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LIGHT-DEPENDENT ACTIVITY OF GLUTAMATE SYNTHASE *IN VITRO*.

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SUMMARY : Glutamate synthase from rice (*Oryza sativa*) green leaves was assayed in a chloroplast reconstituted system. The enzyme activity was totally dependent on externally supplied thylakoid membranes and ferredoxin in the light. Glutamate synthase activity was also detected from etiolated leaves with photoreduced ferredoxin as an electron donor.

INTRODUCTION

In higher plant leaves, the inorganic nitrogen assimilation into amino acids occurs mainly in chloroplasts. This organelle contains nitrite reductase (EC 1.6.6.4) (1,2), glutamine synthetase (EC 6.3.1.2) (3,4,5), and glutamate synthase (EC 1.4.7.1) (2,6).

In vitro, the activity of chloroplastic glutamate synthase is dependent on dithionite reduced ferredoxin; it thus differs in electron donor specificity from pyridine nucleotide: NADPH (EC 1.4.1.13) or NADH (EC 1.4.1.14)-dependent glutamate synthases which were found in young plant tissues (7,8) and non-photosynthetic tissues (9, 10, 11, 12). NADH-dependent glutamate synthase was also shown to be located in the plastid fraction (13, 14).

It is now generally accepted that the incorporation of ammonium into glutamate in green leaves is a light-stimulated process and that it is primarily catalyzed by the coupled reaction of glutamine synthetase and glutamate synthase (15). However so far no-direct evidence exists for the involvement of photoreduced ferredoxin in the light-dependent glutamate synthase reaction: the only experiments carried out with partially purified glutamate synthase used either chemically reduced ferredoxin (16, 17) or methyl viologen (6).

Here we show the light-dependent activity of glutamate synthase in a reconstituted chloroplast system containing a mixture of ferredoxin and thylakoid membranes using an assay method based on a high-pressure liquis chromatography method which permits the detection of very small quantities of glutamate.

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MATERIAL AND METHODS

Plant Materials. *Oryza sativa* L. cv Delta was germinated and grown for 14 days in a growth chamber as described previously (18). Etiolated plants were grown for 10 days in darkness at 29°C.

Enzyme extraction. All procedures were carried out at 4°C. Leaf tissues were homogenated in 10 volumes of grinding medium consisting of 25 mM phosphate buffer (Na_2HPO_4 . KH_2PO_4), pH 7.5 (standard buffer) containing 14 mM β - mercaptoethanol and 1 mM EDTA. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 11,000g for 30 min in a Beckmann J 21 centrifuge. Solid ammonium sulfate fraction was added to the supernatant, and the 30 to 70% saturated ammonium sulfate fraction was taken. The precipitate, dissolved in standard buffer containing 14 mM β - mercaptoethanol was dialyzed for 12 hr against the same buffer.

Preparation of chloroplast thylakoid membrane fraction and isolation of ferredoxin. Spinach leaves were homogenated in 25 mM HEPES buffer, pH 7.5 containing 400 mM sorbitol, 2 mM $MgCl_2$, and 1 mM EDTA. After filtration through 4 layers of cheesecloth, the filtrate was centrifuged at 4,000g for 12 sec. The pellet was washed 3 times with the same isotonic medium, and the chloroplasts were broken by osmotic shock in 10 mM Tris-HCl buffer, pH 7.6. The chloroplast thylakoid membrane fraction was separated by centrifugation at 47,000g for 10 min and heated for 5 min at 55°C to destroy the oxygen evolution capacity (19). Chlorophyll content was determined by the method described (20). Ferredoxin was prepared from spinach leaves by the method of Mayhew (21). The ratio of A 420/A 275 was 0.45. The concentration of ferredoxin was determined using a molar extinction coefficient of 9,680 M^{-1} cm^{-1} (22).

Preparation of chloroplast reconstituted system and enzyme assay. The reaction mixture (100 µl) consisted of 2.25 µmoles phosphate buffer (Na_2HPO_4 . KH_2PO_4), pH 7.3, 0.5 µmole glutamine, 0.5 µmole 2-oxoglutarate, 1 µmole sodium ascorbate, 0.01 µmole 2,6-dichlorophenol indophenol, 1.4 µmole β - mercaptoethanol, thylakoid membranes equivalent to 100 µg of chlorophyll, 0.002 µmole ferredoxin, and 30 µl of enzyme preparation. The reaction was started by adding glutamine while turning on the light which gave a light intensity of 4,000 lux at the level of the assay tubes. The temperature was kept constant at 30°C by incubating the assay tubes in circulation of water. The reaction was stopped by heating at 100°C for 1 min, and after centrifugation at 2,000g for 10 min, the supernatant was used for enzyme assay.

Glutamate synthase was assayed by determining the glutamate formation using high-pressure liquid chromatography (HPLC). The glutamate formed was converted to o-phtalaldehyde (OPA) derivative. The derivatization reagent consisted of 450 mM borate buffer, pH 6.8, 40 mM o-phtalaldehyde, 275 mM β - mercaptoethanol, and 10 % (v/v) methanol. An aliquot (20 to 25 µl) of the reactio mixture was reacted with 100 µl of the derivatization reagent. After 80 sec of the reaction, a sample of 20 µl was subjected to the determination of OPA-amino acids using a model 6000 A chromatograph (Waters Associates, Milford MA 01757 USA). Separation was carried out on a 10 µM silica sphere (30 cm x 4 mm, µBondapak C_{18} column, Waters) with 30 % (v/v) methanol solvent dissolved in 20 mM sodium phosphate buffer, pH 6.8 which was pumped at a flow rate of 1.4 ml/min, giving a pressure of 3,500 p.s.i. Eluted OPA-glutamate and OPA-glutamine were detected at 340 nm. One unit of enzyme activity represents one µmole of glutamate formed per min at 30°C.

RESULTS AND DISCUSSION

Glutamate synthase assay by HPLC. For glutamate synthase assay, previous workers have employed either paper chromatography (2, 6,7) or ion-exchange chromatography (18, 23, 24, 25, 26) in which separated glutamate was determined by its radioactivity or by ninhydrin assay. In this work, we decided to

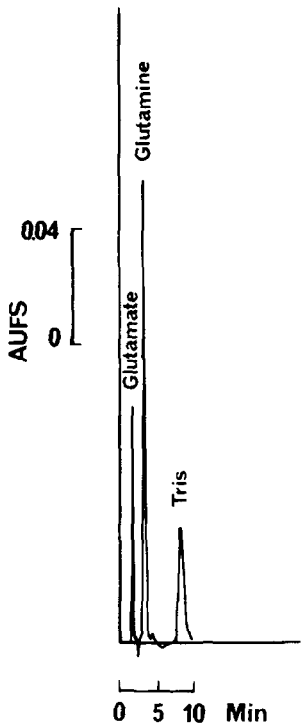


Figure 1 : Elution profile of o-phtalaldehyde derivated glutamate and glutamine on high-pressure liquid chromatography .

use an enzyme assay method based on the HPLC, which is highly sensitive and needs only a small quantity of reaction mixture. Fig. 1 shows an elution profile of OPA-glutamate and OPA-glutamine. The separation was completed within 5 min with retention times of 2 and 4.8 min for OPA-glutamate and OPA-glutamine, respectively. The slight peak appearing after OPA-glutamine corresponds to Tris which was used for thylakoid and ferredoxin preparations. Further details are given elsewhere (27).

Using the high-pressure liquid chromatography method, the stoichiometry of the glutamate synthase reaction was calculated by measuring the peak heights of glutamate formed and glutamine disappeared. Two molecules of glutamate were formed per one molecule of glutamine used (Table I) which is in agreement with the equation of the reaction.

Table I. Stoichiometry of the Glutamate Synthase Reaction.

A	Glutamate formed	nmoles	128.0
B	Glutamine disappeared	nmoles	65.0
	Ratio A/B		1.97

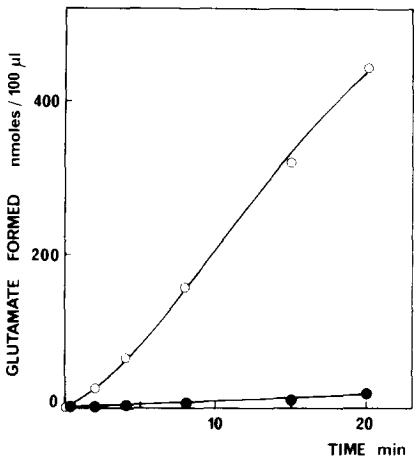
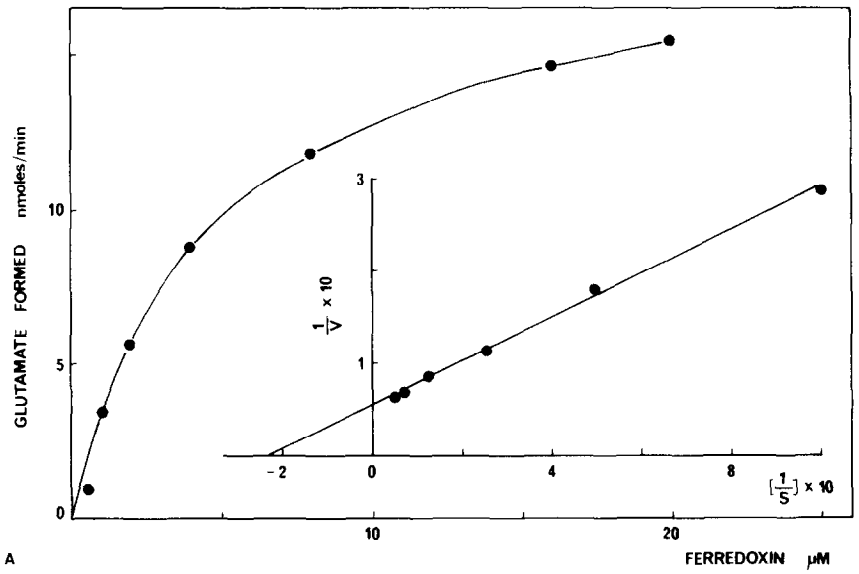


Figure 2 : Time courses of glutamate formation from the extract of rice green leaf tissue in the light and in the dark.

Glutamate synthase activity in chloroplast reconstituted system. The time courses of glutamate synthase activity in the light and in the dark are shown in Fig. 2. The production of glutamate was linear for 20 min in the light after a slight lag period of about 5 min. No significant amount of glutamate was formed in the dark.

Fig. 3 shows the glutamate synthase activity with respect to the different concentrations of two components included in the chloroplast reconstituted system: ferredoxin and thylakoid membrane fraction. It is evident that the increasing concentration of ferredoxin and thylakoik membrane fraction was accompanied with a concomitant increasing amount of glutamate formation (Fig. 3 A and 3 B). With respect to ferredoxin, the Km value was estimated to be 4.3 µM by Lineweaver and Burk plots (Fig. 3 A). The value is therefore similar to that (5.5 µM) of ferredoxin-glutamate synthase in rice green leaf tissue as reported previously (18). Although the light-dependent chloroplast reconstituted system used here for the ferredoxin-dependent glutamate synthase was similar to the system used for the activation of chloroplast enzymes (19), the glutamate synthase did not show any sensitivity towards oxygen; it thus differed from the light actuated enzymes which had to be assayed under anaerobic conditions.

Light-dependent formation of glutamate in the presence of glutamine and 2-oxoglutarate was originally detected in whole intact chloroplasts (28). Later ferredoxin reduced by dithionite was found to be specific electron donor for glutamate synthase in pea chloroplasts (16). The glutamate synthase activity dependent on the chloroplast reconstituted system in the light reported here shows that the light effect is mediated by chloroplast membranes and ferredoxin, indicating that glutamate synthase activity was dependent on the photosystems. In this glutamate synthase assay system, the preparation of thyla-



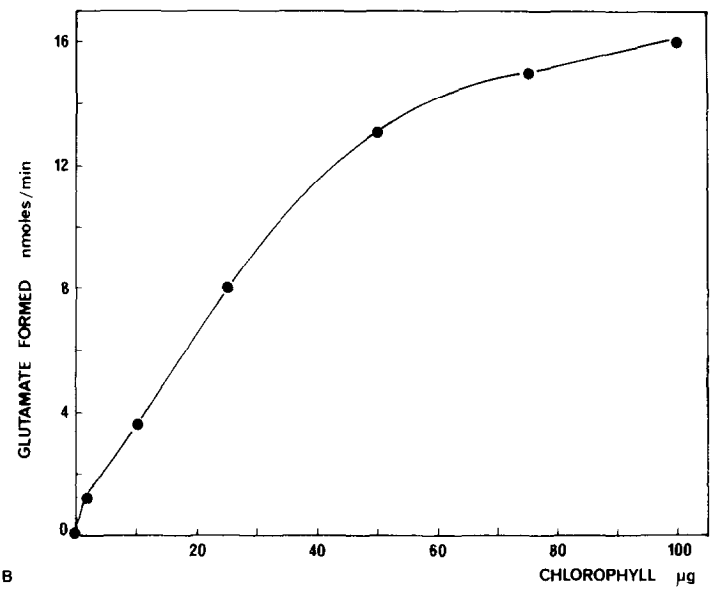


Figure 3 : (A) Influence of different concentrations of ferredoxin on glutate synthase from the extract of rice green leaf tissue.
(B) Influence of different concentrations of thylakoid membrane fraction on glutamate synthase from the extract of rice green leaf tissue.

koid membrane fraction, treated at 55°C for 5 min, was relatively stable compared to non-treated one, so the assay system could be also suitable as one of the routine enzyme assays.

In etiolated rice leaf tissue glutamate synthase dependent on photoreduced ferredoxin was also detected. The kinetics of enzyme activity in the light and in the dark show that the rate of glutamate formation was totally dependent on the light, and only no more than 2 % of the glutamate was formed com-

Table II. Time Courses of Glutamate Formation in the Light and in the Dark in the Extract from Etiolated Rice Leaf Tissue.

Assay condition \ Reaction time (min)	0	5	10	15	20	25	30	35	40
Light	0	69.1	131.8	199.4	277.4	411.6	447.0	498.5	585.3
Dark	0		16.1		24.1		23.3		31.3

The values are given as nmoles glutamate formed.

pared to the activity in the light (Table II). The effect of different concentrations of ferredoxin and thylakoid membrane fraction on etiolated leaf glutamate synthase activity was determined (Table III). It was shown that exogenous ferredoxin and thylakoid membrane fraction were needed for the enzyme activity, and that the enzyme had Km value of 5.4 μM towards ferredoxin, the results being identical to the glutamate synthase from green leaf tissue.

As many other chloroplast enzymes (29), ferredoxin-dependent glutamate synthase activity increases upon greening of the etiolated rice leaf tissue, this increase being accompanied by activation and/or synthesis of the enzyme molecule. Clearly the reducing power for the glutamate synthase in rice green leaf tissue is supplied by the electron transport system. It is also observed that glutamate synthase in etiolated leaf tissue is able to use photoreduced ferredoxin. Although the presence of ferredoxin-dependent glutamate synthase in non-photosynthetic etiolated leaf tissue is established here, the physiological role of glutamate synthase, dependent on ferredoxin in etiolated leaf tissue remains to be clarified; in parallel the elucidation of the electron donor system in etiolated tissue is an exciting problem to be solved.

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Table III. Influence of Different Concentrations of Ferredoxin and Thylakoid Membrane Fraction on Glutamate Synthase from Rice Etiolated Leaf Tissue.

Ferredoxin μM	0	2	3.3	5	10	20	
Activity	0	3.25	5.00	8.60	8.99	9.24	
Chlorophyll μg	0	0.63	1.25	2.5	5	10	100
Activity	0	0.54	0.50	0.86	1.38	2.55	11.31

Activities are given as mU (nmoles glutamate formed per min).

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