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Subunit Composition of Glutamine Synthetase Isozymes from Root Nodules of Bean (*Phaseolus vulgaris* L.)[1]

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ABSTRACT

Glutamine synthetase from bean nodules can be separated into two isoforms, GS_{n1} and GS_{n2}. A purification protocol has been developed. It included protamine sulfate precipitation, ammonium sulfate fractionation, anthranilate-affinity chromatography, Dye-Matrex (Orange A) chromatography, and diethylaminoethyl-cellulose ion-exchange chromatography. GS_{n1} and GS_{n2} have been purified to homogeneity. Subunit structure analysis using two-dimensional polyacrylamide gel electrophoresis revealed that GS_{n1} was composed of two different types of subunit polypeptides. They differed in isoelectric points (6.0 and 6.3) but had the same molecular weights (46,000 Daltons). GS_{n2} was composed of only one type of subunit polypeptide. It had an isoelectric point of 6.0 and a molecular weight of 46,000 Daltons. It was apparently identical to one of the polypeptides found in GS_{n1}. Glutamine synthetase holoenzyme consisted of eight subunits. In the nodule there are two different types of glutamine synthetase subunit polypeptides. Random combinations of the polypeptides should generate nine different isozymes. Our electrophoretic analysis revealed that GS_{n2} was but one of the isozymes, and GS_{n1} was a composite of the other eight. Hence, nodule glutamine synthetase isozymes were homo-octameric as well as hetero-octameric.

GS[2] (EC 6.3.1.2) catalyzes the formation of glutamine from substrates glutamate, ammonia, and ATP. This enzyme and GOGAT (EC 1.4.1.14) coupled together form the GOGAT cycle, which is considered the major ammonia assimilation pathway in most higher plant tissues (17). In legume root nodules, ammonia produced by N_2 fixation in the bacteroids is excreted into the nodule cytosol (9), where it is assimilated into glutamate by the GOGAT cycle (19).

GS has been extensively studied because of its importance in plant nitrogen metabolism. It has been purified and characterized from root nodules of soybeans (15), lupine (14), and alfalfa (8). Cullimore *et al.* (3) reported that bean nodule cytosol has two isoforms of GS (designated GS_{n1} and GS_{n2}), which can be separated from each other by ion-exchange chromatography or native PAGE. GS_{n1} is unique to the nodule whereas GS_{n2} may be identical to the isoform GSr that is found in roots. The physical and kinetic properties of GS_{n1} and GS_{n2} are similar (3). The holoenzyme of both isoforms have identical mol wt (380,000 D), and are both comprised of eight subunit polypeptides of 41,000 D. Lara *et al.* (13) reported that GS_{n1} and GS_{n2} are comprised of two different types of subunit polypeptides (β and γ). The polypeptides have identical mol wt but different isoelectric points.

In an organ such as the bean nodule, there are two different types of GS subunit polypeptides (13). Since the GS holoenzyme consists of eight polypeptides (3), it is not known whether the eight subunit polypeptides of the holoenzyme must be identical (*i.e.* homo-octamer), or they can be different (*i.e.* hetero-octamer). Earlier, we have shown that after GS_{n1} and GS_{n2} had been separated by native PAGE and the gel had been stained with Coomassie blue, we could detect six to nine protein bands in the GS_{n1} region and one protein band in GS_{n2} (23). W suggested that these proteins bands are individual isozymes of the GS isoforms. That is, the isoform GS_{n1} may be composed of six to nine hetero- and homo-octameric isozymes, and GS_{n2} may be composed of one homo-octameric isozyme.

In this report, we shall demonstrate that this suggestion is correct. We purified GS_{n1} and GS_{n2} to homogeneity. Two-dimensional PAGE revealed that GS_{n2} was an isozyme that had only β subunit polypeptide and that GS_{n1} was a composite of eight isozymes. Each of the eight isozymes was made up by different ratio of β and γ subunit polypeptides.

MATERIALS AND METHODS

Plant Culture and Nodule Harvest

Seeds of common bean (*Phaseolus vulgaris* L., cultivar Kentucky Wonder) were purchased from Vermont Bean Seed Co., Bomoseen, VT. *Rhizobium leguminosarum* biovar. *phaseoli* strain 127K14 was obtained from Nitragin Co., Milwaukee, WI. Plastic 15-cm pots were filled with a mixture of perlite and vermiculite that had been wetted with a nitrogen-free plant nutrient solution (16). Four bean seeds were sown in each pot. The pots were inoculated with *Rhizobium* strain 127K14 immediately after sowing at a density of approximately 1×10^9 cells per pot. The *Rhizobium* was cultured in a yeast extract-mannitol medium to the late log phase before using as inoculant. The pots were then placed in a growth chamber (light intensity, 24,000 lux; daytime, 16 h, 27°C; nightime, 8 h, 22°C) and irrigated with nitrogen-free nutrient solution once every 3 d.

Nodulated roots from plants 28 d after sowing were washed free of vermiculite and perlite. The nodules were detached

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[2] Abbreviations: GS, glutamine synthetase; GS_{n1}, nodule-specific glutamine synthetase; GS_{n2}, second form of nodule GS; GSr, root GS; GOGAT, glutamate synthase.

from the roots and kept on ice before being frozen in liquid N_2 and stored at −70°C until use.

Purification of GS

All procedures were carried out at 0 to 4°C. Root nodules (90 g) were macerated with a Sorval Omni Mixer in the presence of 180 mL extraction buffer (0.05 M Tris-HCl [pH 7.5], containing 1.0 mM DTT) as previously described (23). The crude extract was treated with protamine sulfate and fractionated with $(NH_4)_2SO_4$ (3). GS was partially purified by Sepharose-anthranilate affinity chromatography (22, 23). We developed two additional steps to further purify GS. After GS was eluted from the Sepharose-anthranilate affinity column with 30 mM AMP in Palacio's buffer (5 mM K-phosphate [pH 7.2], containing 0.5 mM EDTA, 1 mM $MnCl_2$, and 50 mM K_2SO_4) (22) and dialyzed against the 'running buffer' (10 mM Tris-HCl [pH 7.8], containing 5 mM Na-glutamate, 10 mM $MgSO_4$ and 10% glycerol) (3) overnight as described earlier (23), the enzyme was loaded onto an affinity chromatography column (9 cm long, 1.5 cm diameter) packed with Dye Matrex gel (Orange A) (Amicon Co., Danvers, MA). The column had been previously equilibrated with the running buffer. GS was eluted from the column with the running buffer at void volume because GS did not bind to Orange A, but other proteins did. GS was then loaded onto a column (8 × 1.5 cm) containing DEAE-cellulose, which had been previously equilibrated with the running buffer. This step not only further purified the enzyme but also separated the two isoforms, GS_{n1} and GS_{n2}, from each other. GS_{n1} did not bind firmly to the column and was eluted with 150 mL of running buffer. GS_{n2} was eluted from the column with 200 mL of linear 0 to 0.6 M KCl gradient in the running buffer at a flow rate of 0.3 mL/min. Purified GS_{n1} and GS_{n2} were then frozen in liquid N_2 and stored in −70°C until use.

PAGE

Four PAGE systems were used: (a) a discontinuous native gel system conducted according to Davis (4), (b) an SDS-PAGE system carried out according to Laemmli (11), (c) an isoelectric focusing in tube gel system carried out according to O'Farrell (18), (d) an isoelectric focusing in slab gel system conducted by the method of Kung *et al.* (10).

The experimental conditions and the composition of acrylamide and cross-linker of the first two systems were described in detail earlier (23). O'Farrell's method for isoelectric focusing in tube gel system was used except our pH range was 4 to 7. The conditions and chemical compositions of the isoelectric focusing in slab gel system were similar to that of Kung *et al.* (10) except: (a) 1.5 mL of 40% pH range 5 to 7 and 0.1 mL of 40% pH range 3 to 10 ampholytes (Bio-Rad Laboratories, Richmond, CA) were used and (b) the dimensions of our slab gel were 1.5 mm × 14 cm × 12 cm.

The native gel system was used to detect isozymes of GS (23). The SDS-PAGE system was used to determine the mol wt of GS subunit polypeptides. The O'Farrell's isoelectric focusing system coupled with the SDS-PAGE system were employed to determine the isoelectric point and mol wt of the GS subunit polypeptides. The native gel system coupled with the isoelectric focusing in slab gel system were used to show the subunit composition of the individual GS isozymes.

The subunit analysis of individual isozymes was accomplished by electrophoresizing GS, which had been purified after Dye Matrex chromatography, on the native gel system. We used 5% polyacrylamide analyzing gel (23). After electrophoresis, one lane of the gel was stained for GS activity (1) in order to localize GS_{n1} and GS_{n2} on the gel. In the unstained portion of the gel, the area of a lane containing GS_{n1} and GS_{n2} was sliced into 10 to 11 1.5-mm pieces with a gel cutter. Each gel piece was placed in a small tube and incubated with 50 μl of treatment buffer (8 M urea, 2% pH range 5–7 ampholyte, 5% mercaptoethanol) for 1 h at room temperature. The tubes were gently and constantly shaken during incubation. After incubation, each gel was inserted onto separate lanes of the isoelectric focusing slab gel. After electrophoresis, the proteins in the slab gel were electrophoretically transferred onto a sheet of nitrocellulose (24). The nitrocellulose blot was blocked by immersion in 3% gelatin dissolved in Towbin Tris-saline buffer (10 mM Tris-HCl [pH 7.4] with 0.9% NaCl) for 1 h. The blot was incubated in 100 mL of Towbin Tris-saline buffer, which contained 0.1 mL of rabbit antibody against purified GS_{n1}, overnight at 4°C. The blot was washed five times for 15 min each in Towbin Tris-saline containing 0.05% Tween 20. It was then incubated in 1:2000 diluted goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad Laboratories) for 4 h at room temperature. The blot was then washed five times. The GS subunit polypeptides were visualized by incubating the blot in a Tris-saline buffer solution (20 mM Tris, 500 mM NaCl [pH 7.5]) containing substrates of the peroxidase (0.05% 4-chloro-1-naphthol and 0.015% H_2O_2).

Preparation of Rabbit Antibody Against Purified GS

Purified GS_{n1} (86 μg) was dissolved in about 1 mL of running buffer. The solution was mixed vigorously with 1 mL of Freund's complete adjuvant until the formation of a stable emulsion. The mixture was injected subcutaneously into a 3-month-old New Zealand white rabbit. Three weeks later, the rabbit received another injection of 86 μg of GS_{n1} mixed with Freund's incomplete adjuvant. A third booster injection was made 3 weeks later. A month after the third injection, rabbit serum was collected. The serum contained antibody that reacted with native GS_{n1} and GS_{n2} as well as the subunit polypeptides of GS.

Determination of GS Activity

GS activity on native gels was detected by the transferase assay of Barratt (1). Incubation time was 30 min, and temperature was 30°C. The gels showing activity bands were photographed through a blue filter. Kodak Technical Pan 2415 film was used. The gels were subsequently stained with Coomassie brilliant blue R-250.

GS activity in crude extract and during purification was determined colorimetrically by the transferase reaction (5). One unit of enzyme activity was the amount causing the formation of 1 μmol of γ-glutamyl hydroxamate/min at 25°C.

Protein content was measured colorimetrically by the method of Bradford (2).

RESULTS

Purification of GS_{n1} and GS_{n2}

Table I shows that the protocol resulted in 55- and 66-fold purification of GS_{n1} and GS_{n2}, respectively. The final recovery was 16% of the total activity for GS_{n1} and 4.2% for GS_{n2}. The actual yield was 2.74 mg of GS_{n1} and 0.59 mg of GS_{n2} or a total yield of 3.33 mg. If the purification protocol worked unbiasly for GS_{n1} and GS_{n2}, the ratio of GS_{n1} to GS_{n2} in the crude nodule extract should be 2.74/0.59 or 4.7 to 1. Furthermore, if 3.33 mg of protein was 20.2% of the total activity, the crude nodule extract must have had 16.5 mg of GS. The amount of GS in crude nodule extract was then 16.5 mg of protein out of 925 mg or 1.8%. This value is similar to those reported by Cullimore *et al.* (3) for bean nodules, McCormack *et al.* (14) for lupin nodules, and McParland *et al.* (15) for soybean nodules.

GS_{n1} and GS_{n2} have been purified to homogeneity. Figure 1 shows that in native PAGE, there were no other protein bands except in the areas corresponding to GS_{n1} and GS_{n2} activities. Figure 1A also shows that GS_{n1} had multiple protein bands and GS_{n2} had one band. This was also observed in our earlier report (23). SDS-PAGE demonstrated that the purified GS_{n1} and GS_{n2} were made of subunit polypeptides that have identical M_r of 46,000 D (Fig. 2). The homogeneity of GS_{n1} and GS_{n2} was again demonstrated as there were no other protein bands except the 46,000 D polypeptide (Fig. 2).

Subunit Analysis of GS_{n1} and GS_{n2} by Two-Dimensional PAGE

Figure 3 shows that GS_{n1} were comprised of two subunit polypeptides. They had the same M_r of 46,000 D but different pIs at 6.0 and 6.3. Following Lara *et al.*'s (13) nomenclature, the polypeptide that had pI of 6.0 is designated β and that had pI of 6.3 is designated γ. GS_{n2} was comprised of only β. When GS_{n1} and GS_{n2} were mixed, the mixture showed the presence of β and γ as expected.

Isozyme Composition of GS_{n1} and GS_{n2}

Figure 1A shows that GS_{n1} had multiple protein bands and GS_{n2} had one band. These results indicated that GS_{n1} may be comprised of different isozymes. To explore this possibility, GS, which had been purified after the Dye Matrex chromatography (Table I), was subjected to 5% native PAGE. After electrophoresis, one lane of the gel was stained for GS activity (Fig. 4A), and another lane was stained with Coomassie blue (Fig. 4B). Coomassie blue staining revealed eight protein bands in the GS_{n1} region and one major band in the GS_{n2} region (Fig. 4B). Activity staining showed one large band in the GS_{n1} region and one band in the GS_{n2} region (Fig. 4A). Individual small bands, however, were only barely visible within the large GS_{n1} band (Fig. 4A). In a separate experiment, when we used more protein for PAGE and photographed the gel after activity staining through a blue filter, the individual activity bands within the GS_{n1} region were distinctly visible (Fig. 5).

Both the activity and Coomassie blue staining showed that the first, second, and eighth bands in GS_{n1} were very light and the fifth band was most intense (Figs. 4B and 5). Figure 6 shows a densitometric trace of the Coomassie blue stained gel (Fig. 4B). It clearly demonstrates that the intensity of the bands increased from first to fifth and decreased from sixth to eighth. The intense ninth band was GS_{n2}. The area under the first to eighth peaks and the area under the ninth peak were cut out and weighed. The ratio of the valules was 280 to 50 or 5.6 to 1. The first to eighth peaks represented GS_{n1} and the ninth was GS_{n2}; therefore the ratio of the two isoforms is 5.6 to 1. This value is about 20% different from the 4.6 to 1 ratio calculated based on results shown in Table I.

Subunit Analysis of the Isozymes

Figure 3 shows that GS_{n1} consisted of β and γ subunit polypeptides and GS_{n2} of only β. Knowing the subunit composition of the individual bands shown in Figures 4B and 5 should be interesting. This analysis was accomplished by coupling the native and isoelectric focusing PAGE as described in "Materials and Methods." Figure 7, A and B, show the results of two separate experiments. The bands from the top of the GS_{n1} region consisted mainly of γ polypeptide. The bands from the middle had about equal amounts of β and γ. The bands from the bottom had mainly β and little γ. The GS_{n2} region had only β.

Table I. *Purification of GS_{n1} and GS_{n2} from Bean Root Nodules*

GS was assayed by its transferase activity. One unit (U) of enzyme activity catalyzed the formation of 1 μmol of γ-glutamyl hydroxamate/min at 25°C.

Purification Step	Total Protein	Total Activity	Specific Activity	Purification Factor	Recovery
	mg	*U*	*U/mg protein*		%
Crude extract	925	2035	2.2	1.0	100
Protamine sulfate	735	2058	2.8	1.3	101
$(NH_4)_2SO_4$ (35–55%)	242	1452	6.0	2.7	71
Anthranilate affinity	8.4	612	73.0	33.0	30
Dye Matrex (Orange A)	6.1	494	81.0	37.0	24
DEAE-cellulose					
GS_{n1}	2.74	329	120.0	55.0	16
GS_{n2}	0.59	86	145.0	66.0	4.2

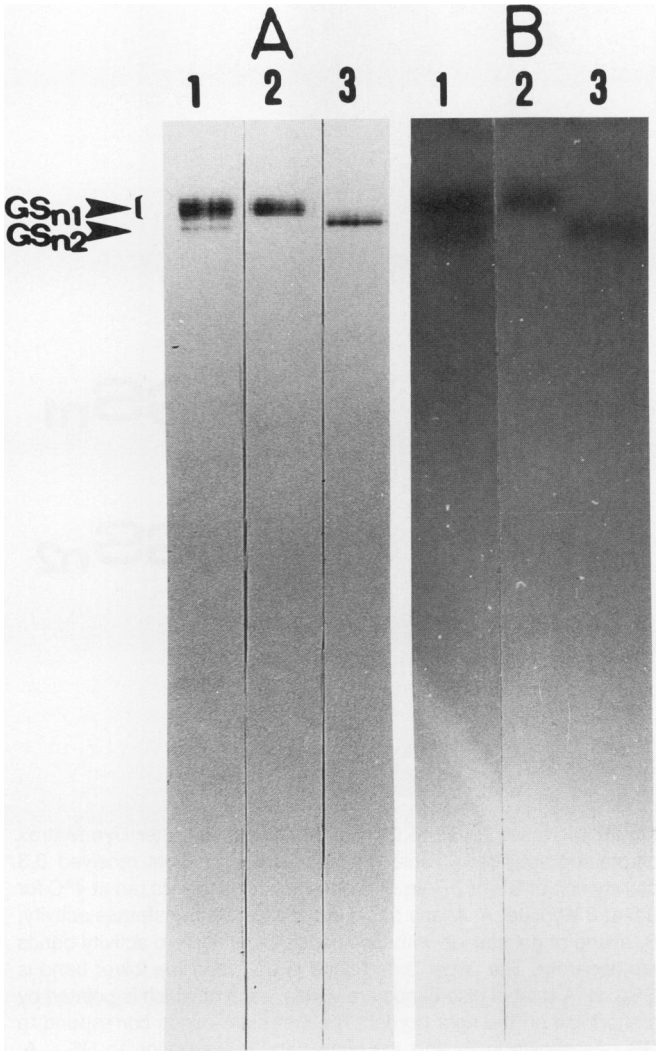

Figure 1. Native PAGE (7.5% gel) of purified GS_{n1} and GS_{n2}. Lane 1, mixture of GS_{n1} (1.8 μg) and GS_{n2} (0.8 μg); lane 2, GS_{n1} (1.8 μg); lane 3, GS_{n2} (1.6 μg). Electrophoresis ran at 4°C for 4 h at a constant power of 8 W/plate. A, The gel was stained with Coomassie blue. Note the multiple protein bands in GS_{n1}; B, the gel was stained for GA transferase activity.

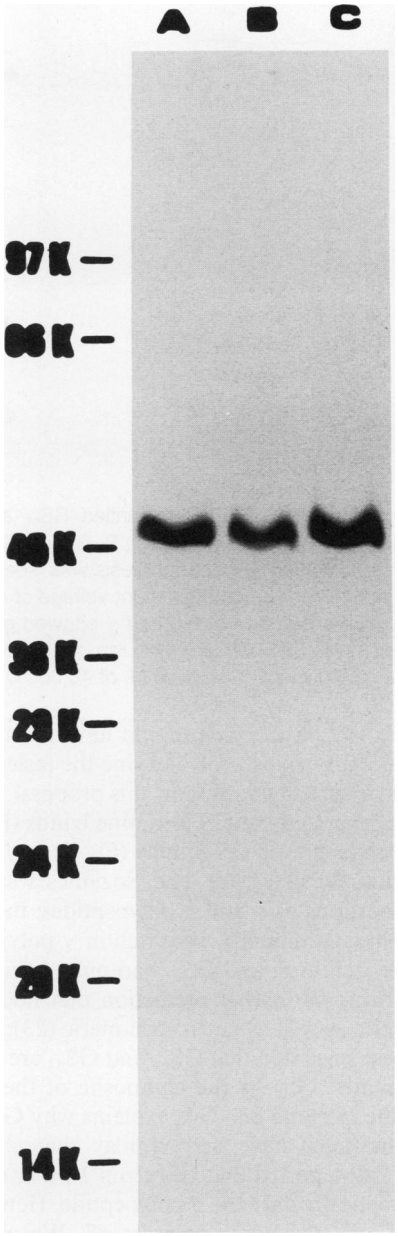

Figure 2. SDS-PAGE of purified GS_{n1} and GS_{n2}. A, Mixture of GS_{n1} (1.8 μg) and GS_{n2} (1.0 μg); B, GS_{n1} (2.9 μg); C, GS_{n2} (2.3 μg). Mol wt markers were phosphorylase β (97 kD), bovine albumin (66 kD), egg albumin (45 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), trypsinogen (24 kD), trypsin inhibitor (20.1 kD), and α-lactalbumin (14.2 kD). Electrophoresis ran at 4°C for 4 h at a constant power of 4 W/plate.

DISCUSSION

GS_{n1} and GS_{n2} from root nodules of common beans (cv Kentucky Wonder) have been purified to homogeneity (Figs. 1 and 2). GS_{n1} consisted of β and γ subunit polypeptides, which differed in pIs (6.0 and 6.3, respectively) but had the same M_r (46,000 D) (Figs. 2 and 3). GS_{n2} consisted of only β subunit polypeptide. Lara *et al.* (13) reported that both GS_{n1} and GS_{n2} have β and γ subunit polypeptides, although the amount of γ in GS_{n2} is relatively small in comparison to β. This discrepancy between their and our results may be because we have purified GS_{n2} to homogeneity. Lara *et al.* also reported that the M_r for β and γ subunit polypeptides was 43,000 D and that pIs for the two polypeptides were 6.2 and 6.6, respectively. These discrepancies between their and our results may be due to the use of different cultivars of beans or variations in analytical techniques.

The native GS consists of eight subunit polypeptides, and in the root nodules they are of two different types (3, 13). We proposed earlier that a total of nine isozymes can be generated assuming the assembly of β and γ subunit polypeptides into the holoenzyme is a random process (23). The proposal predicted that each of the nine isozymes has different proportions of β and γ (*i.e.* $\gamma 8$, $\gamma 7\beta 1$, $\gamma 6\beta 2$, $\gamma 5\beta 3$, $\gamma 4\beta 4$, $\gamma 3\beta 5$, $\gamma 2\beta 6$, $\gamma 1\beta 7$, and $\beta 8$). Since the pI of γ is greater than that of β, the isozymes should be separable from one another by

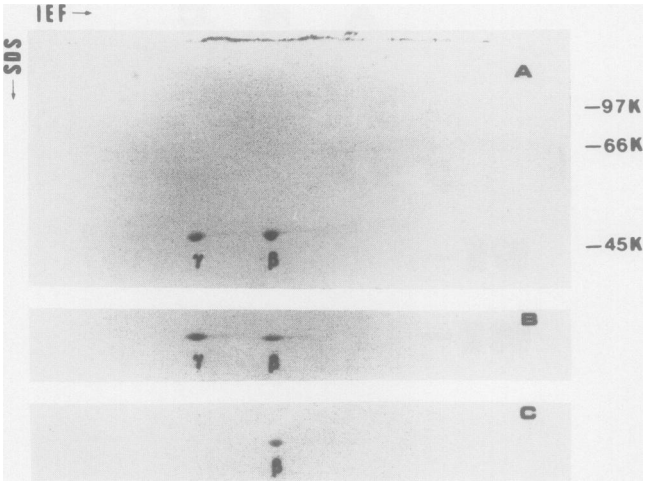

Figure 3. Two-dimensional PAGE of purified GS_{n1} and GS_{n2}. A, Mixture of GS_{n1} (1.8 μg) and GS_{n2} (1.0 μg); B, GS_{n1} (1.8 μg); C, GS_{n2} (1.2 μg). The first-dimensional electrophoresis was isoelectric focusing, which ran at 4°C for 12 h at a constant voltage of 400 V. The β subunit polypeptide showed pI of 6.0 and γ showed pI of 6.3. The second dimension was SDS-PAGE, which ran at 4°C for 5 h at 4 W/plate. Both subunit polypeptide showed M_r of 46,000 D.

native PAGE. The $\gamma 8$ isozyme should have the slowest electrophoretic mobility and the $\beta 8$ isozyme the fastest.

The results reported here support this proposal. (a) The 5% native PAGE separated the GS into nine bands (Fig. 4B). (b) Each of the bands showed GS activity (Fig. 5), indicating they were individual isozymes. (c) The isozymes were made of different proportions of γ and β polypeptides; the ones with slow electrophoretic mobility were rich in γ polypeptide, the fast ones were rich in β, and GS_{n2} had only β (Fig. 7, A and B). This confirms our earlier prediction that nodule GS are homo-octameric as well as hetero-octameric (23).

Our proposal suggested that GS_{n1} and GS_{n2} are collectively the nine isozymes. GS_{n1} is the composite of the first eight, and GS_{n2} is the isozyme $\beta 8$. This explains why GS_{n2} and GS from the root (GSr) have very similar characteristics (3). Ortega *et al.* (20) reported that GSr from 14-d or older plants consists of practically only the β polypeptide. Hence, the root can generate only the homo-octamer $\beta 8$. We propose that GS_{n2} and GSr are identical because they are both the $\beta 8$ isozyme.

Our proposal, however, cannot explain why $\beta 8$ is a predominant isozyme in the nodule. If we assume that the assembly of GS is completely random, the binomial distribution probability predicts the relative ratio of the nine isozymes as 1, 8, 28, 56, 70, 56, 28, 8, and 1. That is, for example, the chance of forming the isozyme $\gamma 8$ or $\beta 8$ is 70 times smaller than that forming the isozyme $\gamma 4\beta 4$. The ratio of the first eight isozymes (GS_{n1}) to $\beta 8$ (GS_{n2}) should be the sum of the first eight numbers to 1 or 255 to 1. However, our calculations based on results shown in Table I and Figure 6, the ratio was 4.6 to 1 and 5.6 to 1, which are very different from the predicted value of 255 to 1.

We can assume, as we did earlier (23), that the assembly of β into the holoenzyme is preferred. However, the results shown in Figure 6 did not strongly support this assumption.

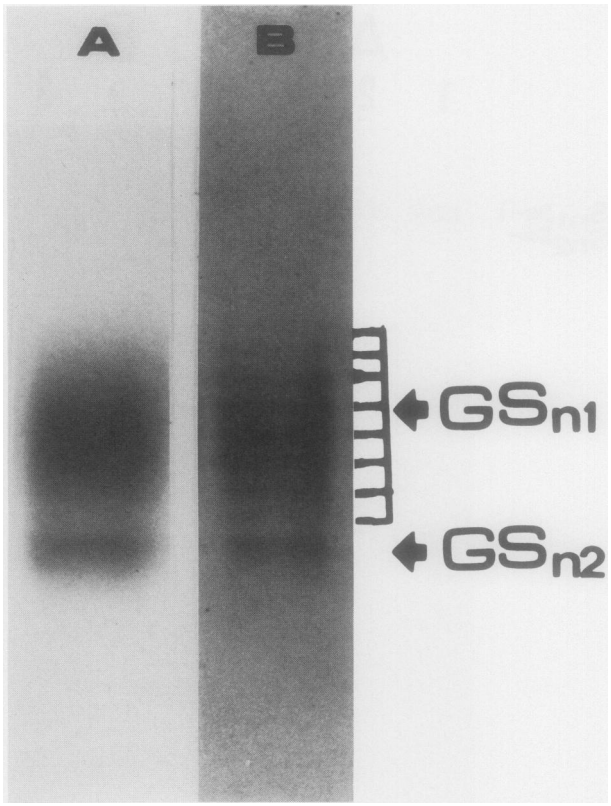

Figure 4. Native PAGE (5.0% gel) of GS purified after Dye Matrex chromatography (see Table I). Each lane of the gel received 0.3 activity unit of GS or 3.7 μg of protein. Electrophoresis ran at 4°C for 3 h at 8 W/plate. A, A lane of gel stained for GS transferase activity; B, a lane of gel stained with Coomassie blue. A, Two activity bands are apparent. The upper broad band is GS_{n1} and the lower band is GS_{n2}. B, A total of nine bands are visible, each of which is pointed by a short line on the right border. The first eight bands correspond to GS_{n1} as shown in B, and the ninth band corresponds to GS_{n2}. A, Note the faintly visible small individual bands within the GS_{n1} area.

The binomial distribution probability predicts that peaks 1 and 9 shown in Figure 6 should be of the same size. Similarly, peaks 2 and 8, 3 and 7, 4 and 6 should have equal size. Peak 5 should be the largest. Figure 6 shows that peaks 6, 7, and 8 were somewhat larger than their counterparts, which indicated the assembly of β may be slightly preferred. However, peak 9 was too disproportionally larger than peak 1 to be accounted for by the slightly preferred assembly of β.

We now believe that GS_{n2} ($\beta 8$) is predominant isozyme because there are two sources of $\beta 8$ in the nodule. A small source is in the infected cells, and a large source is in the uninfected cells, which for the purpose of this discussion are all nodule cells except the infected ones. The β polypeptide is found in the leaves, roots, and nodules (7, 13). Its gene appears to be constitutively expressed (7). Since β polypeptide is ubiquitous, it seems reasonable to assume that β is present in the infected as well as in uninfected nodule cells. The γ polypeptide, on the other hand, is nodule-specific. The expression of its gene seems to be regulated by the developmental events of the nodule (7, 12, 21). Therefore, it is possible that γ polypeptide is found only in the infected cells. If this

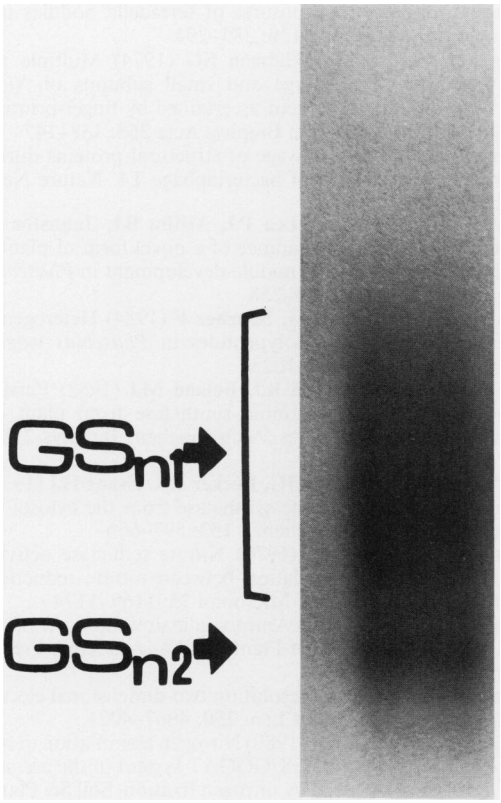

Figure 5. Native PAGE (5.0% gel) of GS purified after Dye Matrex chromatography. Each lane received 0.4 activity unit of GS or 5 μg of protein. Conditions for electrophoresis were the same as in Figure 4 except the run time was 3.5 h. The gel was stained for GS transferase activity and photographed through a blue filter. Individual bands showing GS activity are visible. The first eight bands are GS_{n1}, and the ninth heavy band is GS_{n2}.

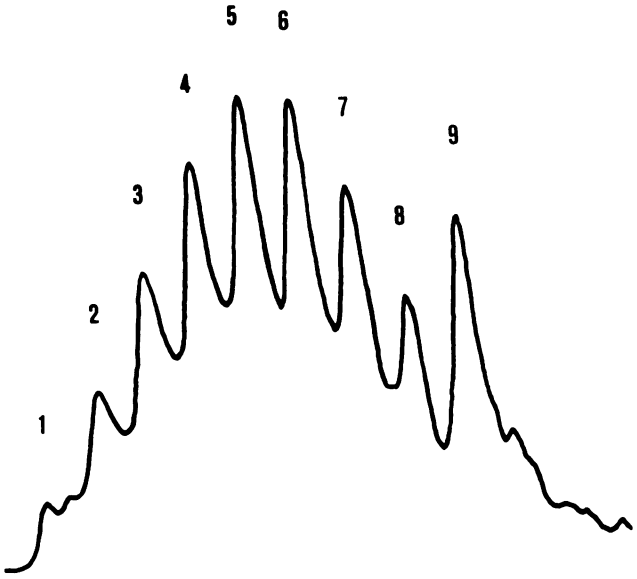

Figure 6. Densitometric trace of Figure 4B. Peaks 1 to 9 correspond to isozymes $\gamma 8$, $\gamma 7\beta 1$, $\gamma 6\beta 2$, $\gamma 5\beta 3$, $\gamma 4\beta 4$, $\gamma 3\beta 5$, $\gamma 2\beta 6$, $\gamma 1\beta 7$, and $\beta 8$, respectively.

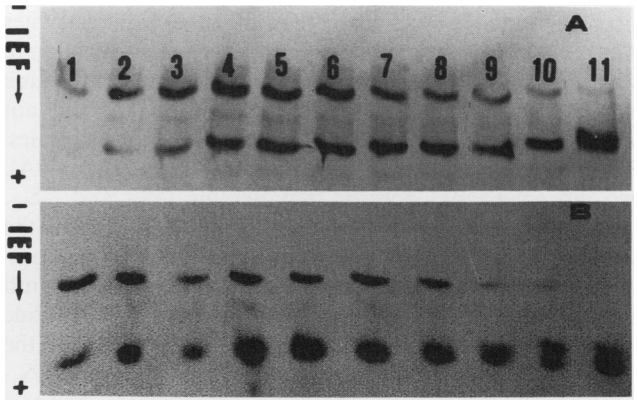

Figure 7. Two-dimensional electrophoresis of individual isozymes. The first dimension was native PAGE (5% gel). The experimental conditions of which were identical as described in Figure 4. The second dimension was isoelectric focusing, which ran at 4°C for 15 h at a constant voltage of 260 V. A and B are the results of two experiments. The numbers in both A and B indicate the position of the 1.5-mm gel pieces cut from the area of an unstained gel that corresponded to GS_{n1} and GS_{n2} as shown in Figure 4, A and B. For example, gel piece No. 1, which probably contained isozymes $\gamma 8$ and $\gamma 7\beta 1$, was cut from top of the gel whereas gel piece No. 10 or 11, which probably had isozyme $\gamma 1\beta 7$ and $\beta 8$, was cut from bottom of the gel. The results shown in A and B do not look identical because the positions of the isozymes in each of the unstained gel are difficult to localize precisely each time.

hypothesis is correct, the uninfected cells, which like the root cells have only β subunit polypeptide, could generate only the $\beta 8$ isozyme. The infected cells, which have β and γ, could generate $\beta 8$ as well as the other eight isozymes. The amount of $\beta 8$ relative to the other eight isozymes in the infected cells is, however, only 1 to 255. During the preparation of nodule extract all types of cells were macerated. The two sources of $\beta 8$ were mixed, resulting in a disproportionately larger amount of $\beta 8$ in the nodule extract.

Recently, Forde *et al.* have shown using transgenic *Lotus corniculatus* that the gene encoding γ polypeptide is expressed specifically in the infected cells (6). They showed also that the gene encoding β polypeptide is expressed widely and evenly in the newly emerged white nodule. In the intermediate-sized pink nodule, the gene is expressed both in the vascular tissue and the inner layers of the nodule cortex and only faintly in the central infected zone. In the mature nodule, the gene is expressed only in the vascular tissue. These findings indicate that in the mature nodule only two GS isozymes can be generated, $\gamma 8$ in the central infected cells and $\beta 8$ in other cells. As we hypothesized, to generate the nine isozymes, the β polypeptide as well as the γ polypeptide have to be present in the infected cells. The lack of β gene expression in the infected cells as reported by Forde *et al.* indicates that the spatial expression of this gene may be different in the *P. vulgaris* and the transgenic *L. corniculatus* nodules. So, whether β polypeptide is synthesized in the infected cells of *P. vulgaris* nodule still needs to be proven.

To account for the fact that nodule GS can be separated into two distinct isoforms by native PAGE or ion-exchange chromatography (3, 23), we suggested earlier that GS_{n2} ($\beta 8$) is

disproportionately more acidic than the other eight isozymes (GS_{n1}) (23). This suggestion is most likely incorrect. Figure 4B clearly shows that the distances separating each of the nine bands were about equal, which indicates that $\beta 8$ did not migrate disproportionately faster than the other isozymes. When the gel was stained for GS activity (Figs. 1B and 4A), two distinct bands appeared, representing GS_{n1} and GS_{n2}. This observation is an artifact. It is possible only because the amount of the eighth band (isozyme $\gamma 1\beta 7$) is small in comparison to the disproportionately large amount of the ninth band ($\beta 8$) (Fig. 4B). The low activity of isozyme $\gamma 1\beta 7$ made it look as though GS_{n2} can be separated from the rest of the isozymes. The same reasons may explain why GS_{n1} and GS_{n2} are separable by ion-exchange chromatography (3). The two activity peaks can be separated because of the low $\gamma 1\beta 7$ activity and the high $\beta 8$ activity.

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