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Purification and Properties of Glutamate Synthase from Germinating Castor Bean Cotyledons

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Abstract

The activity of glutamate synthase in germinating castor bean cotyledon was found to increase rapidly from the 3rd to 4th day of germination. The enzyme was purified about 220-fold by ammonium sulfate fractionation and chromatographies with DEAE-Sephadex, Sephadex G-200, Affi-Gel Blue and Sephadex G-150. The purified enzyme can accept electrons from NADH for the enzyme reaction, but it cannot use NADPH. The enzyme has a molecular weight of 76,000. The optimum pH was 8.5. The enzyme showed normal Michaelis-Menten kinetics for all substrates. Apparent K_m values for *L*-glutamine, 2-oxoglutarate and NADH were 30mM, 330μM and 8μM respectively. The enzyme activities were inhibited by L-glutamate, ATP, GTP, pyrophsphate, citrate and isocitrate. Only phosphate and aspartate enhanced the enzyme activity. Sulfhydryl reagents was strong inhibitor.

Introduction

Glutamate synthase (glutamine: 2-oxoglutarate aminotransferase: NADH oxidizing, GOGAT) is the enzyme first found in bacteria which catalyzes the second step of ammonia assimilation into glutamate *via* glutamine[1]. Subsequent studies have shown that ammonia assimilation into glutamate occurs by those two enzymatc steps rather than by glutamate dehydrogenase in bacteria[2,3], bule green algae[4,5], green algae[6], and higher plant cell[7,8,9]. In higher plants, GOGAT occurs in two forms, each of required a specific electron donor for the reaction: one utilized the reduced form of ferredoxine (Fd-GOGAT, EC 1. 4. 7. 1.) and other utilizes NADH (NADH-GOGAT, EC 1. 4. 1. 14) as reductant. NADH-liked enzyme has been found in a range of nongreen tissues, as sucu as roots[9], etiolated seedling[10] and cell cultures[11]. Pyridine nucleotide (NADH, NADPH)-dependent GOGAT and ferredoxin-dependent GOGAT, the latter is present only in eukaryotic cell, have a molecular stracture and kinetic properties which differ according to the source of enzyme.

During germination of the castor bean, the storage reserves of the endosperm are hydrolyzed and the metabolic products are transferred *via* the cotyledons to the growing embryo[12]. In germinating castor bean endosperm, the glutamine have been newly formed by glutamine synthetase, in addition to produced from degradation of the reserve protein[13]. Exudate collected from the cut hypocotyl of 4-day seedling contained 120 milimolar soluble amino nitrogen and glutamine was the predominant amino acid present, comprising 35 to 40% of the total amino nitrogen[14,15]. In germinating castor bean, sucrose and glutamine are transpoted from emdosperm to axis as carbon and nitrogen carriers, respectively[12]. The cotyledons are highly effective absorptive organ and

glutamine transported is readily transfer to the amino group of other amino acids depends on the presence of transaminases in the cotyledons and .the growing embryo[14].

We now report the purification and some properties of the NADH-dependent GOGAT (EC 1. 4. 1. 14, *L*-glutamine: NAD^+ oxidoreductase (transaminating)) from germinating castor bean cotuledons. The reaction catalyed is:

L-Glutamine+2-oxogulutarate+NADH+$H^+ \rightarrow$ 2 *L*-glutamate+NAD^+

Materials and Methods

Materials

Castor bean seeds (*Ricinus comminis*, harvested in Thailand) were supplied by Ito Seiyu Co. and germinated as described previously[16]. Ultrafilter UK-50 were purchased from Advantec Toyo Co. Standard proteins for the estimatation of molecular weight were obtained from Boehringer/Mannhaim. Affi-Gel Blue were purchased from Bio Rad laboratries. Other chemicals were commercial preparations of the highest purity available.

Enzyme assay

GOGAT was assayed spectrophotometrically by following the oxidation of NADH at 340 nm. The reaction mixture containd 100mM Tris-HCl (pH 8.5), 25mM glutamine, 5mM 2-oxoglutarate 0.15mM NADH, and an aliquot of the enzyme preparation.

Protein Detemination

Protein contents were estimated according to the method of Lowry et al. with bovine serum albumin as the standard[17].

Results and Discussion

Changes in the activity of GOGAT in germinating castor bean cotyledon

When seeds of castor bean were germinated the activity of GOGAT in the cotyledon increased rapidly from 3rd day of germination, reaching a peak after 6-7days, and then declined (Fig. 1). This change of activity may be refrect the change of transport capacity of nitrogen transfer from endosperm to embryo[13].

Purification of GOGAT

All procedures were carried out at 1-5°C. Germinating castor bean cotyledons of 7-day-old were harvested, and frozen in a mortar with liquid N_2. The frozen pieces were ground in chilled mortars into a fine powder; additional liquid N_2 was added, if needed, to prevent throwing. The powder was added 3 volume of extraction buffer, 100mM Tris-HCl buffer (pH 8.0) containing 5mM β-mercaptoethanol. The suspension was centrifuged at 12,000 rpm for 30 min.

The supernatant was fractionationed with ammonium sulfate. The precipitate between 35 and 55 % saturation of ammonium salfate was dissolved in a 10mM Tris-HCl buffer pH 8.0, containing 5mM β-mercaptoethanol (buffer A) and dialyzed against the same buffer overnight.

The dialysate was centrifuged at 10,000 rpm for 20 min. The supernatant was applied to a DEAE-Sephadex column equilibrated with buffer A. The elution was carried out with a linear gradient of potassium chloride (0-0.2N) in the same buffer. The

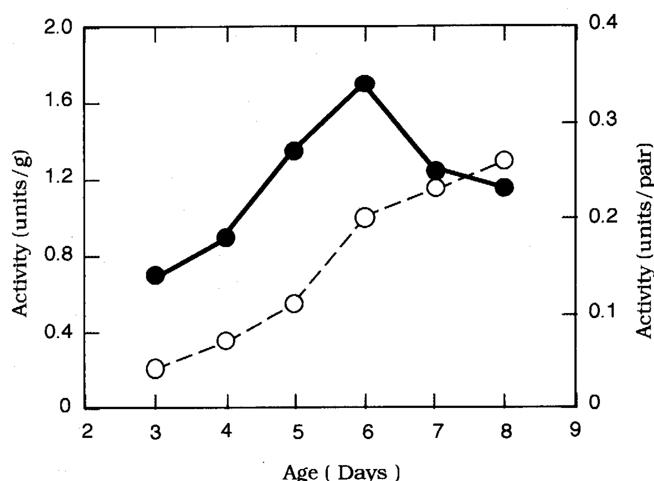

Fig. 1 Change in the Activity of GOGAT in germinaing costor bean cotyledons.
● : Units/g. ○ : Units/Pair.

Table 1 Purification of GOGAT from castor bean cotyledons

Fraction	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Recovery (%)	Fold
Crude extract	1660	9.2	0.006	100	1
$(NH_4)_2$-SO_4 ppt.	590	8.7	0.01	82	2.3
DEAE-Sephadex	91	5.2	0.057	54	9.6
Sephadex G-200	29	4.7	0.16	48	27
Affi-Gel Blue	2.4	1.9	0.8	20	133
Sephdex G-150	0.9	1.2	1.29	12	220

active enzyme fraction was pooled and concentrated with ultrafiltration (Ultrafilter UK 50), and then chromatographed on a Sephadex G-200 column eqilibrated with 50mM Tris-HCl buffer (pH 8.0), containing 5mM β-mercaptoethanol. Actve fraction was pooled and concentrated with ultrafiltration.

The concentrated enzyme was dialyed against buffer A. The active fraction was applied to a Affi-Gel Blue column equilibrated with buffer A. The active fraction was concentrated and Gel filtration step with Sephadex G-150 was repeated. The active fraction was pooled, concentrated and used for the characterization of the enzyme.

The results of the purification are summarized in Table 1. At the final stage of purification , the specific activity of GOGAT was 1.29 units/mg protein, representing 220-fold purification.

Properties of GOGAT

Molecular weight: The molecular weight of the enzyme was estimated by gel filtration on a Sephadex G-200 column calibrated with following globular protein standards: Ferritin (450,000), catalase (240,000), bovin serum albumin (68,000), hen egg albumin (45, 000), chymotorypsinogen A (25,000) and cytochrome C (12,500). The enzyme has a molecular weight of about 76,000. This value is lower than that reported for the enzyme from other higher plant[7,11,18]. It appears that the emzyme from castor bean cotyledons is composed of a single polypeptide chain, as is the enzyme from lupin nodules cytosol[19], alfalfa[9], and cultured rice cells[18]. In contrast, the bacterial enzyme have been reported

to behave as a dimer or tetramer of active spacies[2,7,20].

Effect of pH: The effect of pH on the catalytic activity was studied inthe phosphate and Tris-HCl buffer in the range of 6.0-9.2 (Fig. 2). The optimum pH of the enzyme was 8.5. This value is comparable to that of GOGAT from Lupin nodules cytosol[19], whereas much more lower values (between 7.5 and 7.9) are reported for the enzyme from other higher plants[7,11]. pH optima in the range 7.4-7.8 have been reported for the bacterial emzymes[7]. Ferredoxin-dependent enzymes have been a pH optimum at 6.9-7.5[7].

Substrate specificity: Substrate specifity of the enzyme was determined by assaying the enzyme in the presence of its natural substrates and then in the presence of an analogue of one of them and keeping the other two the same (Table 2). When *L*-glutamate as amino donor was replaced by ammonium chloride, NADH-oxidation was not observed. *L*-asparagine effectively substitutes glutamine as amino donor. Specificity for the amino acceptor is indicated by the inability of pyruvate to substitute for 2-oxoglutarate. Oxaloacetate was also tested but its activity in the glutamate stnthase system could not be evaluated due to the presence of a very active malate dehydrogenase, which caused an almost instantaneous oxidation of the NADH present. Effort to substitute NADPH for NADH in the reaction mixture failed to give any activity. In many bacterial species, GOGAT have been shown to be NADPH-dependent, and are inactive with NADH[7].

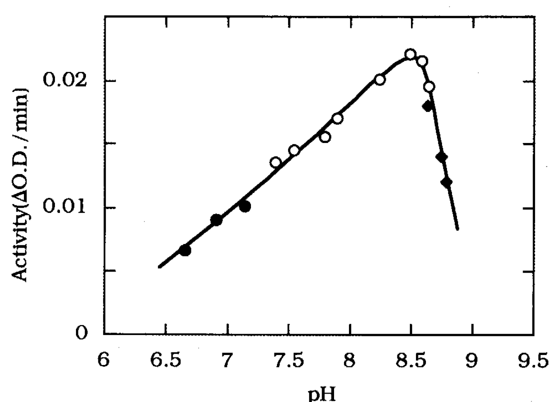

Fig. 2 Effect of pH on GOGAT Activity.
● : 0.1M Phosphate buffer
○ : 0.1M Tris-HCl buffer
◆ : 0.1M Pyrophsphate-HCl buffer

Table 2 Substrate specificity of GOGAT

Reaction mixture	Relative activity (%)
Complete system	100
− Glutamine	0
− Glutamine + 5mM Asparagine	100
− Glutamine + 25mM NH4Cl	0
− 2-oxoglutarate	0
− 2-oxoglutarate + Oxaloacetate	933
− 2-oxoglutarate + 5mM Pyruvate	0
− NADH + 150μM NADPH	0

The complete system was discribed in "Material and Methods."

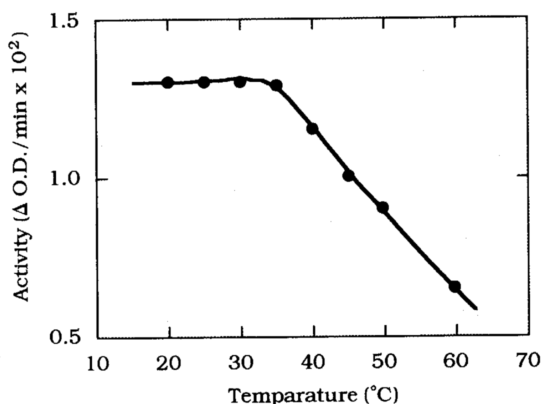

Fig. 3 Heat stability of GOGAT.

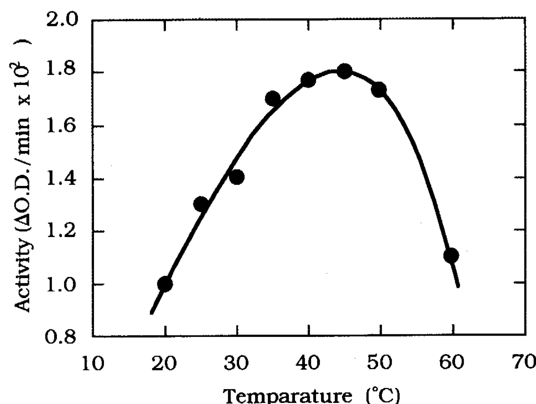

Fig. 4 Effect of temparature on GOGAT activity.

Effect of Temperature: The effect of temperature on the catalytic activity of GOGAT are detemined in 20-60°C (Fig. 3). The optimum temperature for catalysis was 45°C. Activation energy calculated from the Arrhenius Plots was 6.66 Kcal/mol.

Thermostability of the enzyme was investigated by incubating the enzyme in Tris-HCl buffer, pH 8.5, for 10 min at the temprature, and then the residual activity was assayed at 25°C under standard conditions. GOGAT were very heat labile. The enzyme was satble at temparature up to 30°C, but above 35°C gradually inactivated (Fig. 4).

Effect of subatrate concentration: The effects of substrate concentrations on the catalytic rate of GOGAT were determined at optimum pH. K_m values for substrates were determined by Lineweaver-Burk Plots. Since the reaction is a multisubstrate one, the Km value obtained for the various substrates are only apparent. The apparent Km for *L*-glutamate, 2-oxoglutarate and NADH was 20mM, 33μM and 8μM, respectively. These values for 2-oxoglutarate and NADH compare favorably with those obtained for the enzyme from other plant souces[9,21,22]. Although the K_m value for glutamine of GOGAT from castor bean cotyledon is very high, the emzyme is appeared to active sufficiently, in germinating castor bean cotyledons contained the high level of glutamine (e. g. 4-day cotyledon conteined 100 milimolar glutamine).

Effects of various metabolites: The activity of GOGAT was assayed in the presence of 5mM concentrations of TCA cycle metabolites, amino acids and nucleotides. Of the metabolites tested, citrate, isocitrate, glutamate, ATP, GTP and pyrophosphate inhibited the enzyme activity (Table 3). Only phosphate and asparatate enhanced the enzyme activity.

Effect of divalent ions: GOGAT was preincubated with different divalent ions and then assayed for the activity. All the added metal ions inhibited the enzyme activity except Mn^{2+} and Ca^{2+} (Table 4). None of the divalent ion enhanced the activity, which shows that GOGAT requires no metal ion for its activity. Similar results have also been reported from *Pisum sativum*[15].

Effect of SH-brocking reagent: The SH-reagents such as *p*-CMB and $HgCl_2$ inhibited the enzyme activity. Dithiothreitol complately protected this inhibition (Table 5). Thus, it seems possible that SH-groups are important for the activity and the retantion of conformation of the enzyme molecule.

These results indicate that the physical and kinetic properties of NADH-GOGAT from germination castor bean cotyledones is similar to that of enzyme from lupin

Table 3 Effect of various metabolites on GOGAT activity

Metabolite	Activity (%)	Metabolite	Activity (%)
None	100	Histidine	109
Citrate	27	Alanine	109
Isocitrate	45	Leusine	106
Glyoxylate	100	Isoleusine	103
Malate	100	Glutamate	87
Pyruvate	100	Asparatate	154
Succinate	96	ATP	34
Phenylalanine	102	ADP	96
Prorline	108	AMP	103
Asparagine	106	GTP (2mM)	88
Valine	108	Pyrophosphate	35
Glysine	103	Phosphate	143

Final concentration of metabolite was 5mM.

Table 4 Effect of various divalent ions on GOGAT Activity

Divalent ion	Concn. (mM)	Activity (%)
$MgSO_4$	3.0	108
$MnSO_4$	3.0	64
$ZnSO_4$	3.0	60
$CuSO_4$	3.0	80
$CaCl_2$	3.0	115
$CdCl_2$	3.0	59
$HgCl_2$	0.5	39

Table 5 Effect of SH-reagents on GOGAT Activity

SH-reagent	Relative activity (%)
1 mM DTT	107
0.1 mM *P*-CMB	62
0.2 mM *p*-CMB	48
0.1 mM *p*-CMB + 1 mM DT	104

DTT: Ditiothreitol, *p*-CMB: *p*-Chloromercuribenzoate

nodules[19]. It is suggested that glutamate synthase play a key role in the nitrogen nutrition of the germinating castor bean cotyledon providing a means whereby nitrogen transported in the form of glutamine in made available for the synthesis of other protein amino acids *via* transaminase reactions.

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