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Glutamine Synthetase and Glutamate Synthase System of the Marine Green Alga, *Bryopsis maxima**

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The glutamine synthetase (EC 6.3.1.2) and the ferredoxin-dependent glutamate synthase (EC 1.4.7.1) of the marine green macro-alga, *Bryopsis maxima* were investigated. The enzyme system was extracted from chloroplasts, and the activities were quantitatively estimated by thin layer chromatography and with scanners. In addition, the glutamine synthetase activity was measured with an amino acid autoanalyzer. By an indirect method, γ-glutamyltransferase activity of the glutamine synthetase was also confirmed.

Key Index Words: Bryopsis maxima; *glutamate synthase*: *glutamine synthetase*: *thin layer chromatography*.

In place of glutamate dehydrogenase (GDH; NAD-dependent, EC 1.4.1.2 and NADP-dependent, EC 1.4.1.4), it has recently been claimed that glutamine synthetase (GS) and glutamate synthase (GOGAT) mainly catalyze the first step of reductive amination of amino acids in green plants. This is based on the fact that the Km values of GDH for ammonia are very high in general beyond its concentration in chloroplasts.

Historically, the GS (EC 6.3.1.2) from *Escherichia coli* was first extensively purified, and its enzymatic properties were studied in detail (WOOLFOLK *et al.* 1966). The enzyme has, in addition, been known to catalyze the transfer of the γ-glutamyl group of glutamine to hydroxylamine added to the reaction mixture (O'NEIL and JOY 1974). A similar enzyme was also found in fission yeasts (BROWN *et at.* 1973).

On the other hand, a GOGAT by which glutamate and an amino acid are formed from glutamine and a 2-oxo acid in the presence of NADH or NADPH has been found to exist in *Aerobacter aerogens* (TEMPEST and MEERS 1970) and *Escherichia coli* (MILLER and STADTMAN 1972). The GOGAT of the latter bacterium was found, like GS, to be a high molecular weight enzyme whose classification number was first assigned to be EC 2.6.1.53 but is now changed to EC 1.4.1.13 (NADP).

More recently, it has been found that a ferredoxin-dependent GOGAT (EC 1.4.7.1) is existent in the chloroplasts of higher plants and that it catalyzes ammonia assimilation, being coupled with GS (LEA and MIFLIN 1974; ARIMA and KUMAZAWA 1977).

The algal GS and GOGAT system was

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Abbreviations: EDTA, ethylenediaminetetraacetate; DTT, 1,4-dithiothreitol; BSA, bovine serum albumin.

investigated first using extracts from the blue-green algae, *Nostoc ellipsosporum* and *Anabena cylindrica* (LEA and MIFLIN 1975a) and also *Chlorella fusca* var. *vacuolata* (LEA and MIFLIN 1975b), using the whole cell as well as extracts from the latter alga. Most recently, this enzyme system was investigated in a green macro-alga, *Caulerpa simpliciuscula* (MCKENZIE *et al.* 1979).

In the meantime, we have investigated NADP-dependent GDH of *Bryopsis maxima*, which belongs to different family from the Caulerpaceae, and found that its Km value for ammonia is much higher than that of *C. simpliciuscula* GDH (NADP) (GAYLER and MORGAN 1976) but far smaller than those reported in higher plants (NISIZAWA *et al.* 1978). Therefore, it was intersting for us not only to determine the kind of GS and GOGAT system in this macroalga, but also to compare its enzymatic property, if possible, with those of algae already reported. However, determination of the products by this enzyme system has been carried out after separation by paper electro-phoresis which seemed to be very complicated work (LEA and MIFLIN 1975a, LEA and MIFLIN 1975b, MCKENZIE *et al.* 1979).

Thus, we attempted to use the techniques of thin layer chromatography and amino acid autoanalysis without using isotopic technique for the determination of reaction products, and appreciable results were obtained.

Materials and Methods

Algal material: *Bryopsis maxima* was collected, as necessary from the coast of Inubo Cape, Choshi, Chiba Prefecture in January to May, 1981. Fresh fronds were quickly brought back to laboratory in an ice box, and used as an enzyme source soon after or after culturing for several days in a water tank with clean seawater under aeration.

Chemicals: Ferredoxin spinach is the product of Sigma Chemical Comp. All other chemicals were purchased from Wako Pure Chemical Industries, Ltd.

Extraction of enzyme: Firstly, chloroplasts were isolated from algal fronds following the procedure of WRIGHT and GRANT (1978). Approximately 200 g of fresh fronds were chopped with scissors and ground in a mortar with 100 m*l* breaking medium in the presence of a small amount of quartz sand for 10 min. The breaking medium consists of 0.7 M NaCl, 50 mM Tricine, 50 mM $MgSO_4$, 1 mM $MnCl_2$, 2 mM EDTA, 5 mM sodium-ascorbate, 0.1 mM DTT and 0.2% (w/v) BSA, at pH 7.5. The debris was squeezed by hand through 4 layers of gauze, and the extract was centrifuged at 1000×g for 1 min. The dark green precipitate was washed with 100 m*l* resuspension medium, pH 7.5, and centrifuged at 750×g for 10 min. The same wash was repeated twice. The resuspension medium was the same composition as the breaking medium except that it contained 0.5% (w/v) BSA and no DTT. It was confirmed by microscopic examination that the precipiate thus obtained consists of clear chloroplast particles.

The chloroplast pellets were then suspended in 50 m*l* of 50 mM Tricine buffer, pH 7.5, containing 5 mM EDTA, 12.5 mM 2 mercaptoethanol, and were sonicated under icecooling 30 times for 30 sec each with a M.S.E. 150 W sonicator (Otake type 5202 PZT). The broken chloroplast suspension was centrifuged at 12,000×g for 20 min and the supernatant was dialyzed in a cellophane tube against 4 changes of 300 m*l* of 50 mM Tricine buffer, pH 7.5, containing 12.5 mM 2-mercaptoethanol for 1 hr each. To the dialyzed solution was added 40 mM $MgCl_2$ and it was used as the enzyme solution for investigation.

This enzyme solution was available for experiment after storage at-40°C for several days, though the activity decreased gradually. The enzyme was, however, mostly inactivated by storage for more than a week.

Measurement of chlorophyll content in enzyme solution: The enzyme solution (1 m*l*) was concentrated to dryness under reduced pressure, and extracted with 15 m*l* acetone at room temperature. The mixture was

centrifuged at 1,000×g for 5 min. The supernatant dissolving chlorophylls and other pigments in it was analyzed for total chlorophylls with a Shimadzu Photoelectric photometer UV-200 basing on the following equation of ARNON (1949).

Total chlorophylls ($\mu g/ml$)=$20.2A_{645}+8.02A_{663}$, where the absorbance A was obtained by subtraction of the absorbance at 750 nm from the absorbances measured at 645 nm and 663 nm, respectively. The enzyme solutions prepared by the procedure described above contained ordinarily around 80 μg total chlorophylls per m*l*.

Enzyme assays. a) GS activity: The reaction mixture consisted of 33.3 mM Tricine buffer, pH 7.5, 1.3 mM NH_4Cl, 3.3 mM L-glutamate, 26.7 mM $MgCl_2$, 10 mM ATP, and enzyme in a total volume of 3 m*l*. The enzyme reaction was carried out at 30°C together with the control which lacked L-glutamate. The reaction mixture contained around 80 μg of total chlorophylls. The enzyme reaction was initiated by addition of ATP to the reaction mixture lacking ATP.

b) GOGAT activity: The reaction mixture consists of 30.8 mM Tricine buffer, pH 7.5, 4.8 mM L-glutamine, 4.8 mM 2-oxoglutarate, 8 mM amino oxyacetate, 0.16 mg ferredoxin, 9.6 mM sodium-dithionite, 19.2 mM $NaHCO_3$ and enzyme in a total volume of 2.6 m*l*. The enzyme reaction was carried out at 30°C together with control which lacked L-glutamine. The reaction mixture contained around 80 μg of total chlorophylls. The mixture of sodium-dithionite and $NaHCO_3$ must be prepared just before the experiment. The enzyme reaction was initiated by addition of the dithionite mixture to the reaction mixture lacking this reagent.

c) γ-Glutamyltransferase activity of GS: According to the method of HUBBARD and STADTMANN (1967) for bacterial enzymes, the reaction mixture was prepared. It consisted of 0.1 M L-glutamine, 0.3 M $NH_2OH\cdot HCl$, 20 mM Na_2HAsO_4, 0.4 mM, ADP 3 mM $MnCl_2$, 20 mM imidazole and enzyme in a total volume of 3 ml at pH 7.0. The mixture was incubated at 30°C together with a blank with lacked the enzyme, and it contained around 80 μg of total chlorophylls. Then, aliquots of the reaction mixture were removed at intervals and γ-glutamylhydroxamate formed was estimated by reading the absorbance at 540 nm with a Shimadzu Model UV-200S double-beam spectrophotometer.

d) GDH activity: The assay procedure was entirely identical to that used in a previous work (NISIZAWA *et al.* 1978). The enzyme reaction was carried out with an enzyme extract containing about 0.65 μg protein.

General procedure of thin layer chromatography (TLC): Kieselgel 60 F_{254} glass plates (20×20 cm) manufactured by the Merk Corp. were used, and the solvent was a mixture of phenol: water (3:1, v/v). Developing was stopped when the front of the solvent reached 10 cm in height. The TL chromatogram was dried at 80°C for 60 min. The spots were colored with ninhydrin or Fluorescamine (MENDEZ and LAI 1975) reagent by heating at 120°C for 5 min for the former or by standing at room temperature for the latter. Then, the chromatogram was subjected to autoanalysis by a Shimadzu Model CS-920 high speed TLC scanner for the ninhydrin-staining spots. For this, a 496 nm wavelength and a light bundle of 1.25×1.25 mm were used. When the Fluorescamine reagent was used, the analysis was made with a Shimadzu Model CS-910 double-beam TLC scanner at 365 nm wavelength and a light bundle of 9.0×0.8 mm. These scanners were in either case connected with a Shimadzu Model RIA chromatopack to record the data automatically.

Determination of reaction products in GS and GOGAT reaction mixtures.

a) By TLC: The reaction mixture together with the control soon after and 120 min after the incubation were heated at 100°C for 2 min. Then, they were concentrated in an Evapo Quick under reduced pressure to a syrup, and 10 m*l* acetone were added to each sample. After mechanical shaking for 5 min, the mixtures were centrifuged at 1,000×g for 10 min and the yellowish green superna-

tants were discarded. The precipitates were extracted with 1 m*l* water each at 80°C for 5 min and centrifuged at 1,000×g for 10 min. The supernatants were analyzed by TLC for the reaction products.

b) *With amino acid autoanalyzer*: The final aqueous solution of the reaction products, parts of which were applied to TLC analysis as described above, was diluted to an appropriate volume to contain 0.2-0.4 μM per m*l* of amino acids to be analyzed and subjected to analysis with a Hitatchi Model KLA-5 amino acid autoanalyzer.

Determination of protein: Using crystalline bovine serum albumin as a standard, the protein in enzyme solution was estimated by LOWRY-FOLIN method (LOWRY *et al.* 1951).

Results

TL chromatograms of the reaction products by GS and GOGAT: The spots of amino acids were colored with ninhydrin reagent. The resulting chromatograms are shown in Fig. 1. A large spot corresponding to L-glutamine occurs among the products after incubation with GS while the spot of L-glutamate appears among the products by GOGAT. In addition, faint spots like glycine and alanine are visible below the spots of glutamine in blanks as well as samples.

GS activity measured by TLC scanner method: From the two series of reaction mixtures of the same ingredients, TL chromatograms were prepared as in Fig. 1. Each was colored with ninhydrin or fluorescamine reagent and analyzed by TLC scanning. The results are shown in Tables 1 and 2. It is clear in Table 1 that the area of L-glutamate used as substrate decreased notably after incubation, whereas a fairly large area of L-glutamine appeared as a new spot of amino acid. A similar result was obtained in Table 2. However, far smaller amounts of L-glutamine were also found in blanks after incubation. This may be due to reactions between endogenous substrates preexistent in enzyme preparation.

GOGAT activity measured by TLC scanning method: Two series of TL chromatograms were prepared in the same way as with GS, and each was stained with ninhydrin or Fluorescamine reagent. The results from their scanning analysis are shown in Tables 3 and 4. In contrast to the GS series, L-glutamine used as substrate is decreased after incubation whereas L-glutamate is increased far beyond the amount of the one endogenously formed in both Tables.

γ-Glutamyltransferase activity of GS: The result is shown in Table 5. The activity was clearly detectable after incubation

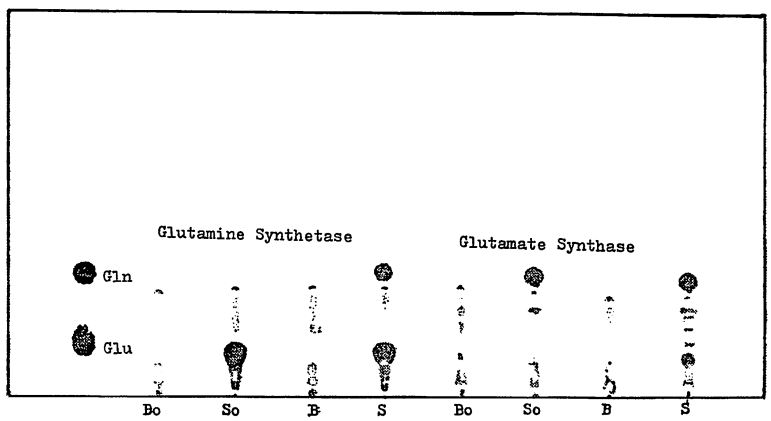

Fig. 1. TL chromatograms of reaction products by GS and GOGAT after staining with ninhydrin reagent. B_0 and B are blanks before and after (120 min) incubation, respectively. S_0 and S are sample reaction mixtures before and after (120 min) incubation.

Table 1. Reaction products* by GS measured with a TLC scanner after coloring with ninhydrin reagent.

Marker	Amino acid	Scanning distance (mm)	Area coefficient
Glu	—	25.2	52725
Gln	—	45.3	42203
Spot			
B_0			
Blank before incubation	—	—	—
B			
Blank after incubation for 120 min	Glu	26.0	160
S_0			
Sample before incubation	Glu	24.6	50095
S			
Sample after incubation for 120 mim	Glu	24.8	44005
	Gln	46.8	5162

* Data for amino acids other than Glu and Gln are omitted in this Table.

Table 2. Reaction products* by GS measured with a TLC scanner after coloring with Fluorescamine reagent.

Marker	Amino acid	Scanning time (min)	Area coefficient
Glu	—	1.04	25857
Gln	—	1.98	20723
Spot			
B_0			
Blank before incubation	—	—	—
B			
Blank after incubation for 120 min	Glu	0.96	1924
	Gln	2.01	233
S_0			
Sample before incubation	Glu	0.9	26465
S			
Sample after incubation for 120 min	Glu	0.94	21725
	Gln	1.94	4376

* Data for amino acids other than Glu and Gln are omitted in this Table.

for 30 min under the present reaction condition. The reaction mixture lacking ADP and arsenate also produced a remarkable amount of γ-glutamylhydroxamate, while that lacking L-glutamate showed no appreciable increase in the amount of this hydroxamate. Therefore, L-glutamine seemed to form under the present reaction condition some compound which shows a high absorbance at 540 nm or produce to γ-glutamylhydroxamate

Table 3. Reaction products* by GOGAT measured with a TLC scanner after coloring with ninhydrin reagent.

Marker	Amino acid	Scanning distance (mm)	Area coefficient
Glu	—	28.8	50540
Gln	—	44.3	51423
Spot			
B_0			
Blank before incubation	—	—	—
B			
Blank after incubation for 120 min	Glu	29.6	358
	Gln	46.4	48
S_0			
Sample before incubation	Glu	28.8	132
	Gln	46.0	40498
S			
Sample after incubation for 120 min	Glu	28.1	775
	Gln	44.5	33030

* Data for amino acids other than Glu and Gln are omitted in this Table.

Table 4. Reaction products* by GOGAT measured with a TLC scanner after coloring with Fluorescamine reagent.

Marker	Amino acid	Scanning time (min)	Area coefficient
Glu	—	0.97	41288
Gln	—	1.80	39532
Spot			
B_0			
Blank before incubation	—	—	—
B			
Blank after incubation for 120 min	Glu	0.98	547
S_0			
Sample before incubation	Glu	1.0	1306
	Gln	1.85	11383
S			
Sample after incubation for 120 min	Glu	0.93	2835
	Gln	1.77	9751

* Data for amino acids other than Glu and Gln are omitted in this Table.

Table 5. γ-Glutamylhydroxamate production* from L-glutamine and hydroxamine during incubation (120 min) with GS. B; blank**, S; sample reaction mixture.

Incubation time (min)	B	S	S−B
0	11.9	14.3	2.3
15	12.9	15.2	2.3
30	13.6	16.7	3.1
45	14.6	18.1	3.5
60	15.8	20.0	4.2
75	16.7	21.4	4.7
90	17.6	22.9	5.3
105	18.1	24.3	6.2
120	19.0	26.2	7.2

* μM/mg protein in reaction mixture.

** Lacks enzyme; other blanks which lacked glutamine or lacked glutamine, ADP and arsenate gave productions of only 0.5 to 1.0 μM γ-glutamylhydroxamate in reaction mixture. The blank that lacked ADP and arsenate, however, gave productions of 10.5 to 16.7 μM γ-glutamylhydroxamate during the same incubation periods.

Table 6. Amino acids* determined with an autoanalyzer in reaction mixtures of GOGAT. B_0, B, S_0 and S are same as in Fig. 1.

Amino acid	B_0	B	S_0	S
Lys	0.82	1.05	—	1.90
His	—	0.19	9.62	—
NH_3	3.44	26.98	7.28	25.66
Asp	0.24	0.72	1.06	0.93
Gln	0.38	1.43	406.30	322.85
Thr	0.44	1.18	—	—
Glu	1.03	1.82	3.38	10.15
Gly	1.13	3.03	1.28	14.86
Ala	0.41	1.53	0.62	24.90

* μg/ml of aqueous solution containing the reaction products extracted from the reaction mixture.

itself.

GOGAT activity measured with amino acid analyzer: The result is shown in Table 6. It is clear that far more L-glutamate was produced after incubation than before whereas L-glutamine was notavly decreased after incubation. Moreover, it is noticeable that glycine and alanine were also increased remarkably after incubation while no appreciable change was found with aspartate. Ammonia was increased, too, but the extent was almost ildentical in both sample and blank.

Km value of GS for ammonia: Changing the concentration of NH_4Cl in the reaction mixture of GS from 1 mM to 4 mM, the extents of L-glutamine production were determined by TLC scanner method. The amounts obtained were treated by the LINEWEAVER-BURK procedure and the Km of GS

for ammonia was estimated graphically to be 0.7 mM.

GDH activity: An enzyme solution extracted from chloroplasts of *B. maxima* in the same way as the extraction of GS and GOGAT was used as the enzyme source. The specific activity of GDH was 0.08 in terms of absorbance per μg protein per min.

Discussion

In place of the electrophoretic separation (ATFIELD and MORRIS 1961) of reaction products hitherto employed in most cases, TLC scanner method was introduced into the detection of the GS and GOGAT system in the present work. The method seemed rather simple since the TLC scanner is disposable. In addition, the activity of this enzyme system was defected readily with the amino acid autoanalyzer, too. Although the applicability of this method was not examined further in the present work, it seemsd to be useful for the investigation of GS and GOGAT system in detail. In any case, however, it is shown by the present investigation that the GS and GOGAT system is present in the chloroplast of *B. maxima*. This is the second proof of the presence of these enzymes in macroalgae. However, we succeeded only in detecting the enzyme activity in the chloroplasts of this alga, so that use of such newly designed procedures remains in future for further enzymatic study.

The Km for ammonia of GS from *B. maxima* was estimated to be 0.7 mM by TLC scanner method. The value is, however, very high as compared with that of pea leaves in which the values of 1.9×10^{-5} M was obtained. However this has been estimated by an indirect method in which liberated phosphate from ATP was measured by the FISKE-SUBBAROW assay (O'NEAL and JOY 1974). In contrast, the Km of *Bryopsis*-GS was about 34 times lower than that was obtained for the GDH (NADP) of the same alga (NISIZAWA *et al.* 1978). In addition, the GDH activity of chloroplasts of this alga was 0.08 per μg protein per min when expressed by decrease in the absorbance at 340 nm. The value suggests that the activity may be approximately 1/8 that of an extract from the whole algal fronds, by rough estimate from the data reported in our previous paper (NISIZAWA *et al.* 1978). Therefore, it is reasonable to assume that ammonia is reductively assimilated into amino acids catalyzed mainly by the GS and GOGAT system in the chloroplasts of *B. maxima*.

However, the above reasoning is inconsistent with that which has been proposed by GAYLER and MORGAN (1976) for the GDH (NADP) of *Caulerpa simpliciuscula*. From the fact that this enzyme localizes in the chloroplast and has a very low Km for ammonia such as 0.4 to 0.7 mM, the authors have assumed that the enzyme plays a role in reductive amination independent of the GS and GOGAT of the alga. On the other hand, there is a report in which the *de novo* synthesis of GDH (NADP) of *Chlorella* was conspicuously enhanced when it was grown in high ammonia and low nitrate media (KRETOVICH *et al.* 1970).

Taking into account these results as well as our results from *B. maxima*, not only GDH but also GS and GOGAT seem to work under a complex relationship in ammonia assimilation. The mode of their physiological roles, therefore, seems more or less different from each other depending on plant species and/or on its physiological condition.

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西澤一俊*・大内正人*・高橋　研*・奥　忠武:** **緑藻オオハネモのグルタミンシンテターゼ (GS)-グルタメートシンターゼ (GOGAT) 系**

マクロ藻における GS/GOGAT 系 (NH_3 の初期固定酵素系) の研究は極めて少ないので，オオハネモにおけるその存在を新しい検出法により証明した。まず，既に高等植物などで報告されている方法により，その新鮮藻体から葉緑体を抽出し，適当な条件下で超音波破壊をして酵素液を作った。酵素反応条件も従来の文献に従ったが，反応生成物 (GS の作用では Gln の生成，GOGAT の作用では Glu の生成) は，反応液からアセトンで沈殿させ，同時に光合成色素を除いた後，その水溶液から薄層クロマトグラフィーにより得たスポットを，ニンヒドリンやフルオレッサミンにて染色してからクロマトスキャンナーにかけて定量した。一方，反応生成物を自動アミノ酸分析でも定量した。薄層法で測定した GS の NH_3 に対する Km は 0.7 mM であった。

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