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CHARACTERISTICS OF TWO GLUTAMINE SYNTHETASE ISOZYMES IN SOYBEAN

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Abstract—Soybean plants [*Glycine max* (L.) Merr. cv. Williams] contained two chromatographically distinct isoforms of glutamine synthetase, GS-1 and GS-2. GS-1 was localized in the soluble fraction while GS-2 was in the chloroplasts. GS-2 occurred only in isolated chloroplasts, and etiolation or cycloheximide treatment of light-grown plants resulted in a dramatic decrease of GS-2 activity. Although there were some minor differences in their characteristics, the isoenzymes did not differ significantly. The increase in total activity during the early phases of growth was due largely to the increase in GS-2 activity. The activity in dark-grown plants, when exposed to light, reached almost to the level of light-grown plants within 48 hr. When the two isoenzymes were separated by DEAE-Sephacel chromatography, GS-1 accounted for 20% of total activity recovered. Although this proportion was found in both four- and 16-day-old seedlings, this conclusion may not be extrapolated to intact seedlings because of the activity loss during purification and different stability of the isoenzymes. On a whole seedling basis, the activity of the enzyme increased during the three-week period of germination and early growth. The reverse was true on a unit weight basis. Specific activities of roots and primary stems were much higher than any other parts of the plant.

INTRODUCTION

Glutamine is the first form of organic nitrogen fixed from atmospheric nitrogen. The importance of glutamine has also been indicated by its role as a nitrogen donor for the synthesis of various metabolites. Since glutamine synthesis is catalysed by glutamine synthetase (EC 6.3.1.2), the importance of the enzyme in determining the overall nitrogen economy of the plants is self-evident. In addition to its role in nitrogen assimilation, the enzyme has also been implicated in the recycling of ammonia released during photorespiration [1,2].

Although glutamine synthetase (GS) has been studied in a number of plant species for some time, only recently it has been shown to exist as at least two different isoenzymes designated as GS-1 and GS-2 in a limited number of plant species [3, 4]. Studies on cellular localization of the isoenzymes have indicated that GS-1 is present in the cytoplasm while GS-2 is present in the chloroplasts.

In this paper, we report the presence and cellular localization of two chromatographically distinct isoenzymes of GS in soybean. Special attention was given to the relative proportion of GS-1 and GS-2 in the whole plant, and development of their activity during the early phases of growth. Unlike most studies which have dealt with a particular tissue of a plant, mostly leaves, we believe that it is important to understand GS in the whole plant.

RESULTS

Enzyme purification

Table 1 shows the results from an enzyme purification. Although a 25% loss of activity resulted, ammonium sulphate precipitation of the enzymes was useful in removing the bulk of proteins in soybean seeds and seedlings.

Separation of GS into two isoenzymes was easily achieved at the DEAE-Sephacel step (Fig. 1A) at the expense of *ca* 60% loss of activity from the crude extract. They were eluted from the column at 0.16 and 0.27 M NaCl respectively. The activity peak fractions of GS-1 and GS-2 were rechromatographed individually on a hydroxyapatite column. GS-1 and GS-2 were eluted from the column at 0.065 M and 0.165 M phosphate respectively (Fig. 1B and C). Recovery was 4 and 9% for GS-1 and GS-2 and they were purified 94- and 143-fold respectively (Table 1). GS-1 recovery of the DEAE-Sephacel fraction from the hydroxyapatite step was 55%; the recovery of GS-2 activity was only 26%.

Characteristics of the two isoenzymes

The pH optima of GS-1 activity were 7.5 and 5.5 in the presence of Mg^{2+} and Mn^{2+}, respectively. Those of GS-2 were 0.5 pH unit higher than GS-1. There was a sharp decline in the Mg^{2+}-dependent activity of both isoenzymes below pH 5.0, while the Mn^{2+}-dependent activity declined sharply above pH 8.0. The Mn^{2+}-dependent activity was 32 to 34% of the Mg^{2+}-dependent activity with both isoenzymes.

There were no significant differences between GS-1 and GS-2 in their apparent K_m values for various substrates. The values of GS-1 and GS-2 respectively were in mM: glutamate, 7.56 and 6.45; ATP, 0.94 and 0.87; NH_2OH, 0.48 and 0.52; NH_4^+, 0.038 and 0.029; Mg^{2+}, 13.14 and 14.47; Mn^{2+}, 0.77 and 1.15.

Both GS-1 and GS-2 showed maximum activity at 42°, with a sharp decline in their activity above this temperature. GS-1 and GS-2 retained only 27 and 7% of their

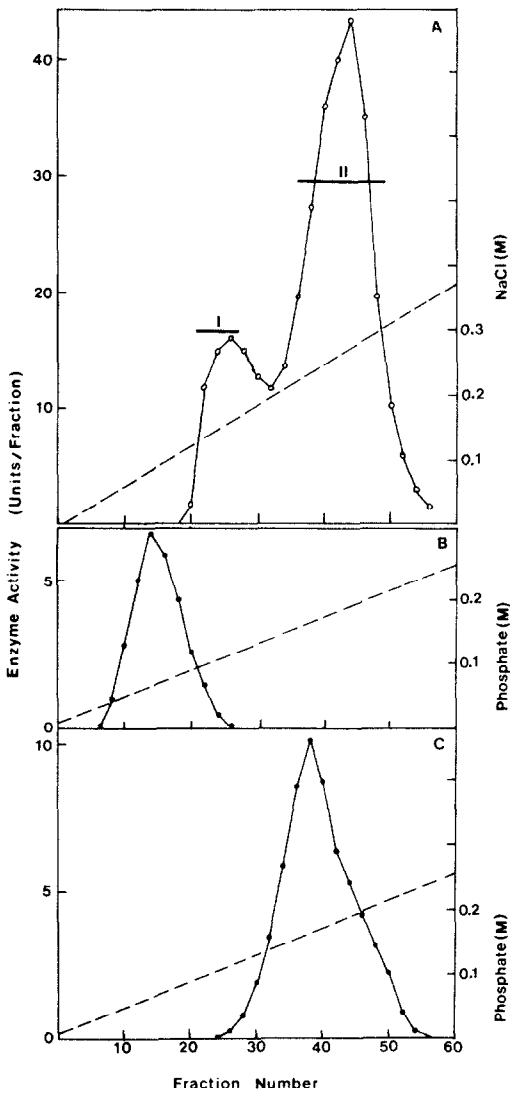

Fig. 1. Elution profiles of two glutamine synthetase isoenzymes on a DEAE-Sephacel (A) and hydroxyaptatite column (B, C). The two peak fractions of I and II in A were rechromatographed as shown in B and C, respectively. Proteins were eluted with a linear gradient of NaCl for A and K-Pi for B and C, with 5 ml (A) and 1.5 ml fractions (B and C). Every other fraction was assayed for enzyme activity.

activity, respectively, when they were preincubated at 55° for 10 min. When ATP was added in the preincubating enzyme solutions, both enzymes were quite stable up to 50°. At 55°, the residual activities of GS-1 and GS-2 were 72 and 51% respectively.

Localization of GS-2 in chloroplasts

GS activity of isolated chloroplasts was eluted from a DEAE-Sephacel column with 0.27 M NaÇl, which corresponds to the same strength of NaCl where GS-2 was eluted in Fig. 1A. We therefore concluded that GS-2 in Fig. 1A originated from the chloroplasts. The treatment of light-grown plants with cycloheximide decreased GS-2 activity by 30% when the enzyme was extracted from the isolated chloroplasts. We routinely used 20 g of leaves to isolate chloroplasts and the enzyme therefrom, obtaining 56 units of activity in 5 mg protein. With the cycloheximide treatment, the recovery was 39 units in 3.8 mg protein.

A typical 16-day-old seedling contained *ca* 111 units of total activity. When the enzyme extract was separated by DEAE-Sephacel column chromatography, the recovered activities of GS-1 and GS-2 were 11.3 and 36.7 units. Thus, more than three-quarters of the total activity was from GS-2. When the seedlings were etiolated for six days before harvest, the activities of GS-1 and GS-2 recovered from the DEAE-Sephacel were 9.8 and 19.8 units respectively. Total activity declined 38% following etiolation; the decreases in activity of GS-1 and GS-2 were 13 and 46% respectively.

Activity changes during the early stage of growth

Total enzyme activity increased linearly during the first four-day germination in the light, and thereafter declined until day six (Fig. 2), after which per plant activity again gradually increased (Table 2). By contrast, in dark-grown seedlings, the activity increased to a maximum by 36 hr and then declined during the later sampling period. When dark-germinated seedlings were exposed to light, the activity increased sharply and, within 48 hr, the activity was similar to that of light-germinated seedlings. The activity of two-day-old dark-germinated seedlings, when exposed to light on day two, increased to 94% of the level of light-germinated seedlings by day four.

Distribution of enzyme activity in a whole seedling

Table 2 shows the changes in total activity in various parts of a seedling. In mature seeds, seed coats contained over three times more activity than the rest of the seed on a protein basis, although per seed activity in seed coat was just 4% of total activity. Total activity of cotyledon pairs declined dramatically during the first 10 days, after which it became more or less stabilized at 15–20% of a whole seedling. Specific activity of cotyledon pairs increased sharply in three weeks with some six-fold decline in proteins.

As plant mass increased, total activity in leaves and growing axis also increased; it accounted for 47% of a whole seedling grown for three weeks. However, specific activity of leaves and growing axis declined, suggesting that the rate of protein increase far exceeded that of activity increase during the same period. The relative proportion of root activity was stable at *ca* 20% level in all samples, so that the top/root ratio of total activity was four at the end of experiment. Total activity presented in Table 2 is the sum of the activity measured individually in various parts of a seedling. When the activity was measured from the extracts of whole seedlings without dividing them into various parts, total activity was 3–5% higher than the values presented in Table 2.

DISCUSSION

Soybean seedlings grown in the light contained two chromatographically distinct isoenzymes of GS (Fig. 1). When the two isoenzymes present in four-day-old, light-

Table 1. Purification of two glutamine synthetase isoenzymes

Fraction	Total activity (nkat)	Specific activity	-fold purification	Recovery (%)
Crude extract	1420	2.3	—	100
$(NH_4)_2SO_4$	1060	4.3	1.9	75
DEAE-Sephacel				
GS-1	110	23.6	10.3	7.8
GS-2	476	20.8	9.1	33.6
Hydroxyapatite				
GS-1	60	216	94	4.2
GS-2	126	328	143	8.9

Twenty 4-day-old seedlings were extracted and purified as described in the experimental.

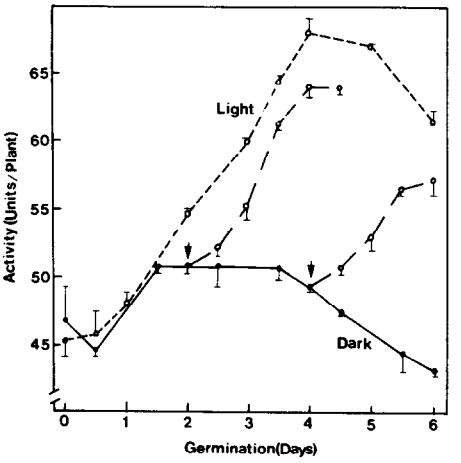

Fig. 2. Changes in total activity during a six day period of germination in a continuous light (--○--) or in continuous dark (——●——). Some dark-germinated plants were exposed to continuous light after two and four days as indicated by the arrows.

grown seedlings were separated at the DEAE-Sephacel step, GS-1 accounted for 20% of total activity recovered (Table 1). This proportion of GS-1 was also found in 16-day-old seedlings. However, it would be difficult to conclude that GS-1 is *ca* 20% of total activity *in vivo*: only 40% of total activity in crude extracts was recovered after the DEAE-Sephacel step, and GS-1 was more stable than GS-2. It is not known whether GS-2 activity was lost preferentially during the purification procedures.

Changes in absolute amount of activity depend on how the results are expressed. For example, when the relative proportion of GS-1 to GS-2 is expressed on a fresh weight basis, the activities of 16-day-old seedlings declined 43 and 60% respectively as compared to those of four-day-old seedlings. But on a whole plant basis, the activities actually increased 2.2-fold and 1.5-fold respectively, during the same period (data not shown). The increased activity was not as much as the increase in fresh weight (3.8 times), even so it was an increase. Although the comparison on a plant basis would reflect the *in vivo* physiology more precisely, unfortunately, most studies have been reported on a unit weight basis.

We were interested in how the activity of GS changed in various parts of a whole plant. We measured the total activity in the first three weeks, at which time the cotyledons turned almost completely yellow. As expected, total activity increased as seedlings growth proceeded, but the relative proportions of the activity showed a different profile in different parts of the seedlings (Table 2). Generally, the roots and primary stems contained the highest specific activity during this period. The younger the plant, the more conspicuous was this trend. At day 21, *ca* one-half of total activity was present in leaves and the newly growing axis even if specific activity in those tissues was lower than any other parts because of growth dilution.

Although GS-1 was clearly separated from GS-2 by DEAE-Sephacel and hydroxyapatite chromatography, their kinetic and physiological characteristics were similar. However, there was a clear difference in their pH optima and their relative activities depending on the cations used. The K_m values for various substrates were in the ranges of other plant species [3,5–7], except that for NH_2OH. The estimated K_m values for both isoenzymes were 0.5 mM $\pm$0.02, and this value was significantly lower than the one reported with soybean hypocotyls [8]. GS-1 appeared to be more stable than GS-2. GS-2 lost 74% of its activity during the hydroxyapatite chromatography (Table 1). Also, GS-2 was more heat labile and ATP had less effect in protecting GS-2 from thermal inactivation.

As has been reported [3,6,7,9–11], GS-2 is localized in chloroplasts, and chloroplast development was found essential to increase GS-2 activity. Etiolation drastically reduced GS-2 activity, while it had no significant effect on GS-1. In a typical example of a 16-day-old seedling, the decline in total activity following etiolation was due to *ca* one-half decrease in GS-2 activity compared with a light-grown normal seedling of the same age. This does not mean, however, that GS-2 is synthesized within the chloroplasts, since cycloheximide decreased the activity of GS-2 only. Therefore, we concluded that although the GS-2 is localized in chloroplasts, it may be synthesized in the cytosol in response to light.

We concluded that the two isoenzymes of soybean GS appear to be very similar in their characteristics, as has

Table 2. Changes of fresh weight (FW), total proteins (TP) and glutamine synthetase

Part	0-day FW (g)	TP (mg)	GS (U)	SA	%	10-day FW (g)	TP (mg)	GS (U)	SA	%
Seedcoat	—	0.3	1.7	5.7	4	—	—	—	—	—
Cotyledons	0.17	23.4	41.1	1.8	96	0.64	15.4	14.7	1.0	23
Leaves + axis	—	—	—	—	—	0.40	4.2	18.3	4.4	29
Roots	—	—	—	—	—	0.35	1.8	13.7	7.6	21
Primary stems	—	—	—	—	—	0.45	1.2	17.6	14.7	27
Total	0.17	23.7	42.8	1.8	100	1.84	22.6	64.3	2.8	100

Specific activities (SA) and relative proportion of total activity (%) in various parts are weeks with tap water only. The average values of three different extractions are presented,

been reported to be the case in other plants [3,5,7]. However, it is not known whether they differ from each other in their fine structures, in addition to their obvious spatial difference within a cell.

EXPERIMENTAL

Plant material. Soybean seeds [*Glycine max* (L.) Merrill cv. Williams] were germinated in a sand-bench of the greenhouse. Unless otherwise indicated, the seedlings were irrigated with tap water only. Some 10-day-old plants were etiolated for 6 days before harvest by covering them with black cloth. The chlorophyll content was 25 ± 5% of the light-grown counterparts when harvested. Cycloheximide (20 μg/ml) was applied directly to the leaves with a hand sprayer, twice a day for 6 days before harvest. To monitor the changes in enzyme activity in the dark, some seeds were dark-germinated in Petri dishes lined with moist filter papers. Some of these seedlings were exposed to continuous light at different times (Fig. 2).

Enzyme extraction. Enzyme was extracted with 25 mM Tris, pH 7.6, containing 1 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 1 mM dithiothreitol. Dry seeds or seedlings were homogenized with a Brinkmann Polytron. After centrifugation for 30 min at 20 000 *g*, the floating lipid materials at the surface of the tubes were scooped away. The crude extracts thus obtained were precipitated with $(NH_4)_2SO_4$. The fraction of 40 to 60% satn was resuspended in a small vol. of the extraction buffer and then desalted through a PD-10 column (Pharmacia). The desalted enzyme preparation was layered on a DEAE-Sephacel column (2.6 × 30 cm) and the proteins were eluted from the column by using a linear gradient of NaCl from 0 to 0.4 M in the extraction buffer. Fractions (5 ml) were collected and every other fraction was assayed for enzyme activity. Two activity peaks were clearly observed at this stage.

The two activity peaks were pooled separately and pptd with $(NH_4)_2SO_4$ to 80% satn. After centrifugation, the ppt. was resuspended and desalted as before, and it was then layered on a hydroxyapatite column (1 × 10 cm). A linear gradient of K-Pi buffer, pH 7, from 10 to 300 mM was used to elute the proteins. Fractions of 1.5 ml were collected and every other fraction was assayed for the activity. All purification steps were conducted at 0 to 4°.

Chloroplast isolation. Chloroplasts were isolated by the method described in ref. [12]. The chloroplasts, suspended in 50 mM Tris, pH 8, containing 0.3 M sucrose and 20 mM EDTA, were incubated in the presence of 0.1% (v/v) Triton X-100 for 10 min [13] and then homogenized with a Polytron. The enzyme purification was then carried out as described above.

Enzyme assay. Enzyme activity was measured as described previously [14] with the following modifications. The standard reaction mixture contained in μmol: L-glutamate, 50; NH_2OH–HCl (freshly prepared and pH adjusted to 7.5), 50; $MgSO_4$, 50; ATP, 5; Tris buffer (pH 7.6), 725; and the appropriate amount of enzyme soln. The final vol was 1.125 ml. The reaction was initiated by adding the NH_2OH, run for 15 min at 35°, and stopped by adding 0.375 ml of $FeCl_3$ soln. After centrifugation, the glutamyl-hydroxamate was measured at 540 nm. When Pi was measured to determine the apparent K_m values for NH_4^+, NH_2OH was replaced by 5 μmol of NH_4Cl. Enzyme activity is expressed in nkat being one unit.

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activity (GS in nkat) in various parts of a seed and seedling

16-day					21-day				
FW (g)	TP (mg)	GS (U)	SA	%	FW (g)	TP (mg)	GS (U)	SA	%
—	—	—	—	—	—	—	—	—	—
0.57	5.9	17.4	2.9	16	0.52	4.0	20.6	5.2	15
0.78	12.9	35.0	2.7	31	1.97	34.2	62.8	1.8	47
0.81	3.0	26.4	8.8	24	1.23	6.5	26.3	4.0	20
0.43	3.4	33.7	9.9	30	0.71	4.8	25.1	5.2	19
2.59	25.2	112.5	4.5	100	4.43	49.5	134.8	2.7	100

also shown. Seeds were germinated in a sandbench and grown for three with 10 seeds or seedlings for each extraction.

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