoxy-1,2-dihydroquinoline (EEDQ) inactivated the purified enzyme. The results suggested the presence of tryptophanyl, lysyl, tyrosyl and carboxyl groups essential for the enzyme catalysis. Double logarithmic plots of the observed pseudo-first order rate constants against modifer concentration revealed modification of only one residue of each group.

Key words: *Cucurbita pepo*, Cucurbitaceae, glutamate synthase, abscisic acid, gibberellic acid, densyl chloride

Introduction

In most prokaryotes, ammonia is assimilated via the combined action of glutamine synthetase (GS, E.C. 6.3.1.2) and glutamate synthase (GOGAT, E.C. 1.4.1.14) (Mengel and Pilbeam, 1991; Lam *et al.*, 1996; Hirose and Yamaya, 1999). Ammonia is incorporated through GS into glutamine, and then GOGAT is responsible for the transfer of the amide group of glutamine to 2-oxoglutarate to yield two molecules of glutamate.

Two different forms of GOGAT are present in higher plants, one utilizes reduced ferredoxin (Fd-GOGAT; EC 1.4.7.1) as a source of reductant (Suzuki *et al.*, 1996) and the other (NADH-GOGAT; EC 1.4.1.14) utilizes NADH (Lea *et al.*, 1990; El-Shora, 1994; Lea, 1997). In addition to the reductant specificity, the enzymes differ in molecular mass, kinetics, and antigenicity (Lea *et al.*, 1990; Temple *et al.*, 1998).

Tissue fractionation studies have shown that Fd-dependent GOGAT is localized in chloroplasts of leaves (Becker *et al.*, 1993; Srivasankar and Oaks; 1996; Sakakibara *et al.*, 1997; Migge *et al.*, 1997). The major role of Fd-GOGAT in green leaves is the assimilation of NH_4^+ released from the photorespiratory pathway which is supported by analysis of mutants lacking Fd-GOGAT in both *Arabidopsis* (Somerville and Ogren, 1997) and barley (Kendall *et al*, 1986).

Cellular and subcellular fractionations studies using density-gradient centrifuging methods have shown that most NADH-GOGAT activity appeared to be in the plastids in roots (Emes and England, 1986), in shoots (Matoh and Takahashi, 1981) and in root nodules (Chen and Cullimore, 1989). On the other hand, Hecht *et al.* (1988) suggested that NADH-GOGAT is located in the cytosol in cotyledons of mustard seedlings. The NADH –dependent enzyme appears to play a major role in the ammonia assimilation in nitrogen fixing nodules (Lea, 1997).

One aim of experiment was to study the effect of some growth regulators on the activity of NADH-GOGAT of marrow cotyledons and abscisic acid, gibberellic acid 2,4-D and kinetin were examined. In addition, the active groups of NADH-GOGAT of marrow as C_3 plant are not established. Therefore, to find out the essential group taking part in the enzyme catalysis was achieved by testing the effect of group-speific modifiers on the enzyme activity. The paper provides experimental evidence for the presence of tryptophanyl, lysyl, arginyl and carboxyl as essential groups for enzyme catalysis.

Materials an Methods

Plant material : Marrow seeds were surface sterilized with 0.1% $CaOCl_2$ for about 5 min and washed with distilled water. The seedlings were raised on moist filter paper in Petri plates, for 5 days in light (irradiance of 70 W m^{-2}) at 25°C and pH 7.5. These were watered with 1/2 strength modified Hoagland's solution. The filter paper was always kept moist.

Effect of growth regulators: Marrow seeds were moistened with 10 ml of distilled water as control or 10 ml of 10^{-6} M ABA, GA_3, 2,4-D or kinetin singly or in combination for 5 days.

Effect of 2,4-D and inhibitors of transcription and translation: Cycloheximide, rifampicin, cordycepin and chloramphenicol are tested *(in vivo)*. Cotyledons from uniformly 5 days old seedlings were collected and then incubated with 10 ml of 10^{-6} M 2,4-D or 0.3 mM of either inhibitors at 25 °C. After 2h of incubation the cotyledons were used for enzyme extraction. 2,4-D or any of the above inhibitors at the same concentration was added to the assay medium of NADH-GOGAT (*in vitro*).

Enzyme extraction: Thirty detached cotyledons were homogenized in a prechilled mortar and pestle using acid washed quartz with 50 mM Tris-HCl (pH 7.5) containing 2 mM $MgCl_2$ and 5 mM 2-mercaptoethanol and 1 mM EDTA. The resulting homogenate was passed through four layers of cheesecloth and the filtrate centrifuged at 10,000 g for 30 min. The supernatant was referred as crude extract.

Enzyme purification: All isolation procedures were carried out at 0-5°C. Enzyme activity as well as protein concentration were determined after each step of purification.

Fifty gm of cotyledons were sliced and transferred with 150 ml of 30 mM Tris-HCl buffer, pH 7.5 to a pre-cooled Warng blender. The cotyledons were homogenized for 5 min. The homogenate was then centrifuged at 10,000 g for 30 min. The resultant supernatant was used as a crude extract. A column of Sephadex G-200 fine, 50 x 2.5 cm was prepared and equilibrated with 30 mM Tris-HCl buffer and pH 7.5. A volume of the crude extract was appled to the column. Elution

was carried out with the same buffer. The flow rate was 15 ml/h and fractions of 2- ml were collected. The combined fractions containing NADH-GOGAT enzyme were applied to a DEAE-Sephacel column (1.5 x 25 cm) equilibrated with 30 mM Tris-HCl bufferand pH 7.5. The column was eluted with the same buffer. The flow rate was 20 ml/h and 2- ml fractions were collected. After about 120 ml buffer had passed through, a linear concentration gradient of 0-50 mM NaCl in the starting buffer was effected over a total volume of 160 ml. The column was eluted with a 5 mM NaCl solution to elute the remaining absorbed protein, and fractions containing the enzyme activity were combined. The combined fractions were concentrated to 5 ml. This concentrated fraction was applied to a Sephadex G-200 column (2.5 x 25 cm), prepared and equilibrated with 150 mM Tris-HCl buffer, pH 7.5. The column was eluted with this buffer. The flow speed was 10 ml/h and 2-ml fractions were collected. Active fractions were then pooled and used for characterization of the enzyme.

Enzyme assay: The enzyme assayed mixture (Dougal, 1974) contained 100 mM sodium phosphate buffer (pH 7.5), 100 mM glutamine, 2 mM 2-oxoglutarate, 0.2 mM NADH and appropriate concentration of the enzyme in a final volume of 1 ml. One unit of activity is the amount of enzyme, which oxidizes 1 μmol of NADH per min.

SDS-Polyacrylamide gel electrophoresis: For subunit molecular determination, sodium dodecyl sulfate –polyacrylamide (10 % wt/vol) slab gel electrophoresis was performed according to Laemmli (1970). Protein standards employed were myosin (200,000), β-glactosidase (116,000), phosphorylase b (97,000), and bovine serum albumin (66,000) and egg albumin (45,000). Following electrophoresis, proteins were stained with Coomassie brilliant blue.

Protein analysis: Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard, and by 280 nm absorbance for the chromatography step.

Oxidation of tryptophan residues by NBS was carried out into cuvettes, one containing pure NADH-GOGAT in 100 mM phosphate buffer (pH 7.5) and another containing buffer. Successive 10 μl aliquots of NBS (5.0 mM) were added to the sample and reference cuvette and the progress of the oxidation reaction was monitored at 280 nm. Simultaneously aliquots of the reaction mixture were withdrawn to assay the residual enzyme activity.

Inactivation by DC: Pure NADH-GOGAT was treated with DC (1-4 mM) in a medium containing 30 mM Tricine-NaOH (pH 7.5) and the pure enzyme at 30 °C for 15 min. Centrifuging the incubation medium for 5 min at 5000 g terminated the modification.

Inactivation by TNM: Pure NADH-GOGAT in 50 mM sodium phosphate buffer, pH 7.5, was incubated at 30°C for 15 min with TNM at various concentrations (1-4 mM). At different time intervals, aliquots were taken and residual activity was measured under standard conditions.

***Inactivation by EEDQ*:** Pure NADH-GOGAT in 50 mM sodium-phosphate buffer, pH 7.0, was incubated for 30 min at 37 °C with varying concentrations of EEDQ (1-5 mM). At various time intervals, aliquots were withdrawn and the residual enzyme activity was measured under standard conditions.

Results and Discussion

Growth regulators at 10^{-5} and 10^{-6} M were chosen in preliminary experiments. Rudnicki *et al.* (1971) also found that abscisic acid at this concentration inhibited the germination of some seeds. The effects of both concentrations were similar. However, the higher concentration caused more pronounced changes in the activity of NADH-GOGAT. Therefore, the results were obtained by applying the 10^{-6} M solutions of hormones namely: abscisic acid, kinetin, GA_3 and 2,4-D. Fig. 1 showed that the activity of crude NADH-GOGAT is strongly suppressed as a result of the treatment of cotyledons with ABA at 10^{-6} M. It is possible that ABA might depress a regulatory gene or interact with a regulator mRNA protein species to inhibit the translation of GOGAT mRNA (Bert *et al.*, 1996). However, the precise mechanism of inhibition remains uncertain.

GA_3 and 2,4-D were able to counteract the inhibitory effect of ABA (Fig. 1) with different rates. The activity of the enzyme was restored in a consistent manner in presence of GA_3 throughout the incubation periods. This is in agreement with other plant enzymes e.g. phosphoenolpyruvate carboxylase (Kumar *et al.*, 1987; El-Shora, 1993; Bihzad and El-Shora, 1996) and dehydrogenases (Jacobsen and Olszewski, 1996). GA_3 may stimulate the synthesis of the enzyme by controlling the transcription and translocation of new mRNA (Jones and Jacobsen, 1991). Contrary to the effect of either GA_3 or 2,4-D, kinetin increased the inhibition of GOGAT activity by ABA. In the presence of ABA plus kinetin, the activity of the enzyme was markedly lower than that of the control sample. This is in accordance with the results of Ryc and Lewark (1982). However, the mechanism of inhibition by kinetin is still unclear.

The effect of antibiotic inhibitors of transcription and translation on the induction of elevated levels of NADH-GOGAT activity by 2,4-D was investigated. The inhibitors reduced mediated-increase by 2,4-D particularly *in vivo* (Table 1). However, when the inhibitors were added directly to the assay mixture (*In vitro*) they showed a little or no effect. In support, 2,4-D expressed marked increase in synthesis of other enzymes like soluble RNA polymerase and chromatin - bound RNA polymerase (Guifoyla *et al.*, 1975), NAD-oxidase from hypocotyls of etiolated *Glycine max* seedlings (Brightman *et al.*, 1988) and peroxidase in mung bean seedlings cuttings (Chen and Poltanick, 1991). Furthermore, 2,4-D showed a very pronounced stimulation of RNA synthesis (Grierson *et al.*, 1982) and resulted in an increase in translatable mRNA (Zurfluh and Guilfoyle, 1982). Thus, it seems likely that 2,4-D counteracted the inhibitory effect of ABA by controlling synthesis or translation of mRNA required for synthesis of the enzyme protein. However, additional work will be needed.

The purification protocol developed for NADH-GOGAT of marrow cotyledons summarized in Table 2. The enzyme was purified to homogeneity as determined by sodium dodecyl sulfate-polyacrylamide gels (Fig. 2). It was free of any glutamate dehydrogenase activity. The subunit Mr of NADH-GOGAT as determined by SDS-PAGE was 200,000. This result is in agreement with the report of Lea (1997). The catalytic activity of the enzyme was determined with NADH as coenzyme. By far the most effective purification step was from DEAE Sephacel chromatography, which removed about 96 % of the contaminating proteins and resulted in 12.7-fold increase in specific activity of NADH-GOGAT activity. Active fractions of four runs were collected and concentrated. After running the Sephadex G_{200} super fine chromatography, 15.8-fold purification was obtained with a yield of 46.3 % of the

Table 1: Effect of antibiotic inhibitors of transcription and translation on the induced increase in NADH-GOGAT activity by 10^{-6} M 2,4-D. Values are means ± S.E. of three estimates.

Treatment	Enzyme activity			
	In vitro		*In vivo*	
	U mg^{-1} protein	% increase by 2,4-D	U mg^{-1} protein	% increase by 2,4-D
Control	8.3 ± 0.8	---	8.3 ± 0.9	---
2,4-D	9.0 ± 0.3	8.4 ± 0.8	14.8 ± 0.6	78.3 ± 0.2
Cycloheximide + 2,4-D	8.4 ± 0.1	1.2 ± 0.9	10.3 ± 0.5	24.1 ± 0.3
Rifampicin + 2,4-D	8.5 ± 0.9	2.4 ± 0.2	11.0 ± 0.1	32.5 ± 0.7
Cordycepin + 2,4-D	8.9 ± 0.3	7.2 ± 0.7	13.3 ± 0.7	60.2 ± 0.5
Chloramphenicol + 2,4-D	8.8 ± 0.2	6.0 ± 0.1	13.0 ± 0.2	56.6 ± 0.4

Table 2: Purification of NADH- GOGAT from marrow cotyledons

Purification stage	Total protein(mg)	Total activity(U)	Specific activity(U mg^{-1})	Purification factor	Yield%
Crude extract	75	633.0	8.3	1.0	100
Sephadex G200 fine Chromatography	15.7	434.2	27.7	3.3	69.7
DEAE Sephacel Chromatography	3.1	328.1	105.8	12.7	52.7
Sephadex G200 super fine chromatography	2.2	288.7	131.2	15.8	46.3

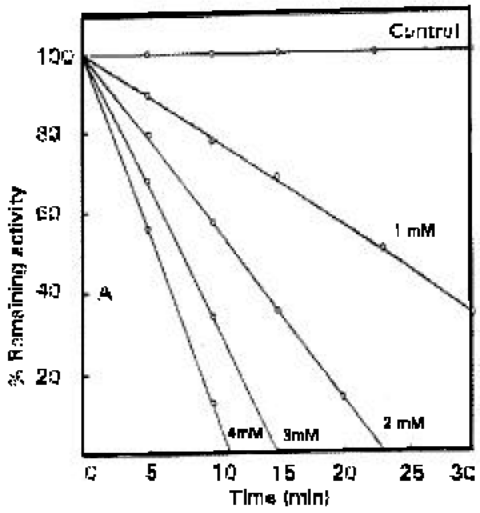

Fig. 6a: First order plot of the inactivation of pure NADH-GOGAt by tetranitromethane

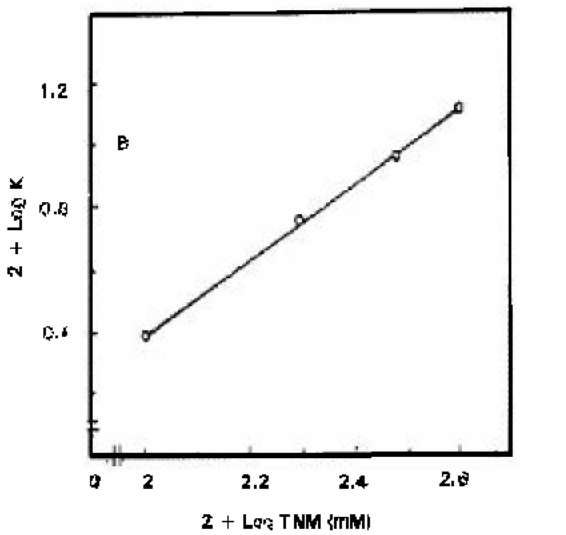

Fig. 6b: Double logarithmic plots of the observed pseudo-first order constants (k) against TNM concentrations. (Data from Fig. 6a)

loaded activity. The last step of purification protocol displayed a specific activity of 131.2 U mg^{-1} protein, which is higher than that reported by Schreier and Bernlohr (1984).

NBS is a tryptophan specific reagent (Ronan *et al*., 1996). Therefore, it was decided to study its effect on NADH-GOGAT activity (Fig. 3). After each addition of NBS there was a progressive decrease in absorption at 280 nm with concomitant loss in activity. These results implicate that tryptophan(s) of NADH-GOGAT are essential for maintaining the integrity of the enzyme. Fig. 4 shows a plot of Ln and versus time for inactivation of NADH-GOGAT at fixed concentration of NBS. The inactivation of the enzyme by NBS was found to be biphasic, which may be due to dimeric form of the enzyme. Densyl chloride was used successfully to study the presence of lysine residues in the mechanism of a vast number of proteins (El-Shora and El-Naggar, 1994).

Densyl chlorideresulted in inhibition of NADH-GOGAT activity (Fig. 5a). The reaction order for densyl chloride was determined from a plot of log K ($K = \ln 2 / t_{0.5}$) against log of densyl chloride concentration. The values of $t_{0.5}$ are calculated from Fig. 5a. The slope was nearly 1 (Fig. 5b), suggesting modification of only one residue of lysine per active site. The presence of essential lysyl group for the catalysis of NADH-GOGAT is in consistent with the results of Vigara *et al.* (1996). The reaction of TNM with proteins is specific for tyrosyl residues (Skoubas and Georgatsos, 1997). Treatment of NADH-GOGAT with TNM led to inactivation, which was dependent on both the time and reagent concentration. The loss of NADH-GOGAT activity followed pseudo first order kinetics (Fig. 6a), and double logarithmic plots of the observed

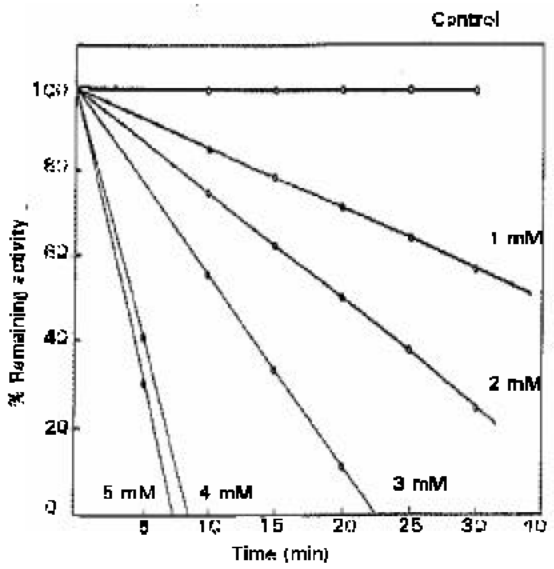

Fig. 7a: First order plot of the inactivation of pure NADH-GOGAT by EEDQ

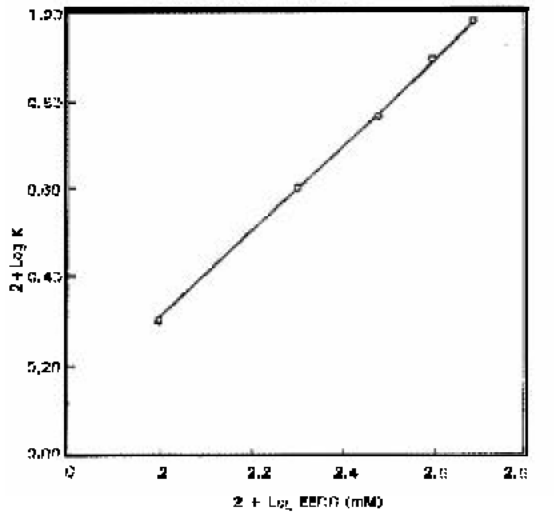

Fig. 7b: Double logarithmic plots of the observed pseudo first order rate constants (k) against EEDQ concentrations. The data are obtained from Fig. 7a.

pseudo-first order rate constants against TNM concentration (Fig. 6 b) yielded an order of 1, indicating the modification of a single residue resulted in the loss of enzyme activity EEDQ is known as a reagent of the carboxyl group (Skoubas and Georgatsos, 1997). Thus, treatment of NADH-GOGAT at various concentrations (1-5 mM) resulted in loss of activity with different rates (Fig. 7a) indicating that inactivation follows pseudo-first order kinetics. This inactivation indicates the involvement of residues with a carboxyl side chain (possibly aspartate and / or glutamate) in the catalytic activity and confirms the results of Vigara *et al*. (1996). From the slope of the line resulting from plotting log of pseudo-first order rate constants (K) vs log of EEDQ (Fig. 7b), it is deduced that the reaction order is 1 and suggests modification of a single residue of aspartate or glutamate inactivates the enzyme.

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