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Purification and Properties of Glutamine Synthetase from Germinating Castor Bean Endosperm

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Abstract

The activity of glutamine synthetase in germinating castor bean endosperm resides in the cytosol. The activity was found to increase rapidly the 3rd to 4th day of germanating in the dark and to be a peak at 5th day. The enzyme was purified from 5-day-old endosperm approximately 100-fold to apparent homogeneity. Electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate yeilded a single band with a molecular weight of 55,000, suggesting that subunits of GS have a identical molecular weight. The Km is 4.9 mM for L-glutamate, 0.65 mM for NH_2OH, 0.92 mM for NH_4Cl (biosynthetic assay), 34.1 mM for L-glutamine and 6.28 mM for NH_2OH (transferase assay). ATP acts as a sigmoidal substrate with a "$S_{0.5}$" of 1.4 mM. Inhibition of the enzyme is observed with AMP, ADP, CTP, GTP, ITP, L-glycine, L-histidine, citrate, carbamyl phsphate, and glucoseamine-6-phosphate. The energe charge significantly affected the activity of GS.

Introduction

In castor bean seedling, the reserve stored in the endosperms is enzymatically broken down to produce energy as well as carbon skeltons during the course of germinaton and evetually utilized for the growth of young plant tissues[1]. It is known that in castor bean glutamine serves as a carrier of nitrogen and during germination about 45% of the total nitrogen in the endosperm of castor bean seedling is transported to the embryonic axis in the form of glutamine[2]. A large increase of glutamine content in the cotyledons during germination suggests that the glutamine be newly formed in the endosperm, in addition to that produced from hydrolysis of reserve protein, and it is conceivable that glutamine synthetase (L-glutamate ammonia ligase (ADP), EC 6. 3. 1. 2) plays an important role of synthesizing glutamine from the storage proteins.

GS is an enzyme of singular importance in metabolism since it links the myriad catabolic processes by which ammonia and α-ketoglutarate are produced with diverse biosynthetic processes that lead ultimately to the formation of protein, nucleic acid, complex polysacchrides, and some vitamins[3]. Purification subunit structure, and kinetic and physicochemical properties of GS have been investigated in various bacteria[4,5], cyanobacteria[6], algae[7], and mammals[8]. In higher plants as well as in many other organisms, an increasing number of studies have been made on the identification, characterization and subcellular localization of GS in the leaves and roots of higher plants[3, 9-12]. Various GS have recently been studied at molecular level by genetic techniques[13, 14].

In this report, we describe a purification procedure for glutamine synthetase from germinating castor bean endosperm and some properties of the purified enzyme.

Materials and Methods

Materials

Castor bean seeds (*Ricinus communis*, harvested in Thailand) were supplied by Ito Seiu Co. and germinated as described previously[15]. Standard proteins for the estimation of molecular weight were obtained from Boehringer/Mannheim. DEAE-cellulose and Sephrose 6B were purchased from Pharmacia Fine Chemicals. Other chemicals were commercial preparations of the highest purity available.

Differential centrifugation

The homogenates of germinating castor bean endosperm were prepared as discribed previously[15, 16]. The homogenates was centrifuged at 800×g for 15 min. The supernatant was again centrifuged at 10,000×g for 30 min. The pellet was carefully washed by being resuspended in the homogenizing buffer, then was recentrifuged. The combined preparation of the supernatant and washings and the pellet are hereafter referred to as the 10,000× g supernatant and the pellet, respectively.

Enzyme assay

GS was assayed by three methods: (a) biosynthetic assay which involves measuring the release of phosphate from ATP in the presence of glutamate, NH_4Cl, and $MgCl_2$[17], (b) biosynthetic assay which involves measuring the production of γ-glutamyl hydroxamate from glutamate in the presence of ATP, NH_2OH and $MgCl_2$[17], (c) transferase essay which involves measuring the presence of ADP, NH_2OH, $MnCl_2$, and arsenate[17]. The assay methods for marker enzymes were described in the previous report[18].

Determination of protein

Protein contents were estimated according to the method of Lowry et al. [19] with bovine serum albumin as the standard. Protein in column eluates was monitored by absorbance at 280 nm.

Electron microscopy

After the purified enzyme was dialyzed against 10 mM imidazole-HCl buffer, pH 7.8, containing 2.5 mM $MgCl_2$, and the droplets of the enzyme were placed on carbon-coated collodion membranes supported on 200-mesh copper grids. The molecules were stained in a 1% aqueous solution of potassium phosphotungstate, pH 7.0. Photographs were taken with a Hitachi H-300 electron microscorpe at an initial magnification of 50,000, using 70 Kv.

Gel electrophsresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was used to estimate the subunit molecular weight of the enzyme according to the procedure of Weber and Osborn[20].

Table 1 Subcellular distribution of GS in germinating caster bean endosperm by differential centrifugation

Enzyme	Total cativty (%)		
	Crude extract	Supermatant	Particulate
Fumarase	7.25	0.88(12)	6.05(83)
Catalase	304	87 (29)	253 (83)
Phosphoenolpyruvate carboxykinase	7.88	7.45(95)	0.23 (3)
Gultamine synthetase	1.27	1.25(98)	0.03 (2)

Enzyme assays are described in Matrials and Methods. The value in parenthesis shows the enzyme activity recovered in the supernatant or particulate fraction as a percent of the crude extract.

Results and discussion

Changes in the activity of GS in germinating castor bean endosperm.

When seeds of castor bean were germinated the activity of GS in the endosperm increased rapidly between the 3rd and 4th day of germination and declined from the 6th days. This change of activity may reflect the change of transport capacity of nitrogen transfer from endosperm to embryo.

Subcellular distribution of GS

The results of differential centrifugation of organelle suspension obtained from germinating castor bean endosperm are shown in Table 1. About 98% of GS activity in the homogenate was recovered in the supernatant fraction, indicating that the enzyme is localized in the cytosol.

GS have been found as isoenzymic forms in bacteria[21], *Neurospora*[22], and higher plants[23]. In pumpkin, the cytosolic enzyme has been found in the dark-grown cotyledons, whereas two isoenzymes of the enzyme, cytosolic and chloroplastic GS, have been found in the green cotyledons[24].

Purification of GS

All procedures were carried out at 1-5°C. Crude extract was obteined from 5-day-old castor bean endosperm as described previously[15,16]. Curde extract was fractionated with ammonium sulfate. The precipitate between 30 and 40% saturation of ammonium sulfate was dissolved in a small volume of Tris-HCl buffer, pH 7.9, (buffer A) and dialyzed against the same buffer overnight at 4°C.

The dialysate was centrifugated and the supernatant was applied to a DEAE-cellulose column equilibrated with the buffer A. The elution was carried out with a linear gradient of sodium chloride (0-1.0N) in the same buffer. The active enzyme fraction was pooled and concentrated with ultrafiltration (Ultrafilter UK-200). Concentrated enzyme was dialyzed against Tris-HCl buffer, pH 8.5, (buffer B) overnight at 4°C.

The enzyme solution was reapplied to a DEAE-cellulose column equilibrated previously with buffer B and eluted with a linear gradient of sodium chloride (0.05-0.3N) in buffer B. Active fraction was pooled and concentrated with ultrafiltration, and then chromatographed on a sepharose 6B column equlibrated with Tris-HCl buffer, pH 7.9.

Table 2 Purification of GS from germinating castor bean endosperm

Fraction	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Recovery (%)	Fold
Homogenate	646	7630	0.085	100	1.0
$(NH_4)_2SO_4$ 30-60% sat.	531	2750	0.193	82.2	2.3
DEAE-Cellulose Chromatgraphy	406	360	1.13	62.8	13.3
DEAE-Cellulose Rechromatography	391	230	1.70	60.5	20.1
Spepharose 6B Gel filtration	233	28	8.29	36.1	98.0

The active fraction was pooled, concentrated and used for the characterization of the enzyme.

The results of the purification are summmarized in Table 2. At the final stage of GS was 8.29 units/mg protein, representing 98-fold purification.

Properties of GS

Molecular weight of subunits: As estimated from its mobility with respect to protein standards, the single band observed upon electrophoresis in polyacrylamide gels containing SDS had a molecular weight of about 55,000. This result suggests that all subunits of native enzyme may be the same. An electron micrograph of GS shows the enzyme molecules in their characteristic 'H' shape. Hence, this enzyme, like the one from *Curcubita pepo*, *Glycine max*, *Pisum sativum*[3], sheep spleen[25], rat and Chinese hamster[8], appears to have the same arrangement of eight subunits in the molecule.

Effect of temperature: The temperature optimum the biosynthetic activity was 45°C. The energy of activation in the temperature range of 20–50°C was 13.6 Kcal/mol.

Thermostability of the enzyme was investigated by incubating the enzyme in cacodylate-HCl buffer for 30 min at the temperature, and then the residual biosynthetic activity was assayed at 35°C under standard conditions. GS were vety heat labile was stable at temperature up to 35°C, but above 35°C gradual inactivation and at 45°C after 30 min almost complete denaturation occurred.

Nucleoside triphosphste specificity: The specificty of GS reaction with Mg^{2+} was examined various nucleoside triphosphates. The maximal rate of reaction was obtained with ATP, whereas GTP had about 28% activity of the maximum. ITP and CTP were poor substrats in the catalysis of GS.

Effect of magnesium and manganese ions: The enzyme require a divalent cation for its activity. GS shows a marked specificity for Mg^{2+} in the biosynthetic and Mn^{2+} in the transferase assay. The enzyme is activated by either Mg^{2+} or Mn^{2+}, but not at all by Ca^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, and Ba^{2+}. Fig. 1 shows that the maximal enzyme activity obtained with Mg^{2+} is at least 1.5 times as much as that with Mn^{2+}, that the optimal concentrations for Mg^{2+} and Mn^{2+} are 14 and 6 mM, respectively, and that at higher concentrations, particularly of Mn^{2+}, inhibition of the enzyme activity occurs.

GS activity was determined over a wide range of Mg^{2+} concentrations at several

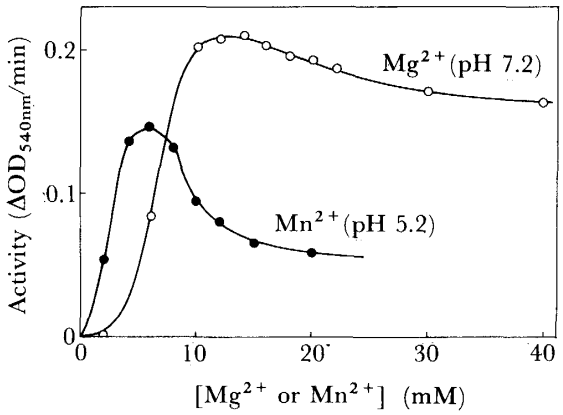

Fig. 1 Effect of Mg^{2+} and Mn^{2+} on the biosynthetic activity of GS. The reaction mixture contained 10 mM ATP, 50 mM L-glutamate, 6 mM NH_2OH, and various concentration of $MgCl_2$(○) or $MnCl_2$(●).

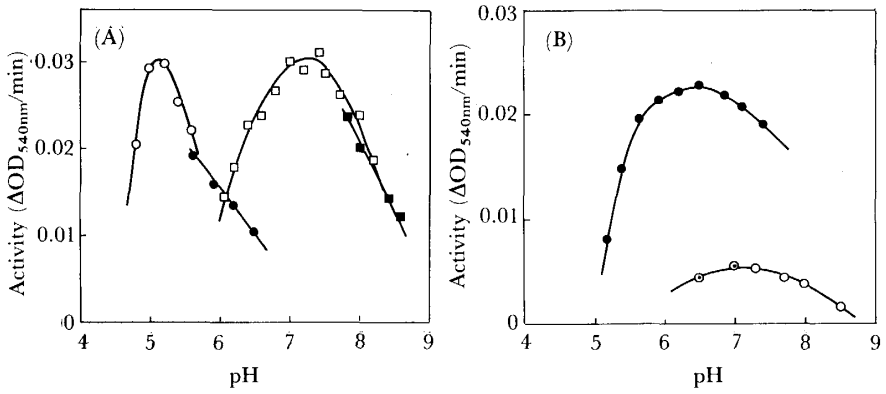

Fig. 2 Effect of pH on the activity of GS with either Mg^{2+} or Mn^{2+}. (A) The reaction mixture contained 50 mM L-glutamate, 10 mM ATP, 6 mM NH_2OH, 15 mM $MgCl_2$ or 10 mM $MnCl_2$, and 60 mM buffer. (○), acetate buffer with Mn^{2+}; (●), cacodylate buffer with Mn^{2+}; (□), HEPES buffer with Mg^{2+}; (■), Tris-HCl buffer with Mg^{2+}. (B) The reaction mixture contained 50 mM L-glutamate, 0.4 mM ADP, 60 mM NH_2OH, 10 mM sodium arsenate, 0.2 mM $MnCl_2$ or 5 mM $MgCl_2$, and 60 mM buffer. (●), cacodylate buffer with Mn^{2+}; (⊙), cacodylate buffer with Mg^{2+}; (○) Tris-HCl buffer with Mg^{2+}.

different ATP concentrations. When Mg^{2+} concentration was varied there was a definite sigmoidal response. As the ATP concentration is increased the response is increasingly sigmoidal and higher concentrations of Mg^{2+} are needed to produce maximal activity. This may indicate that either free ATP is inhibitory or that free divalent cations are necessary to maintain full activity.

Effect of pH: The pH courve for the reaction using eigher Mg^{2+} or Mn^{2+} as divalent cation is shown in Fig. 2. The reaction displayed a broad optimum in the pH. The pH optima with Mg^{2+} and Mn^{2+} were about 7.0 and 6.5, respectively. This value is close to that of GS from other plants[3, 9].

Effect of substrate concentration: The effects of substrate concentrations on the catalytic rate of GS were determined at optimum pH. Km for substrates were determined

Table 3 Km Value for substrates of GS

Reaction	Co-factor	Substrates	Km(mM)
Biosynthetic	Mg^{2+}	L-Glutamate	4.9
	Mg^{2+}	NH_2OH	0.65
	Mg^{2+}	NH_4Cl	0.92
	Mg^{2+}	ATP	1.4*
	Mn^{2+}	ATP	2.1*
Transferase	Mn^{2+}	L-Glutamate	34.1
	Mn^{2+}	NH_2OH	6.3

*$S_{0.5}$ value

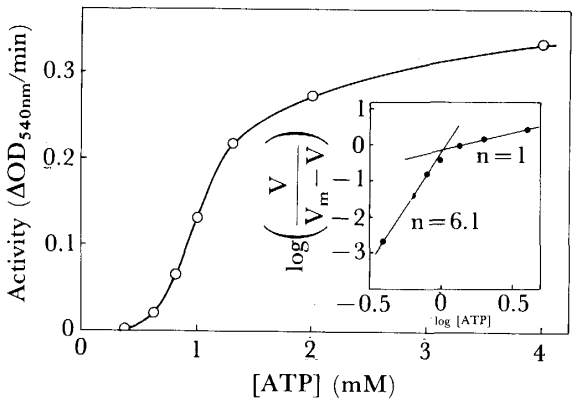

Fig. 3 Substrate saturation curve of GS with ATP as the variable substrate. The insert shows the Hill plots. The reaction mixture contained 50 mM L-glutamate, 6 mM NH_2OH, 15 mM $MgCl_2$, and various concentration of ATP.

by Lineweaver and Burk plots (Table 3). Since the reaction is a multisubstrate one, the Km values determined for the various substrates are only apparent. The enzyme exhibited nonhyperbolic saturation curves for ATP and the $S_{0.5}$ for ATP was 1.4 mM (Fig. 3). All the Km values obtained were within the range of the values reported for GS other plants[3,9].

Effect of SH-blocking reagents: The SH-blocking reagents such as p-CMB and $HgCl_2$ inhibited the GS activity, and 2-mercaptoethanol completely protected this inhibition. Thus, it seems possibly that SH-groups are important for the activity and retention of conformation of the enzyme molecule.

Effect of various Compounds: The effects of several metabolites on biosynthetic activity of GS were estimated. As shown in Table 4, the activities were inhibited by citrate, isocitrate, oxalacetate, glyoxylate, carbamyl phosphate and glucosamine-6-phosphate. Of the many amino acids tested, only glycine, histidene and arginine showed some inhibition. Nucleoties have also been shown to inhibite GS activities, notably ITP, GTP, CTP, and ADP inhibit the enzyme. AMP and IDP were partial inhibitors of the activity. The effect of citrate on GS was further investigated. As shown in Fig. 4, the elevated concentrations of Mg^{2+} reduced the inhibition bycitrate. It has been suggested that the inhibition by citrate may result from a decrease in Mg^{2+} oncentration required for activity by chilation with this citrate of Mg^{2+}. Many studied on inhibition of plant GS enzymes have been reported[3, 9]. In most cases some effects of amino acids noted, however, the amount of

Table 4 Effect of various metabolites on GS activity. Metabolites were added to the assy mixture in the concentrations indicated.

Metabolite (mM)	Relative (%)	Metabolite (mM)	Relative (%)
None	100	Glycine (4)	77
Citrate (20)	31	Histidine (4)	73
Isocitrate (20)	83	Serine (4)	85
α-Ketoglutarate (20)	82	Asparatate (4)	93
Oxaloacetate (20)	82	Ornitine (4)	94
Succinate (10)	95	ADP (5)	41
Pyruvate (10)	98	AMP (5)	76
Glyoxylate (10)	81	ITP (5)	18
Malate (5)	92	IDP (5)	83
Carbamyl-P (5)	71	GTP (5)	33
Glucosamine-6-P (5)	81	CTP (5)	19

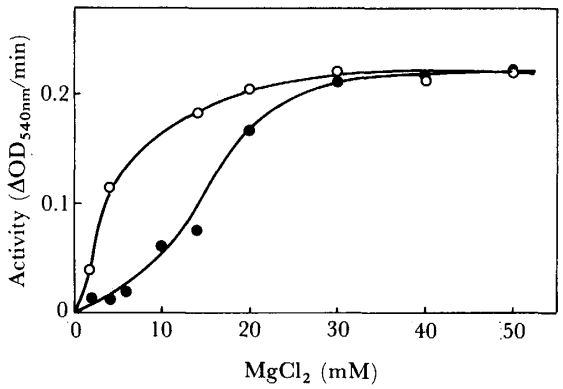

Fig. 4 Effect of Mg^{2+} concentration on the citrate inhibition of biosynthetic activity. (○), no citrate; (●), 10 mM citrate.

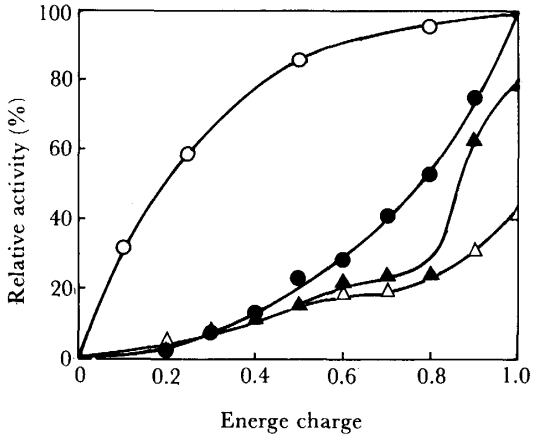

Fig. 5 Effect to energy charge on the biosynthetic activity of GS. The desired energy charge was obtained using myokinase by the method of Klugsoyr et al[29]. The reaction mixture contained 50 mM L-glutamate, 5 mM NH_4Cl, 15 mM $MgCl_2$, 16 units myokinase, and 60 mM Tris-HCl buffer at pH 7.2. The total adenylate concentration in each reaction mixture was maintained at 10 mM. (●), AMP+ATP; (○), ATP alone; (★), AMP+ATP+20 mM glycine; (▲), AMP+ATP+5 mM histidine.

inhibition has generally been less than complete and the concentrations of amino acids required have been large.

The activity of GS from several plant sources has also been shown to be regulated by energy charge of the system[26, 27]. Since ADP and AMP were inhibitory, it was expected that castor bean GS would be significantly affected by the energy charge[28]. Energy charge controls were futher modified in the presence of glycine and histidine. The desired energy charge was obtained using myokinase by the method of Klugsoyr et al[29]. As shown in Fig. 5, a marked variation in inhibition by histidine occurs in the region of energy change value of 0.80-1.0. The enzyme is considerably more sensitive to histidine control in the presumed physiological range of energy chage than under experimental condition (Fig. 6; 5 mM histidine caused about 80% inhibition at an energy charge of 0.8, but only about 20% at a charge of 1.0).

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