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A COMPARATIVE STUDY OF TWO FORMS OF GLUTAMINE SYNTHETASE FROM RICE LEAVES[1,2]

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Abstract

Two forms of glutamine synthetases designated as GSI and GSII were separated by DEAE-Sephacel chromatography from rice leaves at different growth stages. The activity of GSI could maintain a rather constant level at all growth stages, but the activity of GSII decreased markedly after tillering stage. By using 2′5′ADP-Sepharose 4B affinity chromatography and hydroxyapatite absorption, GSI was purified to apparent homogeneity and GSII was nearly homogeneity. GSII was more heat-labile than GSI, and GSII electrophoretically migrated faster then GSI on 5% polyacrylamide gel at pH 8.3. Km values of GSI for NH_4Cl, L-glutamate and ATP were 0.15 mM, 1.89 mM and 1.39 mM, respectively; Km values of GSII for NH_4Cl and ATP were 0.75 mM and 1.72 mM, respectively. The Km value of GSII for L-glutamate showed biphasic with 6.45 mM and 4.17 mM at high and low concentrations, respectively. In the presence of Mg^{2+}, the pH optima for GSI and GSII were 7.1 and 7.4, respectively, GSI and GSII had similar molecular weight of 367,000, and both enzymes consisted of eight identical subunits with molecular weight of 45,800. In the presence of Mg^{2+}, the activities of GSI and GSII were strongly inhibited by Mn^{2+}. Moderate inhibition was observed by Fe^{2+}, Ca^{2+} and Zn^{2+}. However, Co^{2+} stimulated the activities of GSI and GSII markedly.

Key words: Glutamine synthetase; ammonia assimilation; rice leaf; divalent cation.

Introduction

Glutamine synthetase of higher plants is postulated to play a crucial part in assimilation, translocation and storage of ammonia nitrogen (Miflin and Lea, 1980); and it is also thought to play a role in re-assimilation of ammonia released in the photorespiratory nitrogen cycle and the catabolism of nitrogenous storage and

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transport compounds (Keys, 1980). These multiple functions may be catalyzed by different forms of a enzyme. In previous studies, it has been shown that two molecular forms of glutamine synthetase occur in photosynthetically active tissue of many higher plants (Guiz *et al.*, 1979; Mann *et al.*, 1979; Stasiewicz and Dunham, 1979; Kretovich *et al.*, 1981; Hirel and Gadal, 1980; 1982; Winter *et al.*, 1982; Admad *et al.*, 1982; NcNally *et al.*, 1983a, b, c). These two forms are present in different intracellular compartments and can be separated by ion exchange chromatography. One designated as GSI is located in cytoplasm, and the other one designated as GSII is found in chloroplasts (Mann *et al.*, 1980; Hirel and Gadal, 1980; Hirel *et al.*, 1982). It has been suggested that GSII is responsible for ammonia assimilation in the chloroplast and may be regulated by light (Hirel and Gadal, 1980). In constrast, GSI exhibits different regulatory properties and it has been suggested to play a role in the re-assimilation of ammonia during photorepiration (Keys *et al.*, 1978) as well as in glutamine synthesis in the dark (Hirel and Gadal, 1980; NcNally *et el.*, 1983b). The properties and physiological roles of different forms of the enzyme present in different intracellular compartments of the same cell are not clearly understood. Purification and characterization of the two forms of the enzyme should provide further insight into their modes of function and regulation.

In rice plants, the total activity of glutamine synthetase in leaves varied with the growth stages (Yuan, 1982). Therefore, a detailed comparison of purified GSI and GSII from rice leaves during rice growth is of interest considering the important roles played by both enzymes in the same photosynthetic tissue. This report includes the variations of GSI and GSII activity in rice leaves during growth, the procedures for purification of GSI and GSII from rice leaves and their properties such as affinity for substrates, pH optima, molecular size, divalent cation effect and heat stability. The differences between the two enzymes are compared and discussed.

Materials and Methods

Reagents

L-Glutamate, L-glutamine, γ-glutamyl hydroxamate, ATP, ADP and hydroxyapatite were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); Sepharose 4B, DEAE-Sephacel, Sephacryl S-300, 2′5′ADP-Sepharose 4B and high molecular weight calibration kit from Pharmacia Fine Chemicals (Uppsala, Sweden.), Ammonium chloride and hydroxamine hydrochloride from Wako Puro Chemical Industries (Tokyo, Japan). All other chemicals used in these investigations were of analytical grade or of the highest quality commercially available.

Plant Material

In order to meet the actual growth situation, rice variety of *Oryza sativa* L., cv. Tainung 62 was cultivated in the paddy field at the farm of the Institute of Botany, Academia Sinica located at Nankang, Taipei. Rice seedlings at four-leaf stage were transplanted to the paddy field with 5 to 7 seedlings a hill and with spaceing density of 30×25 cm (20 hills/m^2). Field management including basal and top dressings of fertilizer and irrigations, followed the general methods used by farmers. Rice leaves were collected at the tillering, booting and ripening stages. The collected leaves were usually made by noon between 10 to 12 A.M. The leaves were washed with distilled water and sliced to small pieces about 0.5 to 1.0 cm in length, then placed in a plastic bag and kept in a deep freezer at -60°C until extraction of glutamine synthetase. The activity of glutamine synthetase in leaves was stable at least three months at -60°C.

Extraction and Purification of Glutamine Synthetase

Frozen rice leaves were ground to powder with liquid nitrogen using a mortar and a pestle, and all further operations were carried out at 4°C. One hundred grams of the resulting powder was homogenized with 500 ml of 0.05 M Tris-HCl buffer (pH 7.5) containing 2 mM 2-mercaptoethanol, 2 mM EDTA (buffer A) for 10 minutes in an ice bath using a Polytron homogenizer at full speed. The homogenate was filtered by squeezing through a nylon net (200 mesh) and the filtrate was centrifuged at 12,000×g for 20 minutes. The supernatant was brought to 30% saturation with solid ammonium sulfate. After centrifugation, the supernatant was then brought to 60% saturation with ammonium sulfate and the 30-60% fraction was collected. The precipitate was dissolved in a small volume of buffer A and loaded on a Sepharose 4B column (2.6×80 cm) equilibrated with 0.01 M Tris-HCl buffer (pH 7.5) containing 2 mM 2-mercaptoethanol, 2 mM EDTA and 5 mM $MgCl_2$ (buffer B). The column was eluted with buffer B and the fractions with enzyme activity were pooled and loaded on a DEAE-Sephacel column (2.6×20 cm) equilibrated with buffer B. The column was washed with buffer B to remove the unabsorbed substances, then eluted with a linear gradient of 0-0.5 M KCl in buffer B in a total volume of 500 ml. Fractions (6 ml each) showing GSI and GSII activity were pooled separately. GSI was dialyzed against 0.01 M Tris-HCl buffer (pH 7.5) containing 2 mM 2-mercaptoethanol, 2 mM EDTA, 5 mM $MgCl_2$ and 10 mM L-glutamate (buffer C), then subjected to affinity chromatography on a column (0.9×10 cm) of 2′5′ADP-Sepharose 4B equilibrated with buffer C. The column was washed with buffer C to remove unbound protein. GSI was eluted with a linear gradient of 0-2 mM ATP in buffer C in a total volume of 100 ml. GSII was dialyzed against 0.01 M imidazole-HCl buffer (pH 7.0) containing 2 mM 2-mercaptoethanol, 1 mM $MnCl_2$, and 10 mM L-glutamine (buffer D), then subjected to the 2′5′ADP-Sepharose 4B column

equilibrated with buffer D previously. The column was washed with buffer D to remove unbound protein. GSII was eluted with a linear gradient of 0-10 mM ADP in buffer D in a total volume of 100 ml. The pooled fractions (3 ml each) showing GSI or GSII activity was dialyzed againt 0.01 M Na, K-phosphate buffer (pH 7.0), then loaded on an hydroxyapatite column (2.6×3 cm) equilibrated with 0.01 M Na, K-phosphate buffer (pH 7.0). The column was washed with 100 ml of 0.1 M Na, K-phosphate buffer (pH 7.0). GSI or GSII was eluted with a linear gradient of 0.1 to 0.3 M Na, K-phosphate buffer (pH 7.0) in a total volume of 100 ml. The pooled fractions (3 ml each) showing GSI or GSII activity was concentrated and dialyzed against buffer B.

Enzyme Assay

The biosynthetic activity of glutamine synthetase was assayed either based on γ-glutamyl hydroxamate formation in the presence of NH_2OH or based on the release of inorganic phosphate in the presence of NH_4Cl.

The biosynthetic assay based on γ-glutamyl hydroxamate formation was the procedure of Kanamori and Matsumoto (1972). The assay mixture with a final volume of 3 ml contained 33 mM Tris-HCl buffer (pH 7.5), 3.3 mM ATP (pH 7.0), 83 mM sodium glutamate, 33 mM $MgSO_4$, 10 mM NH_2OH (pH 7.0, freshly prepared), 3.3 mM cysteine (pH 7.0, prepared daily) and appropriate amount of enzyme solution. The reaction was initiated by the addition of NH_2OH, and glutamate was omitted in the blank test. After incubation at 30°C for 15 minutes, the γ-glutamyl hydroxamate formed was determined by adding 1.0 ml of ferric chloride reagent which was made by mixing equal volume of 10% $FeCl_3 \cdot 6H_2O$ in 0.2N HCl, 24% TCA, and 6N HCl. The absorbance at 540 nm was then measured. The commercial γ-glutamyl hydroxamate was used as standard. One unit of glutamine synthetase was defined as the amount of enzyme catalyzing the formation of 1.0 nmole of γ-glutamyl hydroxamate per minute at 30°C.

The biosynthetic assay based on release of inorganic phosphate was the procedure of Shapiro and Stadtman (1970). The assay mixture with a final volume of 0.4 ml contained 50 mM imidazole-HCl buffer (pH 7.0), 7.5 mM ATP, 100 mM sodium glutamate, 50 mM NH_4Cl, 50 mM $MgCl_2$, and appropriate amount of enzyme solution. The reaction was started by adding enzyme solution, and the enzyme solution was omitted in the blank test. After incubation at 30°C for 15 minutes, the reaction was stopped by adding 3.6 ml of ferrous sulfate reagent (0.8% $FeSO_4 \cdot 7H_2O$ in 0.015 N H_2SO_4, prepared freshly), followed by adding 0.3 ml of ammonium molybdate reagent [6.6% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 7.5 N H_2SO_4]. After several minutes, the absorbance at 660 nm was measured. The sodium dihydrogen-phosphate dihydrate was used as standard. One unit of glutamine synthetase was defined as the amount of enzyme catalyzing the release of 1.0 nmole inorganic

phosphate per minute at 30°C.

Glutamine synthetase also can be assayed for its transferase activity according to the procedure of Shapiro and Stadtman (1970). The assay mixture with a final volume of 3 ml contained 40 mM imidazole-HCl buffer (pH 7.0), 30 mM L-glutamine, 3 mM $MnCl_2$, 0.4 mM ADP (pH 7.0), 20 mM sodium arsenate (ph 7.0), 60 mM NH_2OH (pH 7.0, freshly prepared), and appropriate amount of enzyme solution. The reaction was initiated by adding NH_2OH, and L-glutamine was omitted in the blank test. After incubatiuon at 30°C for 15 minutes, the γ-glutamyl hydroxamate formed was determined by adding 1.0 ml of ferric chloride reagent as described as above.

Determination of Protein

Protein concentrations were determined by various methods according to the stages of enzyme purification. In the crude extracts and ammonium sulfate preciptate fraction, protein concentration was determined by micro-biuret methods (Itzhaki and Gill, 1964). At the step of Sepharose 4B chromatography, protein was determined by the method of Lowry *et al.* (1951). At the steps of DEAE-Sephacel, 2′5′ADP-Sepharose 4B and hydroxyapatite chromatography, protein was quantitated by turbidimetric tannin micromethod (Mejbaum-Katzenellenbogen and Dobryszycka, 1959). Crystalline boving serum albumin was used as the standard.

Determination of Molecular Weight

The molecular weight of GSI and GSII was estimated by a calibrated Sephacryl S-300 column (2.6×70 cm) previously equilibrated with 0.05 M Tris-HCl buffer (pH 7.5) containing 2 mM 2-mercaptoethanol, 2 mM EDTA and 0.1 M NaCl. Aldolase (158,000), catalase (232,000), ferritin (440,000) and thyroglobulin (669,000) were used as the standards. Kav was calculated from the formular Kav = (Ve-Vo)/(Vt-Vo). Where Ve is the elution volume of each protein, Vo is the void volume and Vt is the total bed volume.

The subunit weight of GSI and GSII were estimated by SDS-polyacrylamide gel eletrophoresis according to Weber and Osborn (1969) on 12.5% polyacrylamide gel containing 0.1% SDS. α-lactalbumin (14,400), trypsin inhibitor (20,100), carbonic anhydrase (30,000), ovalbumin (43.000), albumin (67,000) and phosphorylase b (94,000) as standards.

Polyacrylamide Gel Electrophoresis

Disc-gel electrophoresis was performed on 5% acrylamide gels using Tris-glycine buffer (pH 8.3) with stacking gel according to Davis (1964). Protein bands on rod gels were stained with Coomassie Brilliant Blue R. Glutamine synthetase activity bands on the slab gel were visualized immediately after electrophoresis by bathing the gel in the tranferase assay mixture at 30°C for 1 h and subsequently placing

them in the ferric chloride-trichloroacetic acid solution. The red-brown color of the ferric hydroxamate developed where glutamine synthetase activity pressent.

Results

Variations of Two Forms of Glutamine Synthetase Activity in Rice Leaves during Growth

The total activity of glutamine synthetase in rice leaves varied with the growth stages of rice plants. Usually, glutamine synthetase showed the highest level of activity at the tillering stage, and decreased to a lower level at booting, then increased again at the flowering stage (Yuan, 1982). However, there are two

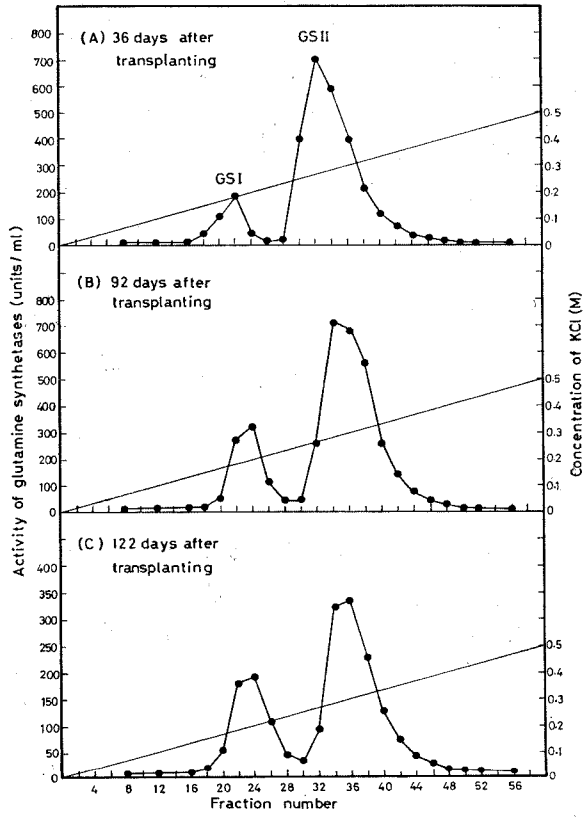

Fig. 1. Variations of GSI and GSII activity in rice leaves of Tainung 62 in the first crop season during growth. The elution patterns were from DEAE-Sephacel column, and 6.4 ml per fraction was collected. Enzyme activity was determined by biosynthetic reaction and expressed as nmole γ-glutamyl hydroxamate produced per minute.

forms of glutamine synthetase GSI and GSII in rice leaves. GSI and GSII in leaves of Tainung 62 were separated by DEAE-Sephacel column chromatography at the tillering (36 days), flowering (92 days), and ripening (122 days) stages in the the first crop season; and at the tillering (33 days) and booting (74 days) stages in the second crop season. The elution patterns are demonstrated in Fig. 1. The enzyme activities were determined and calculated as units per gram of fresh weight which is shown in Table 1. The results indicated that the activity of GSI in leaves maintained a rather constant level during rice growth, and increased slightly with the advancement of rice growth; but the activity of GSII decreased markedly with the advancement of rice growth. Obviously, variation of the total activity of glutamine synthetase is owing to the decrease of GSII activity at the later growth stage.

Table 1. *Variations of GSI and GSII activity in rice leaves during growth*

Crop season	Days after transplanting	GSI and GSII activity* (units/gm fresh weight)		Ratio of GSI/GSII
		GSI	GSII	
1st	36	59.61	383.61	6.44
	92	89.28	356.96	4.00
	122	86.25	193.14	2.24
2nd	33	67.94	253.15	3.73
	74	69.78	222.32	3.19

* Biosynthetic activity was expressed as nmole γ-glutamyl hydroxamate produced per minutes.

Purification of Glutamine Synthetases GSI and GSII from Rice Leaves

Typical results in purifying GSI and GSII are summarized in Table 2. GSI and GSII were separated from each other after DEAE-Sephacel column chromatography. The elution pattern is demonstrated in Fig. 2. GSI was eluted at KCl concentration between 0.15 to 0.2 M, and GSII was eluted between 0.25 to 3.0 M.

During 2′5′ADP-Sepharose 4B affinity chromatography, GSI was purified depend upon the property of its biosynthetic activity and GSII was depend upon the property of its fransferase activity. When ADP-Sepharose 4B column was equilibrated with 0.01 M Tris-HCl buffer (pH 7.5) containing 2 mM EDTA, 2 mM 2-mercaptoethanol, 5 mM $MgCl_2$, and 10 mM L-glutamate, and crude GSI was also dialyzed against the same buffer; then the GSI activity could bind to the column and eluted at ATP concentration between 0.6 to 1.5 mM. However, only small amount of GSII could bind to the column under this condition, more than 90% of GSII was in the unbound portion.

Table 2. *Purification of glutamine synthetases GSI and GSII from rice leaves*

Purification step	Total protein (mg)	Total activity (units)[a]	Specific activity (units/mg protein)	Recovery (%)	Purification fold
Crude extract[b]	1372.8	327,043	238.2	100	1
30-50% Ammonium sulfate precipitate	891.3	255,094	286.2	78	1.2
Sepharose 4B eluate	349.3	238,741	683.5	73	2.9
DEAE-Sephacel eluate					
GSI	36.1	32,714	906.2	10	3.8
GSII	90.3	170,062	1,883.3	52	7.9
ADP-Sepharose 4B eluate					
GSI	1.7	26,159	15,387.7	8	64.6
GSII	36.9	104,654	2,836.1	32	11.9
Hydroxyapatite eluate					
GSI	0.18	23,482	103,455.5	7	547.7
GSII	2.50	36,555	14,622.0	11	61.4

[a] Biosynthetic activity was expressed as nmole of Pi produced/min. One unit of enzyme was defined as the amount of enzyme catalyzing the formation of 1.0 nmole Pi/min at 30°C.

[b] Crude extract was prepared from 100 grams of frozen leaves of Tainung 62 at the booting stage.

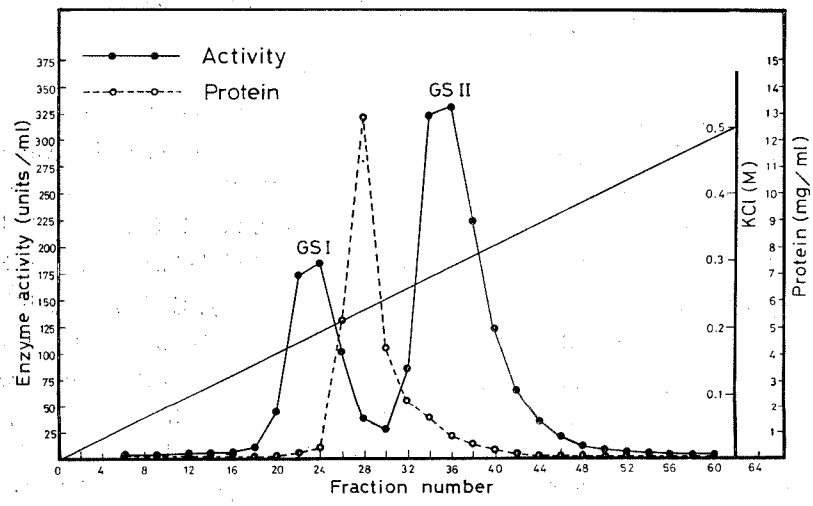

Fig. 2. Elution pattern of glutamine synthetase of rice leaves from DEAE-Sephacel column, and 6.4 ml per fraction was collected. Enzyme activity was determined by biosynthetic reaction and expressed as nmole γ-glutamyl hydroxamate produced per minute.

When ADP-Sepharose 4B column was equilibrated with 0.01 M imidazole-HCl buffer (pH 7.0) containing 2 mM 2-mercaptoethanol, 1 mM $MnCl_2$, and 10 mM L-glutamine, and crude GSII was also dialyzed against the same buffer; then the GSII activity could bind to the column and eluted at ADP concentration between 4.5 to 8.0 mM.

The purified GSI and GSII from hydroxyapatite column were subjected to electrophoresis in 5% polyacrylamide gels, the protein band coincided with the region of glutamine synthetase activity visualized by the transferase reactions. The purified GSI showed a single protein band. GSII revealed a major protein band with transferase activity, and two minor protein bands in which one marked with X showed a little transferase activity (Fig. 3). GSII migrated faster than GSI on 5% polyacrylamide gel electrophoresis at pH 8.3.

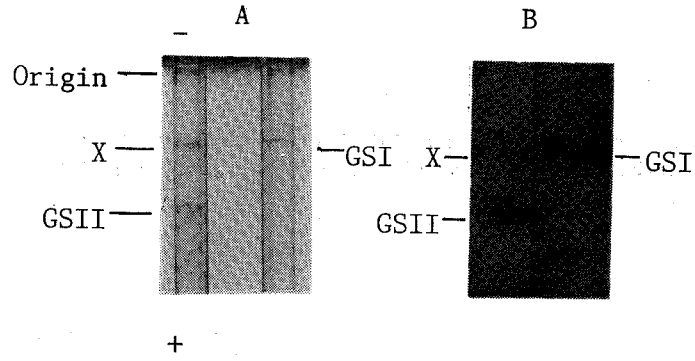

Fig. 3. Polyacrylamide gel electrophoresis of glutamine synthetases GSI and GSII from the hydroxyapatite column. Rod gels (A) were stained with 0.05% Coomassie Brilliant Blue R. The slab gel (B) was stained for glutamine synthetase activity. Activity stains were performed by incubating the gel in the γ-glutamyl transferase reaction mixture for 1 h at 30°C and then treated with ferric chloride-trichloroactic acid stopping solution to visualize the produced γ-glutamyl hydroxamate.

Affinity for Substrates

The relationship between reaction velocity and substrate concentration of GSI and GSII was determined. The substrate saturation curves and double reciprocal plots for NH_4Cl, L-glutamate and ATP were presented in Figs. 4, 5, and 6. The apparent Km value for each substrate was also estimated from reciprocal plots and listed in Table 3. Obviously, GSI showed greater affinity for substrates than GSII.

The Km values of GSI and GSII for NH_4Cl were found to be very low, however, the Km value of GSII was five fold higher than that of GSI. As to the

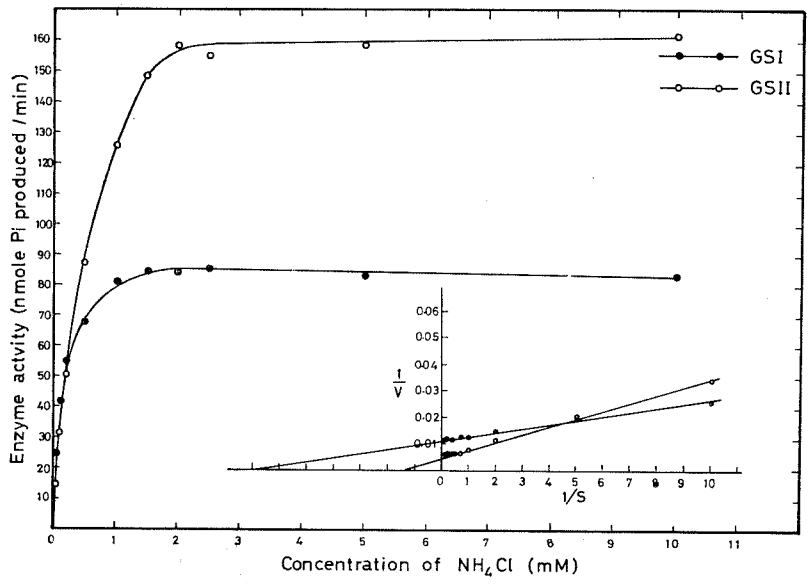

Fig. 4. Substrate saturation curves and double reciprocal plots (inset) with NH_4Cl as the variable substrate for glutamine synthetases GSI and GSII from rice leaves.

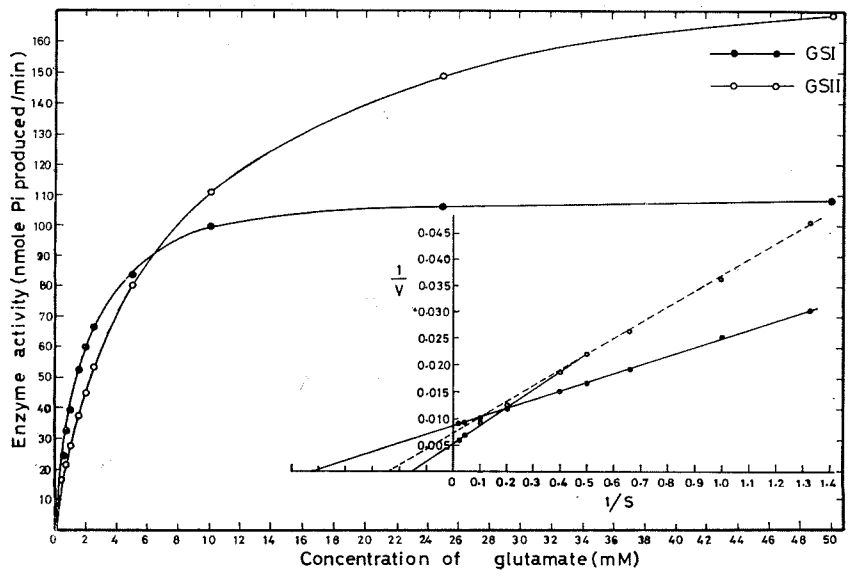

Fig. 5. Substrate saturation curves and double reciprocal plots (inset) with L-glutamate as the variable substrate for glutamine synthetases GSI and GSII from rice leaves.

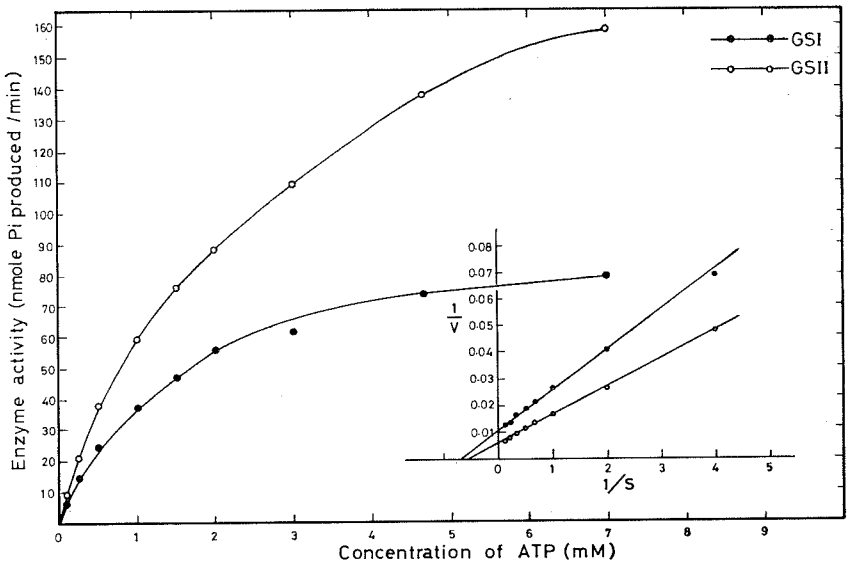

Fig. 6. Substrate saturation curves and double reciprocal plots (inset) with ATP as the variable substrate for glutamine synthetases GSI and GSII from rice leaves.

Table 3. *Affinity for substrates of glutamine synthetases from rice leaves*

Substrates	Km	
	GSI	GSII
	(mM)	(mM)
NH_4Cl	0.15	0.75
L-Glutamate	1.89	6.45(4.17)*
ATP	1.39	1.72

* GSII shows two apparent Km values of 6.45 mM and 4.17 mM at high and low glutamate concentration, respectively.

affinity for L-glutamate, GSI was found to exhibit normal Michaelis-Menten kinetics, while a non-linear reciprocal plot was obtained for GSII and two apparent Km values were obtained (Fig. 5). Both Km values of GSII were about two to three fold higher than that of GSI. The Km values of GSI and GSII for ATP were found to be quite similar although Km value of GSII was still higher than that of GSI.

pH Optimum

The effect of pH on the activity of GSI and GSII is shown in Fig. 7. Enzyme activity was determined by biosynthetic reaction and expressed as μmole γ-glutamyl

hydroxamate produced per 15 minutes. Assays were performed in 0.2 M sodium cacodylate-HCl buffer for pH range from 6.2 to 7.4 and in 0.2 M Tris-HCl buffer for pH range from 7.1 to 8.9. The results showed that GSI exhibited optimal activity around pH 7.1 and GSII had an optimal activity around pH 7.4.

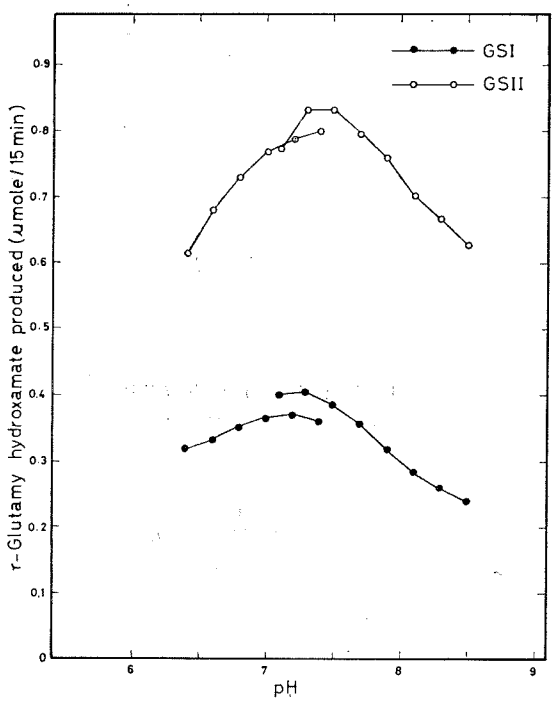

Fig. 7. Effect of pH on the activity of GSI and GSII. Enzyme activity was determined by biosynthetic reaction and expressed as μmole of γ-glutamyl hydroxamate produced in 15 minutes. Assay were performed in 0.2 M cacodylate buffer between pH 6.2 to 7.4 and in 0.2 M Tris-HCl buffer between pH 7.1 to 8.9.

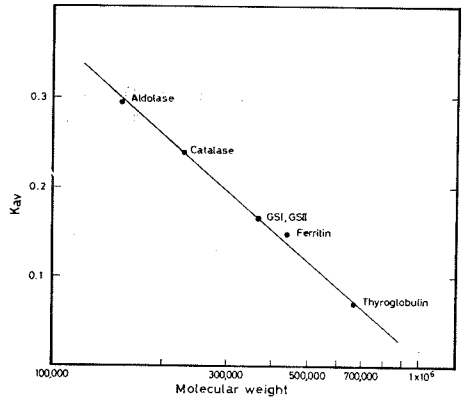

Fig. 8. Estimation of molecular weight of GSI and GSII by gel filtration on Sephacryl S-300 column. Column size: 2.6×70 cm. Elution buffer: 0.05 M Tris-HCl buffer (pH 7.5) containing 2 mM 2-mercaptoethanol, 2 mM EDTA and 0.1 M NaCl. Flow rate: 32 ml/h, 2 ml per fraction was collected.

Molecular Weight

The molecular weight of GSI and GSII were estimated by gel filtration on Sephacryl S-300 column. Both GSI and GSII appeared to have the similar molecular weight about 367,000 (Fig. 8). When two enzymes were subjected to SDS-polyacrylamide gel electrophoresis on a 12.5% polyacrylamide gel containing 0.1% SDS, only one subunit was identified with molecular weight of approximate 45,800 (Fig. 9).

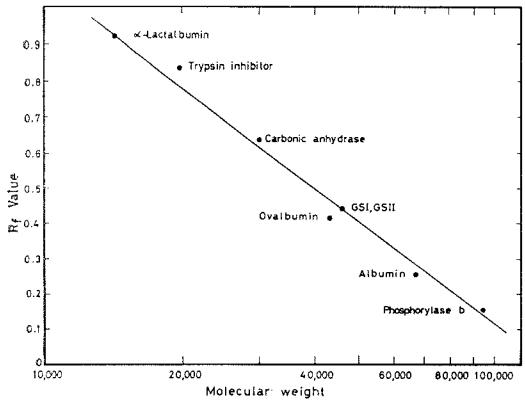

Fig. 9. Estimation of molecular weight of subunit of GSI and GSII by SDS-polyacrylamide gel electrophoresis on a 12.5% poly acrylamide gel containing 0.1% SDS.

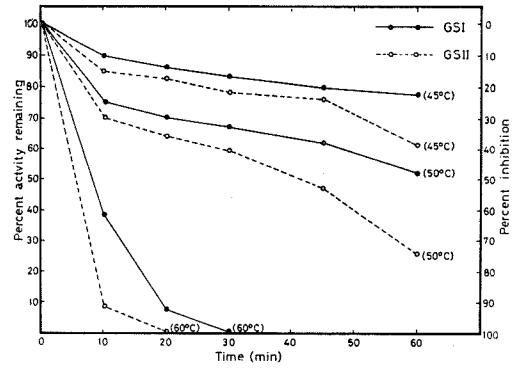

Fig 10. Thermal inactivation of GSI and GSII from rice leaves. Enzyme activity was determined by biosynthetic reaction based on release of inorganic phosphate.

Divalent Cation Effect

The effects of various divalent cations, including Mn^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Ca^{2+}, Zn^{2+} and Ni^{2+}, on the activity of GSI and GSII were studied. When GSI and GSII were preincubated with 25 mM of each divalent cation at 30°C for 10 minutes in 0.2 M Tris-HCl buffer (pH 7.5), and assayed for biosynthetic activity based on γ-glutamyl hydroxamate formation. The results are shown in Table 4. The relative activity of GSI decreased to 6.63%, 7.67%, 28.76%, 47.96%, 63.56%, and GSII decreased to 1.90%, 10.56%, 43.92%, 40.10%, 67.72% due to the effect of Cu^{2+}, Mn^{2+} Fe^{2+}, Zn^{2+} and Ca^{2+}, respectively. However, Ni^{2+} stimulated GSI activity slightly, and did not influence the GSII activity. Co^{2+} stimulated GSI activity up to 156.90% and also stimulated GSII activity up to 123.56%.

Thermal Stability

The effect of temperature on the activity of GSI and GSII is shown in Fig. 10. GSI and GSII were incubated for different time intervals at 45°C, 50°C and 60°C in 0.01 M Tris-HCl buffer (pH 7.5) containing 2 mM EDTA, 2 mM 2-mercaptoethanol and 5 mM $MgCl_2$. After incubation, the enzyme solution were kept in an ice bath, and was assayed for biosynthetic activity based on relase of inorganic phosphate. GSII was more heat-labile than GSI. When the two enzymes were incubated at 45°C and 50°C for 60 mimutes, the activity of GSI was inhibited about 20% and 50%, and GSII was inhibited about 40% and 75%, respectively. At 60°C, the activity of GSII was completely inhibited after 20 minutes of incubation, and the activity of GSI was also inhibited about 90%.

Table 4. *Effect of divalent cations on the activity of glutamine synthetases GSI and GSII from rice leaves*

Divalent cation[a] (25 mM)	Relative activity[b] (%)	
	GSI	GSII
None	100	100
Mn^{2+}	7.67± 0.69	10.56± 0.89
Co^{2+}	156.90± 3.89	1.90± 0.75
Cu^{2+}	6.63± 0.73	123.56±11.26
Fe^{2+}	28.76± 1.03	43.92± 2.52
Ca^{2+}	63.56± 2.48	67.72± 3.48
Zn^{2+}	47.96± 2.99	40.10± 4.65
Ni^{2+}	106.36± 3.89	99.86± 2.61

[a] Enzymes were preincubated in 25 mM of each divalent cation at 30°C for 10 minutes. $Mn^{2+}=MnCl_2$, $Co^{2+}=CoCl_2$, $Cu^{2+}=CuSO_4$, $Fe^{2+}=FeSO_4$, $Ca^{2+}=CaCl_2$, $Zn^{2+}=ZnSO_4$, $Ni^{2+}=NiCl_2$.

[b] Enzymes were assayed for biosynthetic activity based on γ-glutamyl hydroxamate formation in the presence of NH_2OH. Each value is the mean ±S. E. M. of triplicate determinations. 60 units of GSI and 70 units of GSII were used in each assay.

Discussion

The variations of total activity of glutamine synthetase in rice leaves had been surveyed at all stages of rice growth (Yuan, 1982). Usually, it showed the highest activity at the tillering stage, and decreased to a lower level at booting, then increased again at the flowering stage. In this study, two forms of glutamine synthetases GSI and GSII in the rice leaves were separated and their activities were measured at different stages in the two crop seasons (Table 1). The results indicated that the variations of total activity of glutamine synthetase during rice growth is due to decrease of the GSII activity and increase of GSI activity at the later growth stage. The decrease of GSII activity at the ripening stage might be due to senescence of rice leaves. At that time, the *de novo* synthesis of GSII in chloroplasts was slowdown or even ceased, and GSI was responsible for reassimilation of ammonia in the senescing leaves. The results suggest that GSI could play a central role in the photorespiratory nitrogen cycle (Keys *et al.*, 1978) at the ripening stage; and also play this central role in ammonia assimilation in cytosol in the dark; in fact, it might acconut for the light-independent formation of glutamine as described by Ito *et al.* (1978) that the first step of ammonia assimilation in cytosol catalyzed by GSI is independent on light. On the other hand, the increase of GSI activity and decrease of GSII activity in the later growth stage also support an independent phenomanon for the light-dependent balance between

the two forms of the enzyme (Hirel *et al.*, 1982).

The purification of glutamine synthetase from rice leaf extract by DEAE-Sephacel column chromatography showed two forms of glutamine synthetases GSI and GSII (Fig. 2). Our improved method in purifying GSI and GSII (Table 2) from rice leaves permited us to obtain an apparent homogenous GSI preparation judged by polyacrylamide gel electrophoresis. GSII preparation was nearly homogenous, although it was still contaminated with a small amount of impurities, more than 90% of protein is GSII. In Fig. 3, the minor protein band marked with X showing a little transferase activity might be the contamination of GSI or could be another form of glutamine synthetase. The two enzymes differ in net charge in higher pH. GSII electrophoretically migrated faster than GSI at pH 8.3.

According to the apparent Km values listed in Table 3, it showed that GSI had greater affinity for NH_4Cl, L-glutamate and ATP than GSII. The apparent Km value of GSI for L-glutamate was found to be 1.89 mM; whereas the activity curve of GSII was biphasic with Km values of 6.45 mM and 4.17 mM at high and low L-glutamate concentrations, respectively. Similar negative co-operativity had been found for GSII in leaves of the other rice variety (Guiz *et al.*, 1979), barley (Mann *et al.*, 1980) and halophytes (Ahmad *et al.*, 1982). The negative co-operativity with respect to L-glutamate exhibited by GSII implies that large changes in cellular L-glutamate concentration would have relatively little effect on the rate of glutamine synthesis. This characteristic might be physiologically significant in relation to the subcellular localization of glutamine synthetase. If as seems likely, GSII is chloroplastic, a mechanism for modulating the possible effects of rapid changes of L-glutamine concentration in chloroplasts could be important with respect to the operation of glutamate synthase cycle.

In the presence of Mg^{2+}, the pH optima for GSI and GSII were found to be pH 7.1 and pH 7.4, respectively (Fig. 7). It was the same as that found in barley (Mann *et al.*, 1979) and pumpkin leaves (Kretovich *et al.*, 1981). Compared to GSI, GSII seemed to be slightly shifted towards a more alkaline pH. Werdan *et al.* (1975). reported that illumination of chloroplasts causes a decrease of the pH in the thylakoid space by 1.5 and an increase of the pH in the stroma by almost 1 pH units. Therefore, it is reasonable for GSI to function in cytosol in the dark, and for GSII to function in chloroplast in the light. Furthermore, GSII was more heat-labile compared to GSI.

GSI and GSII have the similar native molecular weight of about 367,000, and both enzymes consist of identical monomers with same molecular weight of about 45,800. Therefore, both GSI and GSII are composed of eight identical subunits. This is quite similar to the glutamine synthetase isolated from soybean hypocotyl (Stasiewicz and Dunham, 1979) and the other rice variety (Hirel and Gadal, 1980).

It had been found that Mn^{2+} and Ca^{2+} inhibited Mg^{2+}-dependent activity of the

glutamine synthetase from most organism (Tate *et al.*, 1972). Part of the inhibition by Mn^{2+} and Ca^{2+} might be due to the shift in pH optimum (O'Neal and Joy, 1974). On the other hand, it is possible that Mn^{2+}, Ca^{2+} and perhaps Zn^{2+} are competing with Mg^{2+}. These divalent cations may have binding sites distinct or partially distinct from the Mg^{2+} binding site. Segal and Stadtman (1972) found that *E. coli* enzyme exists in a distinct conformational state with each of the four cations (Mg^{2+}, Co^{2+}, Cd^{2+} and Ca^{2+}), and different cations caused different kinetic properties. In the present study, both activities of GSI and GSII were strongly inhibited by Cu^{2+} and Mn^{2+}. Moderate inhibition was observed by Fe^{2+}, Zn^{2+} and Ca^{2+}. On the contrary, Co^{2+} stimulated the activities of GSI and GSII markedly. There is no much difference regarding to divalent cation effect in the two enzymes, which implies that the conformational states of the two enzyme in the presence of each cation might be similar. The precise functions and regulatory properties of the two glutamine synthetases remain to be further investigated.

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水稻葉片中兩種型態的麩醯胺合成酶特性之比較

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使用 DEAE-Sephacel 離子交換層析法，自不同生長時期之水稻葉片中，分離出 GSI 及 GSII 兩種型態的麩醯胺合成酶。GSI 在水稻整個生長期間，能保持較爲平穩的活性强度，但 GSII 之活性强度自分蘖期後即顯著下降。使用 2′5′ADP-Sepharose 4B 親和力層析法以及 Hydroxyapatite 吸法着，GSI 能純化到均一的程度，GSII 亦能純化到接近均一的程度。以熱處理時，與 GSI 相較，則 GSI 之活性較爲不穩定。於 5％之聚丙烯醯胺膠體中，在 pH 8.3 之電解液中進行電泳分析時，則 GSII 之移動速度較 GSI 爲快。對於作用基質之親和力方面，GSI 對於氯化銨，麩胺酸及 ATP 之 Km 值，分別爲 0.15 mM，1.89 mM 1.39 mM。GSII 對於氯化銨及 ATP 之 Km 值，分別爲 0.75 mM 及 1.72 mM；可是 GSII 對於麩胺酸之 Km 值，則出現雙段式的兩個 Km 值，在麩胺酸之高濃度及低濃度時，其值分別爲 6.45 mM 及 4.17 mM。於 Mg^{2+} 存在下，GSI 及 GSII 之活性之最適 pH 值分別爲 7.1 及 7.4。GSI 及 GSII 之分子量相同，皆爲 367,000；兩者具有相同的單分子，且單分子之分子量皆爲 45,800。於 Mg^{2+} 存在下，Mn^{2+} 及 Cu^{2+} 皆能强力的抑制 GSI 及 GSII 之活性。Fe^{2+}, Ca^{2+} 及 Zn^{2+} 對於 GSI 及 GSII 之活性亦皆有相當强的抑制作用。但 Co^{2+} 則顯著地增强 GSI 及 GSII 之活性。