Vol. 87, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1979 Pages 627-634

ISOLATION AND CHARACTERIZATION OF TWO FORMS OF GLUTAMINE SYNTHETASE FROM SOYBEAN HYPOCOTYL

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Received February 23, 1979

Summary: Two forms of glutamine synthetase have been isolated from four-day-old, etiolated soybean hypocotyls by a procedure involving protamine sulfate and ammonium sulfate precipitation, and DEAE-cellulose and Bio-Gel chromatography. The two activities, which eluted from DEAE-cellulose at 0.1 M and 0.8 M KCl, were further purified on Bio-Gel columns to a total purification of 500- and 2000-fold, respectively. The enzymes were characterized by similar molecular weights of 365,000, similar pH and temperature optima, and a similar apparent K_m for hydroxylamine. The enzymes differed with respect to their apparent K_m for glutamine and their degree of association with ADP. Preincubation in the presence of ADP resulted in a stabilization to thermal denaturation.

Glutamine synthetase (L-glutamate:ammonia ligase, ADP, EC 6.3.1.2) activity has been isolated from several plants (1-5). The enzyme, as identified in eukaryotic systems, is regulated by repression and derepression in response to changes in environmental levels of combined nitrogen (6), by purine and pyrimidine nucleotides and several amino acids which are derived from glutamine (2, 7) and by energy charge (2, 8).

Recent studies have shown the existance of two forms of glutamine synthetase (GS) in *Rhizobium japonicum* 61A76 (9). The two enzymes had different isoelectric points as well as differing in stability at 50°C. Two forms of GS from *Bacillus caldolyticus* were shown to have similar molecular weights but slightly different isoelectric points (10). This paper presents the isolation and partial purification of two forms

0006-291X/79/060627-08$01.00/0

of GS from a higher plant. The enzymes have similar molecular weights but were eluted from DEAE-cellulose at 0.1 M KCl and 0.8 M KCl. In addition to exhibiting different apparent K_m values for L-glutamine, the enzymes also varied in stability to prolonged elevated temperatures when preincubated in the presence or absence of ADP.

Materials and Methods: Soybean seeds *(Glycine max* variety Harosoy 63*)* were obtained from William Boyd, Phelps, New York. Seeds were germinated in the dark at 29°C for four days.

Assay procedure. GS activity was determined by the γ-glutamylhydroxamate assay (11).

1. L-glutamine + $NH_2OH \xrightarrow[AsO_4]{Mn^{2+}ADP}$ γ-glutamylhydroxamate + NH_3.

The assay mixture consisted of 30 mM L-glutamine (pH 7.0), 20 mM Na_2HAsO_4, 3 mM $MnCl_2$, 60 mM hydroxylamine (pH 7.0), 0.4 mM adenosine 5'-diphosphate (ADP-pH 7.0), 20 mM imidazole-HCl buffer (pH 7.0), H_2O and enzyme in a total volume of 1 ml. Typical reactions were initiated by the addition of the enzyme, incubated at 37°C for 10 minutes, and terminated by the addition of 0.5 ml of a solution containing 4% (w/v) trichloroacetic acid (TCA), 1 N HCl and 1.67% $FeCl_3$. The absorbance of the solution was measured at 540 nm. One unit of enzyme was defined as that amount required to catalyze the synthesis of 1 μmole of γ-glutamylhydroxamate in 10 minutes at 37°C. Crude enzyme preparations were also assayed by the biosynthetic reaction based on the release of inorganic phosphate (2).

2. ATP + L-glutamate + NH_4 + $\xrightarrow{Mg^{2+}}$ L-glutamine + ADP + Pi.

The assay consisted of 500 mM imidazole-HCl (pH 7.0), 20 mM $MgSO_4$, 1 mM diethylenetriamine pentaacetic acid (DTPA), 80 mM L-glutamate, 4 mM NH_4Cl, 8 mM ATP, H_2O and enzyme in a total volume of 1 ml. The reaction was terminated by the addition of 10% TCA and the Pi released measured by the method of Fiske and Subbarow (12). Protein was determined by the method of Lowry and coworkers (13) using bovine serum albumin as a standard.

Isolation. Cotyledons were removed from four-day-old etiolated plants and approximately 2 cm of the hypocotyl "hook" region were harvested. The tissue was homogenized with a Polytron PCU-2 using a homogenization buffer containing 200 mM imidazole-HCl (pH 7.5), 10 mM $MnCl_2$ and 2 mM 2-mercaptoethanol. Following filtration through cheesecloth and Miracloth, the homogenate was centrifuged at 22,000 x g for 20 minutes and the pellet discarded.

Purification. A 1% (w/v) solution of protamine sulfate was added to the supernatant to obtain a concentration of 7.5% (v/v). The preparation was stirred 20 minutes and then centrifuged at 22,000 x g and the pellet discarded.

Norit A (0.03-0.05g/ml) was added to the supernatant and stirred for 10 minutes. After centrifugation at 27,000 x g for 20 minutes, the supernatant was filtered.

The filtrate was brought to 70% saturation with crystalline ammonium sulfate. Following centrifugation at 22,000 x g for 20 minutes, the pellet was resuspended and desalted using Sephadex G-50 chromatography.

The desalted preparation was applied to DEAE-cellulose and eluted with a step gradient of KCl. Following concentration using membrane filtration, the DEAE-cellulose fractions were separately applied to Bio-Gel A-0.5 columns for further purification and determination of approximate molecular weights.

Results: A comparison of the specific activities of crude fractions using the biosynthetic assay and the transferase assay shows the specific activities to be 0.08 μmoles Pi/mg/10 min. at 37°C and 4.0 μmoles γ-glutamylhydroxamate/mg/10 min. at 37°C, respectively.

As shown in Figure 1, two major peaks of GS activity were separated on DEAE-cellulose and purified 500- and 2000-fold, respectively, following Bio-Gel chromatography (Figure 2, Table 1).

Bio-Gel Peak 1 and Peak 2 enzymes were extensively dialysed against 200 mM imidazole-HCl (pH 7.5) and 2 mM 2-mercaptoethanol to remove any cations. Added enzyme, hydroxylamine, arsenate and L-glutamine were necessary for activity (Table 2). Assays minus ADP for enzymes 1 and 2 showed a 78% and 90% decrease in activity, respectively. In these partially purified enzyme preparations, apparently ADP remains in association with the proteins present in solution. Elimination of cations resulted in a 98% and 97% decrease in activity, respectively, as compared to the complete assay. A substitution of Mg^{2+} for Mn^{2+} resulted in a large decrease in enzyme activities, indicating a Mn^{2+} requirement by both enzyme fractions. Bio-Gel Peak 1 enzyme showed optimum activity at pH 7.0 and Peak 2 had an optimum at pH 6.5.

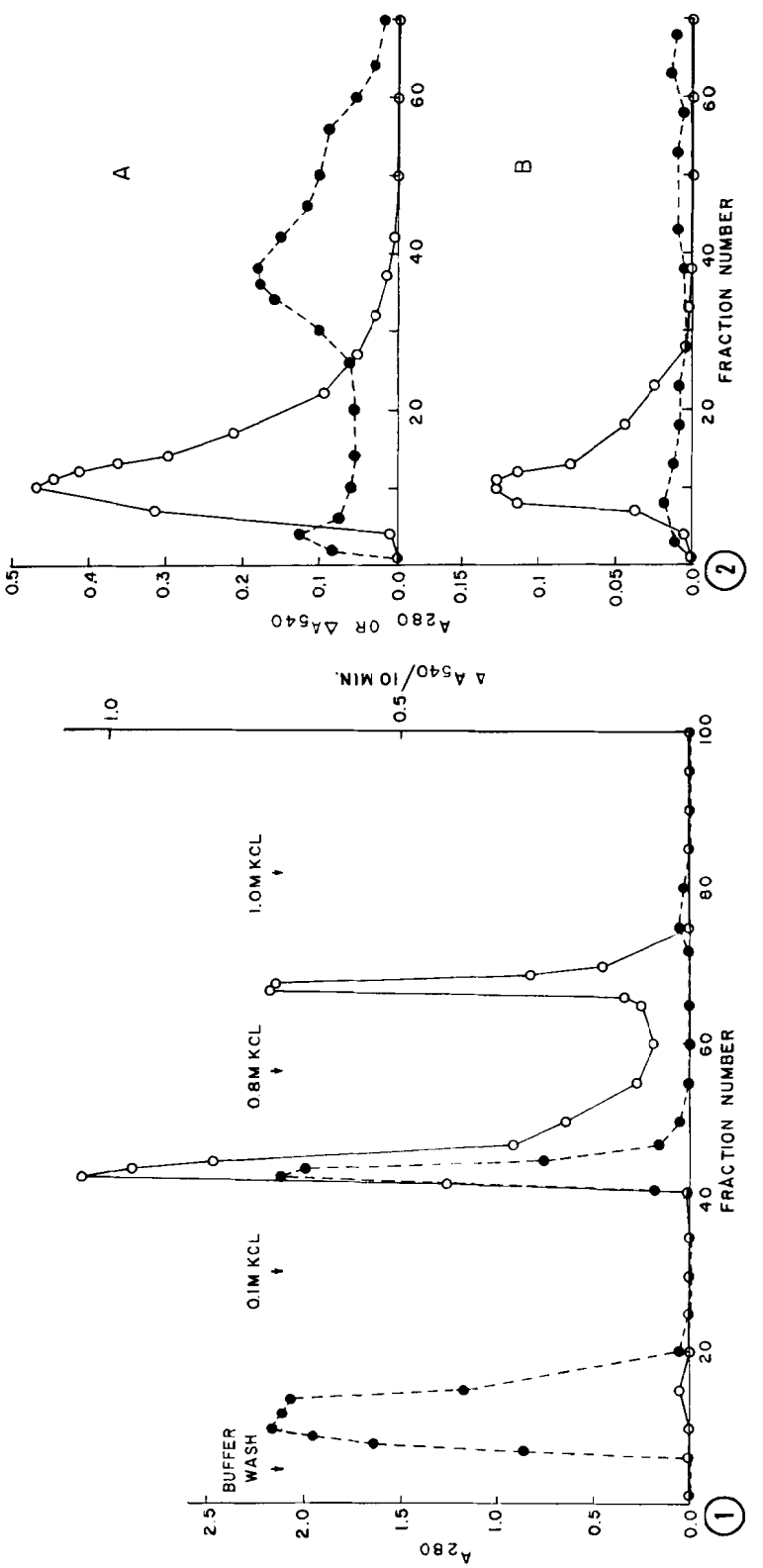

Figure 1. Separation of two GS activities by DEAE-cellulose chromatography. Elution of proteins from the column was by a step gradient of KCl and collected in 4 ml fractions. Protein (●--●) was monitored at 280 nm and enzyme activities (0—0) at 540 nm. Peak 1 consisted of fractions 42-50, and Peak 2 fractions 65-71.

Figure 2. Bio-Gel A-0.5 chromatography of soybean GS activities. The first point on both graphs equals 140 mls (void volume). Elution was with a buffer containing 200 mM imidazole-HCl (pH 7.5), 10 mM $MnCl_2$, 2 mM 2-mercaptoethanol and 100 mM KCl. Two ml fractions were collected. Protein 2 (●--●) was monitored at 280 nm and enzyme activity (0—0) at 540 nm. A = chromatography of DEAE-cellulose Peak 1, B = chromatography of DEAE-cellulose Peak 2.

Table 1. Purification of glutamine synthetase from soybean hypocotyl.

Purification Step	Volume (ml)	Protein (mg/ml)	Total Units*	Specific Activity	Purification
Crude Homogenate	272	6.0	6528	4	1
Protamine Sulfate	283	3.2	7641	9	2
Norit A Absorption	245	1.3	4410	14	3
Ammonium Sulfate	18.6	7.1	2827	22	5
DEAE-Cellulose					
Peak 1	11.5	2.1	846	35	9
Peak 2	7.8	0.013	515	5097	1264
Bio-Gel A-0.5					
Peak 1	5.9	0.016	194	2059	511
Peak 2	10.1	0.001	81	8080	2005

*1 Enzyme Unit = 1 μmole γ-glutamyl hydroxamate/0.05 ml/10 min. at 37°C

Table 2. Assay requirements for soybean glutamine synthetase.

Assay Conditions	Bio-Gel Peak 1 % of Control Activity	Bio-Gel Peak 2 % of Control Activity
Complete	100**	100***
- Enzyme	0	0
- NH_2OH	0	0
- L-glutamine	0	0
- ADP	22	10
- M^{2+}*	2	3
- Mg^{2+}	48	31

* M^{2+} represents any cation

** Control activity of Bio-Gel Peak 1 = 1.53 μmoles γ-glutamyl hydroxamate/10 min. at 37°C.

*** Control activity of Bio-Gel Peak 2 = 0.33 μmoles γ-glutamyl hydroxamate/10 min. at 37°C.

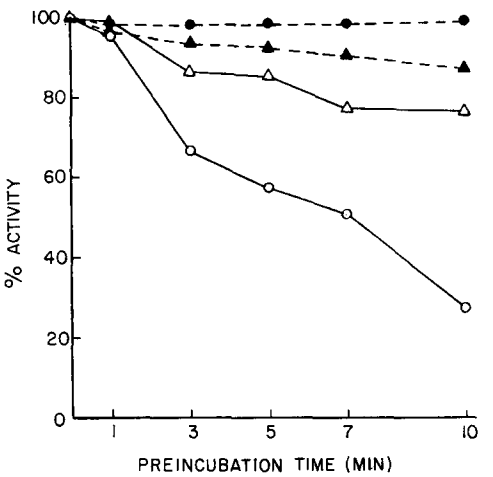

Figure 3. Effects of preincubation at 45°C on GS activities. Preincubation of the enzyme was in the presence and absence of ADP for various times at 45°C. Reactions were assayed for 10 minutes at 37°C. Bio-Gel Peak 1 without ADP (0—0) and with ADP (●---●). DEAE-cellulose Peak 2 without ADP (Δ—Δ) and with ADP (▲---▲). Control activity for Peak 1 without and with ADP was 0.67 μmoles and 0.79 μmoles γ-glutamyl hydroxamate/10 min. at 37°C, respectively; for Peak 2 without and with ADP equaled 4.0 μmoles and 4.8 μmoles γ-glutamyl hydroxamate/10 min. at 37°C, respectively. ADP concentration for preincubation was 40 μM.

Bio-Gel enzyme 1 had an apparent K_m for L-glutamine of 36 mM, whereas enzyme 2 had an apparent K_m of 17 mM. At concentrations of L-glutamine greater than 60 mM, both enzymes were inhibited. The apparent K_m with respect to NH_2OH of Peak 1 and 2 was 4.5 mM and 5.3 mM, respectively. Both enzyme fractions were inhibited by concentrations of hydroxylamine greater than 60 mM. Both preparations were not affected by increasing concentrations of ammonia up to 100 mM.

Approximate molecular weights, as determined by Bio-Gel chromatography with commercially prepared proteins, was 365,000 for both enzymes.

To further investigate the association of ADP with both activities, the enzymes were preincubated in the presence and absence of ADP for various times at 45°C and then assayed at 37°C for 10 minutes. Bio-Gel Peak 1 preincubated without ADP after 10 minutes lost 70% of the initial activity whereas the enzyme preincubated with ADP lost less than 2% of the initial activity (Figure 3). Peak 2 preincubated without ADP after 10 minutes decreased 20% whereas the enzyme preincubated with ADP decreased 10%. These data indicate that preincubation with ADP results in a stabilization of enzyme activity at elevated temperatures.

Discussion: A comparison of the specific activities of crude fractions using both the biosynthetic and transferase assays showed the enzyme to be 50 times more active as assayed by the transferase method. Similar results were observed for GS in soybean root nodule cytoplasm (1).

The differences between the two forms of GS in *Rhizobium japonicum* 61A76 (9) were isoelectric points and stability at 50°C. Wedler and coworkers (10) using *Bacillus caldolyticus* also reported two enzymes on the basis of slightly different isoelectric points. The two enzymes isolated in this study had similar molecular weights but were eluted from DEAE-cellulose at very different salt concentrations. These elution properties indicate major differences between the proteins. The pH (Peak 1 = 7.0; Peak 2 = 6.5) and temperature optima (Peak 1 = 50°C; Peak 2 = 45°C) as well as the apparent K_m values for hydroxylamine for both enzymes are similar. However, the apparent K_m's with respect to L-glutamine of the two enzymes are significantly different.

Based on these studies, it also appears that ADP is associated with and stabilizes the enzymes from thermal denaturation. Preincubation of Peak 1 enzyme with ADP at 45°C for 10 minutes shows a significant retention of activity as compared to the control (-ATP). Peak 2 enzyme with ADP also shows a similar but less dramatic retention of activity.

This study presents evidence for the existance of two forms of GS in the non-nitrogen fixing tissue of the soybean hypocotyl. It has also been shown that the enzymes exist in association with ADP. This association with ADP may be involved in the regulation of the enzymes since ADP:ATP ratios have been implicated in the regulation of GS activity in sunflower roots (8) and pea leaves (2).

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