Purification and partial characterization of glutamine synthetase from root nodules of faba bean

Purificación y caracterización parcial de la glutamina sintetasa de nódulos radicales de haba.

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ABSTRACT

Glutamine synthetase (GS, EC 6.1.3.2) from the plant fraction of *Vicia faba* L. nodules was purified to apparent homogeneity using Sepharose-anthranilic acid affinity chromatography. The enzyme is composed of three 40 kD polypeptides and has a native molecular weight of 310-330 kD, determined by gel filtration chromatography and native gradient polyacrylamide gel electrophoresis, respectively, and showed cross-reactivity with antibodies obtained against *Phaseolus vulgaris* nodule GS. Two isoenzymes, GSn-1 and Gsn-2, were separated in nodules by anion-exchange chromatography on Q-Sepharose, while just one form could be identified in roots, all the isoenzymes being identical in subunit composition and native molecular weight. The transferase to semibiosynthetic activity ratio of all three GS forms was found to be unusually low. Glutamine synthetase abundance in nodules was quantified by densitometry, representing about 5% of total soluble protein extracted. Possible significance of these and other characteristics of *V. faba* GS is discussed.

Key words: Enzyme isoforms. Glutamine synthetase. Nodules. Root. *Vicia faba*.

RESUMEN

La glutamina sintetasa (GS, EC 6.1.3.2) de la fracción vegetal de nódulos radicales de *Vicia faba* L. se ha purificado hasta homogeneidad aparente utilizando cromatografía de afinidad con sefarosa-antranílico. El enzima está compuesto de tres polipéptidos diferentes de 40 kDa y presenta un peso molecular nativo de 310-330 kDa, determinado por cromatografía de exclusión molecular y electroforesis nativa en gradiente de poliacrilamida, respectivamente, y mostró reacción cruzada con anticuerpos obtenidos frente a la GS de nódulos de *Phaseolus vulgaris*. Dos isoenzimas, GSn-1 y GSn-2, fueron separadas en nódulos mediante cromatografía de intercambio aniónico en Q-sefarosa, mientras que sólo una pudo ser identificada en raíz. Todas ellas mostraron idéntico número de subunidades y peso molecular nativo. La relación de actividad transferasa-semibiosintética fue, para las tres isoformas de GS, inusualmente baja. La

abuandancia del enzima en nódulos fue cuantificada mediante densitometría y representó aproximadamente el 5% de la proteína soluble extraída. Se discute la posible significación de estas y otras características del enzima en *V. faba*.
Palabras clave: Glutamina sintetasa. Isoenzimas. Nódulos. Raíces. *Vicia faba*.

Recibido:1-10-1997
Aceptado:28-11-1997
BIBLID [0004-2927(1997) 38:4; 345-356]

INTRODUCTION

In legumes, nodule glutamine synthetase is a major enzyme responsible for the assimilation of the ammonia produced by nitrogen fixation in the bacteroids (for a review see ref. 1). Moreover, in bean, GS activity has been reported to be a rate limiting step in N_2 fixation and then for growth and yield (2). Thus an understanding of functioning, isoforms occurrence, polypeptide composition and regulation of nodule GS will contribute to maximization of nitrogen fixation in legume crops.

GS has been purified from a number of plant organs and species, showing a native MW of about 350 kD and it is composed of 8 subunits of MW in the range of 38-45 kD (3). Multiple GS forms have been separated by both ion-exchange chromatography and native PAGE from several plant tissues and more recently it has been shown that these isoenzymes arise from differential expression of a multigene family (4, 5). *Phaseolus vulgaris* nodules are one of the best characterized GS systems, showing two main isoenzymes designated GSn-1 and GSn-2. GSn-1 is composed by the nodule-specific polypeptide called γ and the GSn-2 isoform is composed by the β polypeptide, which is also expressed in roots and leaves (6). In mature leaves, two forms (GS_1 and GS_2) and six polypeptides (α, β, a, b, c, and d) have been described while in roots just one form and two polypeptides (α and β) were reported (6).

Little, if any, is known about GS from *Vicia faba* nodules. In this work we report its purification and characterization, as well as separation of enzyme isoforms, as a first step in the study of such a key enzyme in this valuable crop.

MATERIALS AND METHODS

Plant material

Vicia faba L. cv. Alborea was inoculated with the wild type *Rhizobium leguminosarum* bv. *viciae* GRL19 (7) and grown in 1-litre sterile Leonard jars with vermiculite and mineral solution (8) containing 2 mM KNO_3. Growth

nditions were previously described (9). Fully functioning nodules and young rtions of roots were harvested at flowering (42-44 DAP), frozen in liquid trogen and stored at –40°C until use.

odule GS purification

All purification steps were carried out at 0-4°C, basically according to ara *et al.* (6) with some modifications. Nodule crude extract (20 g) was epared with 40 ml of extraction buffer, comprising 10 mM Tris-acetate pH 8, $MgSO_4$ $7H_2O$ 8 mM, sodium L-glutamate 5 mM, EDTA 0.5 mM, $NaHSO_3$ 5 mM, 10% (v/v) glycerol and 0.04% (v/v) 2-mercaptoethanol (buffer A). he brei was centrifugated at 20000 *g* for 20 min and the supernatant was aded on a DEAE-Sephacel column (2.5 × 30 cm), equilibrated with buffer . Proteins were eluted with 10-fold concentrate buffer A containing 10% (v/v) lycerol (buffer B). Fractions showing GS (EC 6.1.3.2) activity were pooled, dding $MnCl_2$ up to a final concentration of 2.25 mM, and further purified by ffinity chromatography on a column (1 × 20 cm) of Sepharose-anthranilic cid (10) equilibrated with buffer B. After washing with buffer A, proteins ere eluted with 100 ml of a 0 to 30 mM linear AMP gradient in buffer B ontaining $MnCl_2$. Fractions showing maximum GS activity were pooled, oncentrated with $(NH_4)_2SO_4$, resuspended in 1.5 ml buffer A containing .125 mM $MnCl_2$, and dialysed in the same buffer. After dialysis, protein was ivided into aliquots and stored at –40°C.

GS activity and protein determination

GS activity was measured by the transferase (11) and semibiosynthetic (12) ssays. One unit of activity represents 1 μmol of γ-glutamyl-hydroxamate ormed per minute at 30°C. Protein was determined colorimetrically by the Bradford procedure (13).

Separation of GS isoforms from root and nodules

Nodule proteins were extracted as described above (10 g/20 ml). After chromatography of crude extract on DEAE-Sepharose (2.6 × 16 cm), as described for DEAE-Sephacel, fractions with highest GS transferase activity were pooled and dialysed in buffer A at pH 7.0. The dialysed sample was oaded on a Q-Sepharose column (1.5 × 8.5 cm) equilibrated and washed with he same buffer and eluted with 200 ml of a 0 to 400 mM KCl linear gradient.

From each peak, fractions containing the highest GS activity were pooled, concentrated, and dialysed by ultrafiltration in an Amicon 8050 system (YM-100 membrane) operated at 4°C. Roots (35 g) were ground in a mortar with liquid N_2 and extracted with 100 ml of buffer A containing 1% (v/v) glycerol and 7.5% (w/v) PVPP, using a Sorvall Omni-mixer operated at maximum speed for a total time of 3 min. Further steps were performed as described for nodules.

Sephacryl S-300 chromatography

To determine native MW of the different GS isoforms, DEAE-Sepharose samples were applied to a Sephacryl S-300 column (1.5 × 70 cm) equilibrated with buffer A. Proteins were eluted with the same medium and 1.5 ml fractions were collected. Identical procedure was carried out for MW markers.

Electrophoresis and polypeptide analysis

Non-denaturing PAGE was performed at 4°C using a 4-16% acrylamide linear gradient and GS bands were stained by the transferase assay. Coomassie R-250 stained gels were used to determine GS abundance in nodule crude extracts by scanning densitometry in a Beckman DU-70 spectrophotometer. SDS-PAGE as well as immunoprecipitation, two-dimensional electrophoresis and Western immunodetection for polypeptide analysis were carried out as previously reported (6, 14, 15) using antibodies obtained against *V. faba* and *P. vulgaris* nodule GS.

RESULTS

The GS from the cytosolic fraction of *Vicia faba* nodules was purified to homogeneity by affinity chromatography using anthranilic acid coupled to Sepharose 6B (Tab. 1, Fig. 1A). The low specific activity and the amount of protein obtained could indicate that *V. faba* nodule GS is specifically and irreversibly inactivated during the Sepharose-anthranilic acid chromatography. Due to this fact the final purification factor was only 15, although the amount of protein was 420-fold lower than the initial one in the crude extract.

The nodule GS migrates as a single protein band of about 40 kD on SDS-PAGE (Fig. 1A). The two-dimensional electrophoresis analysis of the purified enzyme shows two different GS polypeptides, called β and γ, with identical MW but different isoelectric points (Fig. 1B). This result was confirmed by

Table 1.—Purification of glutamine synthetase from root nodules of *Vicia faba*.

Step	*Total protein (mg)*	*Total activity (units)*	*Specific activity U.(mg protein)$^{-1}$*	*Purification (-fold)*	*Re*
Crude extract	795.6	490.6	0.62	1.0	
DEAE-Sephacel	53.6	328.6	6.13	9.9	
Sepharose-anthranilate	15.1	63.5	4.20	6.8	
$(NH_4)_2SO_4$ precipitation	1.9	17.2	9.10	14.7	

an immunoprecipitate of *V. faba* nodule crude extract, using antibodies ra against *P. vulgaris* nodule GS, subjected to two-dimensional electrophor (not shown). However, Western immunodetection of the purified nodule revealed the presence of a minor quantity of a third additional polypepti called α (Fig. 1C).

The separation of GS isoforms from *V. faba* nodules and roots was carr out by anion-exchange chromatography. In nodules, two peaks showing activity were clearly distinguishable in the eluate from the Q-Sepharo column, the first one (GSn-1) eluted at 125 mM KCl while the second (GSn- eluted at 270 mM and contained most of the activity (80% of total eluted However, in the case of roots only one peak eluted from the Q-Sepharos also at 270 mM coinciding with GSn-2 (Fig. 2). The ratio transferase semibiosynthetic activity for these three isoforms of GS was 7, 9, and 1 respectively while the same ratio for the crude extracts was 18 for root G and 15 for the nodule enzyme, which indicates that both activities wer differentially affected by the purification process.

The native MW of GS isoforms was estimated by both non-denaturing PAGE and gel filtration chromatography on Sephacryl S-300, using partially purified GS (from Q-Sepharose). Native PAGE stained for transferase activity revealed a single band of MW about 330 kD in all cases (GSn-1, GSn-2 and root GS)(Fig. 3). The estimation by filtration on Sephacryl S-300 for the three isoforms was also identical but a little lower, about 310 kD.

After identification of GS band, scanning densitometry analysis of a Coomassie-stained native gel (not shown) of nodule crude extract revealed that GS represented about 5% of the total soluble protein extracted from *V. faba* nodules.

The specificity of antibodies raised against *V. faba* nodule GS was tested by immunoblot of a nodule crude extract subjected to SDS-PAGE, showing a single band of 40 kD (Fig. 4). An identical result was obtained with the different partially purified preparations from roots and nodules.

Table 1.—Purification of glutamine synthetase from root nodules of *Vicia faba*.

Step	*Total protein (mg)*	*Total activity (units)*	*Specific activity U.(mg protein)$^{-1}$*	*Purification (-fold)*	*Recovery (%)*
Crude extract	795.6	490.6	0.62	1.0	100
DEAE-Sephacel	53.6	328.6	6.13	9.9	67
Sepharose-anthranilate	15.1	63.5	4.20	6.8	13
$(NH_4)_2SO_4$ precipitation	1.9	17.2	9.10	14.7	3

an immunoprecipitate of *V. faba* nodule crude extract, using antibodies raised against *P. vulgaris* nodule GS, subjected to two-dimensional electrophoresis (not shown). However, Western immunodetection of the purified nodule GS revealed the presence of a minor quantity of a third additional polypeptide, called α (Fig. 1C).

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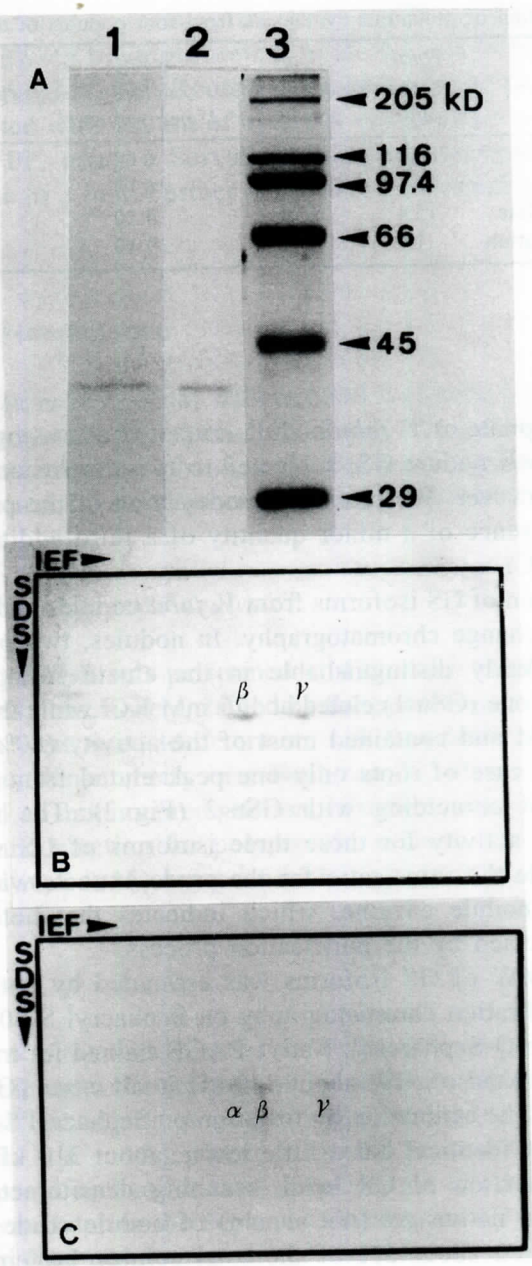


Fig 1.—**A.** Coomassie-stained SDS-PAGE of purified GS from nodules of *V. faba*. (1) 6 μg of GS from Sepharose; (2) 6 μg of pure GS; (3) Molecular weight markers, miosin 205 kD, β-galactosidase 116 kD, phosphorylase 97.4 kD, bovine serum albumin 66 kD, ovalbumin 45 kD, carbonic anhydrase 29 kD. **B.** Two-dimensional electrophoresis of purified *V. faba* GS. **C.** Western immunodetection of GS polypeptides in fig. 1B, using antibodies against *P. vulgaris* nodule GS. α, β and γ are GS polypeptides.

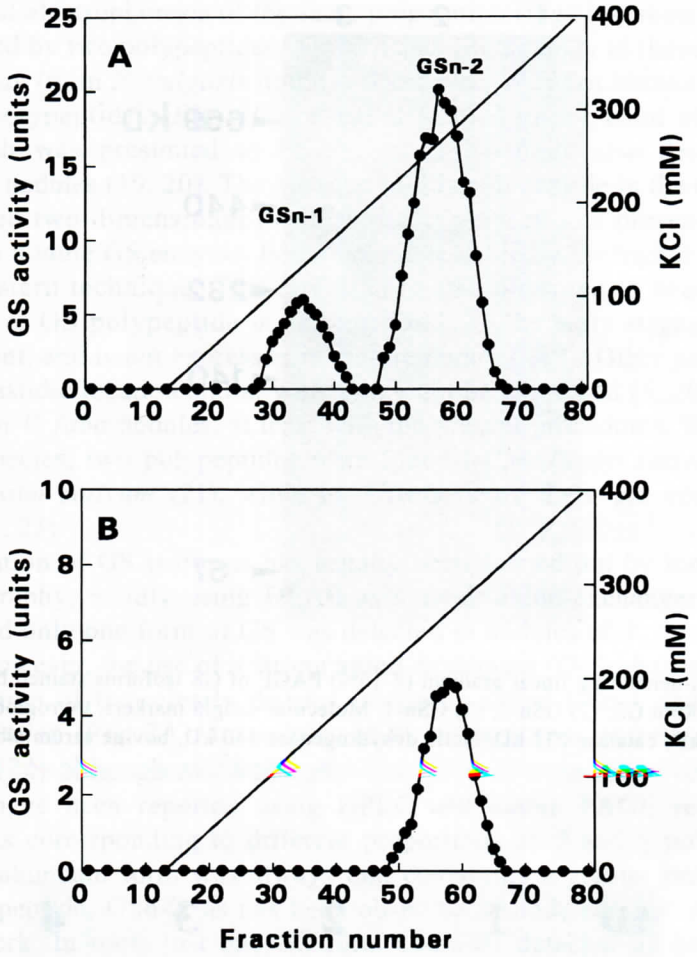


Fig. 2.—Elution profile after chromatography on Q-Sepahrose of partially purified GS from nodules (A) and roots (B). GSn-1 and GSn-2 are nodule GS isoenzymes.

DISCUSSION

The Sepharose-anthranilic acid chromatography has been successfully used to purify GS from such different sources as *Neurospora crassa* (10) or *P. vulgaris* (6, 16). In *V. faba* nodules, however, although GS was purified to homogeneity (Fig. 1A) with this method, the enzyme was irreversiblely inactivated during chromatography (Table 1), indicating possible differences with GS from other sources.

SDS-PAGE of the purified enzyme showed a subunit MW (40 kD) very similar to those obtained for nodule GS from other legumes (17, 6, 18). Two-

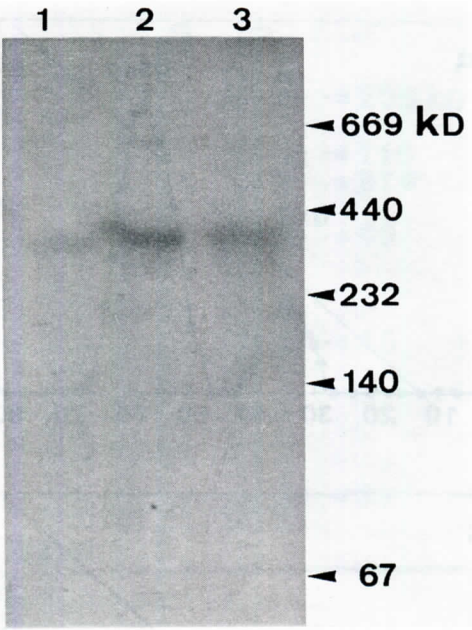


Fig. 3.—Non-denaturing linear gradient (4-16%) PAGE of GS isoforms stained by transferase activity. (1) Root GS; (2) GSn-2; (3) GSn-1. Molecular weight markers, thyroglobulin 669 kD, ferritin 440 kD, catalase 232 kD, lactic dehydrogenase 140 kD, bovine serum albumin 67 kD.

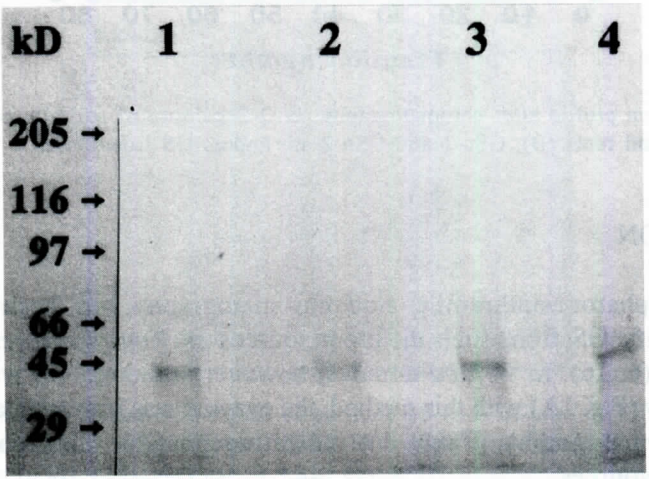
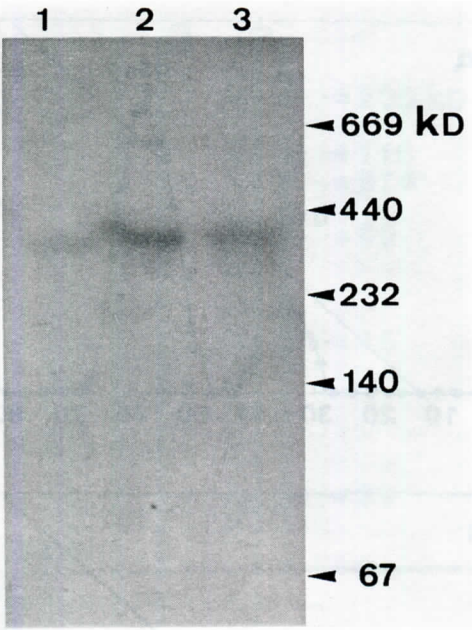


Fig. 4.—Western-blot of the different GS isoforms after SDS-PAGE. (1) GSn-1; (2) GSn-2; (3) Root GS; (4) nodule crude extract. Molecular weight markers as in fig. 1A.



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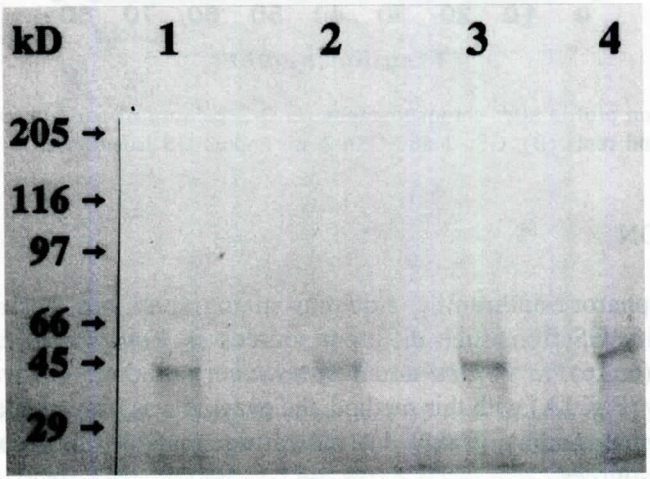

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dimensional electrophoresis of the same preparation (Fig. 1B) showed that GS is composed by two polypeptides, called β and γ in analogy to those described by Lara *et al.* (6) in *P. vulgaris* nodules. However, Western immunodetection of these polypeptides (Fig. 1C) revealed a third minor band of identical MW which was presumed to be the α polypeptide also described for *P. vulgaris* nodules (19, 20). The absence of this polypeptide in the Coomassie blue stained two-dimensional PAGE indicates a very low presence of α in the *V. faba* nodule GS enzyme, being only detectable by the higher sensitivity of the Western technique. This situation is also observed in bean nodules, where the α GS polypeptide is presented only in the early stages of nodule development, and is not expressed in mature nodules (19). Other polypeptides as δ, of plastidic location and ε, which may not be expressed (5, 20) were not observed in *V. faba* nodules, at least with the present procedures. With regard to other species, two polypeptides were found in *Medicago sativa* (17) and five in *Pisum sativum* (21), while in *Glycine max* there are contradictory results (22, 23).

Separation of GS isoforms has usually been carried out by ion-exchange chromatography, mainly using DEAE as a weak anion-exchanger, and with this method only one form of GS was detected in nodules of *V. faba* (data not shown). However, the use of a strong anion-exchanger (Q-Sepharose) allowed the separation of two clearly distinct isoforms, called GSn-1 and GSn-2 in order of elution (Fig. 2A). In nodules of *P. vulgaris* two isoforms were also separated (24) although more recently four (20) or even nine (16) different isoforms have been reported using HPLC and native PAGE respectively, these forms corresponding to different proportions of β and γ polypeptides. The most abundant form was always that containing a greater proportion of the β polypeptide, GSn-2, as has been observed in nodules of *V. faba* in the present work. In roots just one GS form could be detected by both DEAE-Sepharose (data not shown) and Q-Sepharose (Fig. 2B), which eluted at the same ionic strength as GSn-2. A similar situation was found in *P. vulgaris* (24, 25), although in chickpea, root GS eluted at a similar ionic concentration as GSn-1 (26). Cai and Wong (27) proposed that both GSn-2 and root GS are homooctameric (β_8) forms of the enzyme. This circumstance, however, depends on root development stage, as root embryo GS is mainly composed by the α-polypeptide and then its proportion decreases in relation to the β polypeptide during root development in *P. vulgaris* (14).

All the GS forms showed identical MW, by both filtration chromatography (310 kD) and native PAGE (330 kD, Fig. 3), and accordingly they should be structured as octameric enzymes. These forms also share antigenicity, as root GS was recognized by antibodies raised against nodule GS (Fig. 4), probably due to the β-polypeptide. In this sense, *V. faba* and *P. vulgaris* nodule GS presented common antigenic determinants as anti-*P. vulgaris* GS recognized

and precipitated *V. faba* GS. Also deserving attention was the low transferase to semibiosynthetic ratios found for the different GSs in *V. faba* (see above) compared to those of *P. vulgaris* (2, 19, 20).

Densitometric analysis of native PAGE of crude extract showed that nodule GS in *V. faba* is a highly abundant protein, representing up to 5% of total soluble protein extracted. This value is clearly higher than the 1.2 to 2% described for other legumes (17, 28). Nevertheless, the ratio transferase to semibiosynthetic activity in *V. faba* suggests that GS might have been limiting the symbiotic efficiency in this crop. Further studies to ascertain whether differences observed between *V. faba* and *P. vulgaris* GSs result from the tropical-temperate legume difference, represent coincidence, or the fundamental behaviour of the nodules of meristematic and determinate type will be of valuable interest.

ACKNOWLEDGEMENTS

Financial support was obtained through Plan Andaluz de Investigación, Grupo No. 2037. J.M.C. thanks Spanish M.E.C. for a fellowship to work in Dr. Lara's laboratory.

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