Heterogeneity of Glutamine Synthetase Polypeptides in
*Phaseolus vulgaris* L.  

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ABSTRACT

Glutamine synthetases from roots, nodules, and leaves of *Phaseolus vulgaris* L. have been purified to homogeneity and their polypeptide composition determined.

The leaf enzyme is composed of six polypeptides. The cytosolic fraction contains two 43,000 dalton polypeptides and the chloroplastic enzyme is formed by four 45,000 dalton polypeptides. Root glutamine synthetase consists only of the same two polypeptides of 43,000 dalton that are present in the leaf enzyme. The nodule enzyme is formed by two polypeptides of 43,000 dalton, one is common to the leaf and root enzyme but the other is specific for *N*₂-fixing nodule tissue. The two glutamine synthetase forms of the nodule contain a different proportion of the 43,000 dalton polypeptides.

Glutamine synthetase (EC 6.3.1.2.) is the major enzyme for ammonia assimilation in higher plants (15). GS⁵ from different plant tissues has a mol wt in the range of 350,000 to 400,000 (14, 17). The enzyme consists of eight subunit of 43,000 to 47,000 D. Multiple forms of GS have been separated by ion exchange chromatography from a variety of plant tissues. In green tissue of barley (12) and rice (7, 9), cytosolic and chloroplastic GS (called GS; and GS₂) have been described. In root tissue only one form of GS has been found (12, 17). Two forms of GS, GS₃-1 and GS₃-2 have been observed in *Phaseolus* root nodules (3). GS₃-1 is a new form which appears during nodule development (10), whereas GS₃-2 is undistinguishable from the one found in roots (3). Recently, two GS polypeptides have also been identified in alfalfa root nodules (6). One of the best characterized eucaryotic GS is the one from *Neurospora crassa*, in which two nonidentical polypeptides are structured in different oligomeric forms (4, 11, 21). This paper reports the purification and polypeptide composition of GS from leaves, roots, and nodules of *Phaseolus vulgaris*.

MATERIALS AND METHODS

**Plant Material.** *Phaseolus vulgaris* L. cv Ojo de cabra was inoculated with the wild type *Rhizobium phaseoli* strain CFN-

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2 Abbreviations: GS, glutamine synthetase; GS₁, cytosolic glutamine synthetase; GS₂, chloroplastic glutamine synthetase; GS₃-1 and GS₃-2, nodule glutamine synthetases; RB, running buffer; PBS, phosphate buffered saline; IP, isoelectric point.

42 (20) and grown for 4 weeks in a glasshouse under natural daylight (14 h) (mean day temperature, 25 to 30°C; RH, 45%). The plants were watered every other day with nitrogen-free nutrient solution (2), and with water alternatively. Nodules and leaves were harvested after 4 weeks, roots from noninoculated plants after 2 weeks. Etiolated leaves were obtained after 10 d from noninoculated plants grown at 30°C in the dark and watered only with distilled H₂O.

**Root GS Purification.** All purification steps were carried out at 4°C. Root tissue, frozen in dry ice, (100 g) was ground in a mortar with 100 ml of 2-fold concentrated RB (10 mm Tris-HCl (pH 8.4), 10 mm Mg-acetate, 10% (v/v) glycerol). The brei was filtered through four layers of muslin and centrifuged at 20,000 g for 45 min. The supernatant was brought to 70% (NH₄)₂SO₄, centrifuged at 15,000 g for 15 min. The pellet was resuspended in 10 ml RB, desalted on a Sephadex G25 column (2.5 x 40 cm), and loaded on a DEAE cellulose column (2.5 x 30 cm). Both columns were previously equilibrated in RB. After washing with 400 ml RB, proteins were eluted with 400 ml of a 0 to 0.4 M KCl linear gradient in RB. Four-ml fractions were collected and those showing GS activity were pooled and chromatographed on a column (1 x 25 cm) of Sepharose-anthranilic acid as described previously (18), omitting MnCl₂ in the elution gradient. The fractions showing GS activity were concentrated 5- to 10-fold in a dialysis tube immersed in crystalline sucrose and then dialyzed overnight in 10-fold diluted RB containing 10% (v/v) glycerol. The purified enzyme solution was brought to 50% (v/v) with glycerol and stored at -20°C.

**GS Purification from Nodules.** Nodule extract was prepared by grinding 20 g of nodule tissue in a mortar with 100 ml of extraction buffer, 100 mm Tris-HCl (pH 8.4), 10 mm Mg-acetate, 10% (v/v) glycerol, 0.5% (v/v) Triton X-100. The brei was centrifuged at 20,000 g for 45 min. The supernatant (100 ml), was precipitated by adding 1 volume of acetone at -20°C and immediately centrifuged at 16,000 g for 15 min. The collected pellet was dried with filtered air, resuspended in RB, and loaded on a column (2.5 x 40 cm) of DEAE cellulose. Further purification was done following the same steps described for root GS.

**GS Purification from Green and Etiolated Leaves.** Green (50 g) or etiolated (12 g) leaves were ground in a mortar with dry ice to yield a fine powder. Two volumes (w/v) of extraction buffer, 100 mm (Tris-HCl (pH 8.4), 10 mm Mg-acetate, 10% (v/v) glycerol, 10 mm β-mercaptoethanol, 1 mg/ml of BSA (w/v), were added. The extract was filtered through four layers of muslin and centrifuged at 20,000 g for 45 min. The supernatant was fractionated with (NH₄)₂SO₄. The precipitate obtained from 35 to 70% saturation was dissolved in a minimal volume of RB containing 10 mm β-mercaptoethanol. The sample was applied on a column (2.5 x 60 cm for green and 1 x 68 cm for etiolated leaves) of Sephacryl S-300 equilibrated in the same buffer. The volume of the sample applied did not exceed 2.5% of the volume of the
column. Proteins were eluted with RB and fractions containing GS activity were pooled and loaded on a column of Sepharose-anthraniolic acid as described above.

**Glutamine Synthetase Determination.** GS activity was measured by the transferase assay of Ferguson and Sims (5). One unit of activity represents 1 μmol of γ-glutamyl-hydroxamate formed per minute at 30°C.

**Polypeptide Analysis of the GS Forms.** GS forms were isolated by ion exchange chromatography from crude extracts of nodule and etiolated leaf, following the procedure described by Lara et al. (10). The fractions containing the highest activity of each peak were pooled and immunoprecipitated as reported previously (11) using anti-GS serum raised against the purified enzyme from nodule for the nodule GS forms and from green leaf for the leaf GS forms. These immune complexes were analyzed by two-dimensional gel electrophoresis.

**Electrophoresis.** SDS-PAGE was performed in 7.5% (w/v) polyacrylamide gel according to Palmiter et al. (19). Two-dimensional gel electrophoresis was done according to O'Farrell (16), except that electrofocusing gels were run at 400 v for 20 h omitting the final high voltage pulse. Electrophoresis in the second dimension was carried out as previously reported (21) without urea. Proteins were stained with Coomassie blue R (13).

**Protein Determination.** Protein was measured colorimetrically by the Bradford procedure (1).

### RESULTS

The GS from roots, nodules, and leaves was purified using a column of anthranilic acid bound to Sepharose (Table I). This procedure of affinity chromatography was reported previously for *N. crassa* GS (18). SDS-PAGE shows that the enzyme from roots or nodules migrates as a single protein band of 43,000 D (Fig. 1, lanes 1 and 2). GSs from green and etiolated leaves present two protein bands of 45,000 and 43,000 D (Fig. 1, lanes 3 and 4). The 45,000 D band is the major component in the green leaf enzyme, while in etiolated leaves both proteins are present in approximately equal amounts. A band with higher mol wt is still observed in the etiolated leaf (Fig. 1, lane 4).

Subsequently, the polypeptide composition of the purified enzymes was analyzed by two-dimensional gel electrophoresis. Root GS is composed of two polypeptides, called α and β, IP-5.8 and 6.2, respectively (Fig. 2, A). The nodule GS is formed by two polypeptides. The first one (γ) is a specific nodule component and the second one is similar to the β polypeptide observed in roots (Fig. 2, B). Root and nodule enzymes were co-electrophoresed and only three polypeptides were observed indicating that β is the same in both tissues (Fig. 2, C). The 43,000 D GS band in the leaf enzymes is composed of the same α and β polypeptides as present in the root GS (Fig. 2, D and E). This was confirmed when both root and leaf enzymes were co-electrophoresed (Fig. 2, F). The 45,000 D protein band observed in the enzyme of green leaves is composed of four polypeptides, called a, b, c, and d. In the etiolated leaf component 'a' is not observed (Fig. 2, D and E).

To demonstrate if the native GS forms contain the same polypeptides found in the pure enzymes, GSN-1 and GSN-2 from nodule extract and GS1 and GS2 from etiolated leaf extract were separated using a DEAE-Sephalac column (Fig. 3, A and B).

### Table I. Purification of GS from Roots, Nodules, Green, and Etiolated Leaves of Phaselous vulgaris

<table>
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<tr>
<th>Purification Step</th>
<th>Total Protein</th>
<th>Total Units</th>
<th>Specific Activity</th>
<th>Purification Factor</th>
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<td>Crude extract</td>
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<td>(NH4)2SO4</td>
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Fig. 1. SDS-PAGE of the purified GS from different tissues of *P. vulgaris*. SDS-PAGE of the GS purified from (1) root (8 μg), (2) nodule (10 μg), (3) green leaf (12 μg), and (4) etiolated leaf (4 μg) of *P. vulgaris*. Mol wt markers: bovine plasma albumin, 66,000; ovalbumin, 45,000; trypsinogen, 24,000; β-lactoglobin, 18,400; and lysozyme, 14,300. Proteins were stained with Coomassie blue R.
Fractions with the highest enzyme activity from each peak were immunoprecipitated and their polypeptide composition was analyzed. The γ polypeptide from nodules is the main component in the GSn-1 form (Fig. 4, A), while the β polypeptide is present in a high proportion in the GSn-2 form (Fig. 4, B). Analysis of the leaf GS forms indicated that the chloroplastic GS2 form is composed of b, c, and d polypeptides (Fig. 4, D). The GS1 form is mainly composed of α and β polypeptides (Fig. 4, C), but a third component appears weakly between α and β.

Furthermore, the component 'd' is still observed in the GS1 fraction. Studies are in progress to clarify whether 'd' is a real component of the cytosolic GS1, or a cross-contamination between the GS1 and GS2 peaks.

**DISCUSSION**

The results reported here demonstrate that the multiple forms of GS are related to the presence of different polypeptides of this enzyme in *P. vulgaris*.

The polypeptide analysis of the two nodule forms of GS (3) shows that GSn-1 has a higher proportion of the γ and GSn-2 of the β polypeptide (Fig. 4, A and B). Improved chromatographic methods for the separation of the two GS forms are required to elucidate if the presence of β in GSn-1 and of γ in GSn-2 is not an artifact. GSn-1 form, which is expressed only in effective nodules (10), is composed mainly of the nodule specific polypeptide γ. This suggests that this polypeptide is responsible for the assimilation of ammonium produced by the bacteroids during symbiosis.

In the root tissue, the GS is composed of two polypeptides which comigrate with the α and β polypeptides of the leaf GS1 form (Fig. 2, F), indicating that the root GS and the cytosolic GS1 form are identical. Considering our polypeptide analysis of leaf and root extracts, we presume that the component observed between α and β in the GS1 peak (Fig. 4, C) has no relation with the GS polypeptides.

The GS2 form is composed mainly of the 45,000 D polypeptides b, c, and d (Fig. 4, D). The chloroplastic localization of this form (22) and the absence of these higher mol wt polypeptides in the root and nodule GS and in embryo leaves (data not shown), imply that this GS2 form could be encoded by a gene that is only expressed in mature leaves. During the preparation of this paper, the multiple polypeptide composition in the chloroplastic GS2 has been described (8). It is not yet known whether these polypeptides are encoded by one or more different genes or whether they arise by post-transcriptional modification. The a, b, c, and d GS polypeptides are specific for mature leaves and the γ polypeptide is specific for nodules. The physiological and developmental importance of these observations is currently under study.
FIG. 3. Elution profile after chromatography on DEAE-Sephacel columns of the different GS forms from nodule and etiolated leaf extracts. A, Nodule glutamine synthetase forms, GSn-1 and GSn-2; B, etiolated leaf glutamine synthetase forms GS1 and GS2.

FIG. 4. Two-dimensional gel electrophoresis analysis of the GS forms from nodule and etiolated leaf. Two-dimensional gel electrophoresis of the immunoprecipitated GS. A, GSn-1; B, GSn-2; C, GS1; D, GS2. a, b, c, and d are GS polypeptides; SP, serum proteins.
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LITERATURE CITED


