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Short Communication

Isolation and Immunochemical Characterization of Plant Glutamine Synthetase in Alfalfa (*Medicago sativa* L.) Nodules[1]

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ABSTRACT

Host plant glutamine synthetase (GS) has been purified 100-fold from N_2-fixing alfalfa (*Medicago sativa* L.) nodules by a new procedure involving preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as a final step. An SDS-polypeptide fraction corresponding to plant GS was identified and consisted of two major polypeptides of 40,000 to 45,000 molecular weight. Antibodies to the SDS-polypeptide fraction were raised in mice by intraperitoneal injection, and antisera were collected as ascitic fluid. Crude extracts of soluble protein from the plant fraction of nodules were resolved by SDS-PAGE and then subjected to electrophoresis in the second dimension into antibody-containing agarose gel. A single immunochemically active protein species was observed using this crossed immunoelectrophoresis method, even though both major GS SDS-polypeptides were apparently resolved in the first (SDS-PAGE) dimension. Plant GS protein in crude nodule extracts was quantitated immunochemically by comparison with immunoprecipitin arcs of similarly treated amounts of pure antigen. Using this technique, it was determined that plant GS was present at 150 micrograms per gram fresh weight or 1.2% of total plant soluble protein in N_2-fixing alfalfa nodules.

Results suggest that alfalfa nodule plant GS consists of two major subunit polypeptides, but only a single immunochemically active native protein was observed. The crossed immunoelectrophoresis procedure described here should be generally applicable for immunochemical detection of lower abundance components of crude plant extracts.

GS[2] (EC 6.3.1.2) is the major enzyme for ammonia assimilation in higher plant tissues (7, 13). Host plant GS in the cytoplasm of infected cells of N_2-fixing legume nodules assimilates ammonia generated as the product of symbiotic N_2 fixation (9). High relative levels of GS specific activity are induced in the plant fraction of N_2-fixing nodules (3, 4), typically 10- to 100-fold greater than in other plant tissues. Legume nodules therefore present a convenient system for studying the biosynthesis of this plant protein. The objectives of this work were to isolate and characterize host plant GS in alfalfa nodules and to develop immunochemical methods for quantitation of GS (independent of enzymic activity) in crude nodule extracts.

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[2] Abbreviations: GS, glutamine synthetase; PAGE, polyacrylamide gel electrophoresis; IgG, immunoglobulin G.

MATERIALS AND METHODS

Plant Material. Tetraploid alfalfa (*Medicago sativa* L., line IC 4X, derived as described earlier [10]) was propagated by means of stem cuttings (from E. T. Bingham, University of Wisconsin, Madison, WI) and grown in pots of sand in a glasshouse. Roots were inoculated with a commercial preparation containing *Rhizobium meliloti* (Nitragin Co., Milwaukee, WI). Plants were watered daily with tap water and were treated weekly with a nitrogen-free nutrient solution (50 ml/plant) consisting of 6 g K_2HPO_4, 6 g K_2SO_4, 1.3 g $CaCl_2$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.6 g $MnCl_2 \cdot 4H_2O$, 0.1 g $CuSO_4 \cdot 5H_2O$, 0.2 g $CoSO_4 \cdot 7H_2O$, 0.2 g $NaMoO_4$, and 0.2 g Fe-chelate (Sequestrene 330, CIBA-Geigy, Ardsley, NY) per liter. Nodules were harvested at the onset of flowering, rinsed, blotted, and immediately immersed in liquid N_2. Excised nodules were kept at −140°C until extraction.

Purification of Plant GS from Nodules. Nodules were ground to a fine powder under liquid N_2 in a mortar with pestle. The powder was immediately extracted (5 ml/g) in 50 mM Tris-HCl (pH 7.4 at 25°C), 2 mM $MgCl_2$, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 15% (v/v) ethylene glycol (buffer A) at 4°C with magnetic stirring. Bacteroids were removed by centrifugation (30,000*g*, 30 min) and GS was purified from the soluble plant fraction of nodules by pH 4.0 precipitation for 30 min on ice, DEAE-Sephadex A-25 column chromatography (step-elution with 100 mM NaCl in buffer A), Reactive Blue-2 agarose column chromatography (step-elution with 200 mM NaCl in buffer A), and preparative SDS-PAGE (12% acrylamide, discontinuous system of Laemmli [6]).

Measurement of GS Activity. Ammonia-dependent GS activity was determined using a radioisotopic assay as previously described (3). Substrate concentrations for GS assays were 10 mM NH_4^+, 10 mM L-glutamate (30 μCi/mmol L-[U-^{14}C]glutamate), 10 mM ATP,

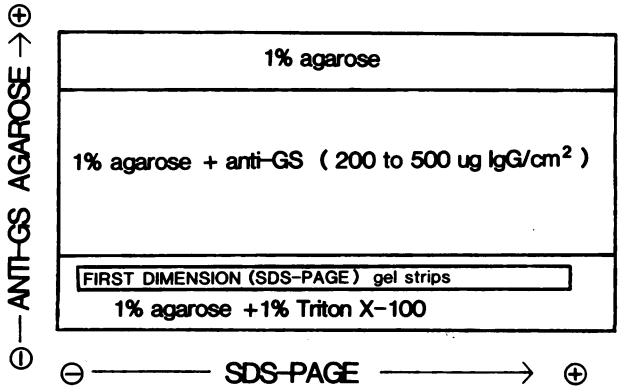

FIG. 1. Schematic of crossed immunoelectrophoresis.

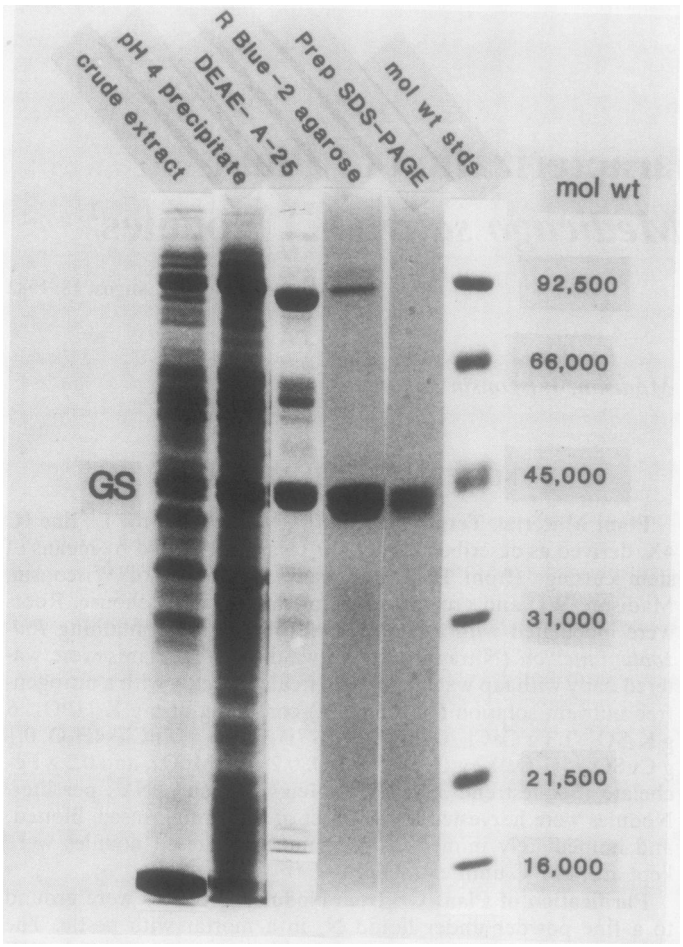

FIG. 2. Purification of plant GS from alfalfa nodules. Active fractions from each step in the purification procedure were resolved by SDS-PAGE (10% acrylamide) and stained with Coomassie Brilliant Blue-R.

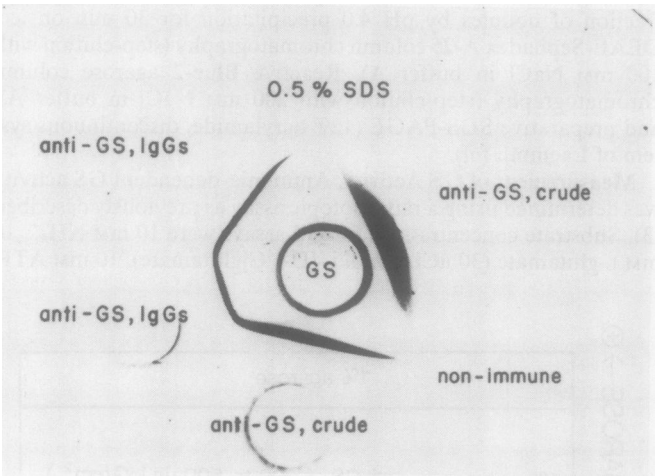

FIG. 3. Ouchterlony double immunodiffusion analysis of antibodies raised to alfalfa nodule GS. The central well contained partially purified GS (DEAE-Sephadex A-25 fraction).

11 mM Mg^{2+}, 100 mM Tricine-NaOH, pH 8.3 at 25°C.

Preparation of Antibodies. Antibodies to the purified SDS-polypeptide fraction were raised in white Swiss mice by intraperitoneal injection. Antisera were collected as ascitic fluid following injection of 180/TG cells (12). Mouse IgGs were isolated by protein A-Sepharose affinity chromatography (5).

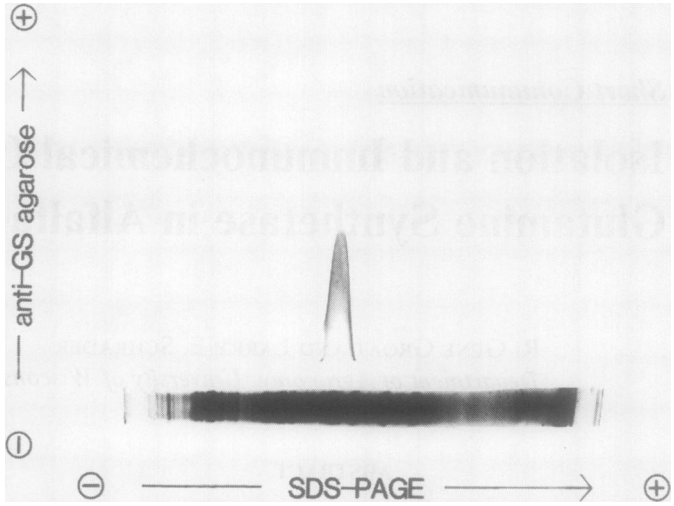

FIG. 4. Crossed immunoelectrophoresis of GS in a crude extract of the plant fraction of alfalfa nodules. First dimension: SDS-PAGE (10% acrylamide). Second dimension: electrophoresis into 1% agarose containing 250 μg anti-GS IgGs/ml.

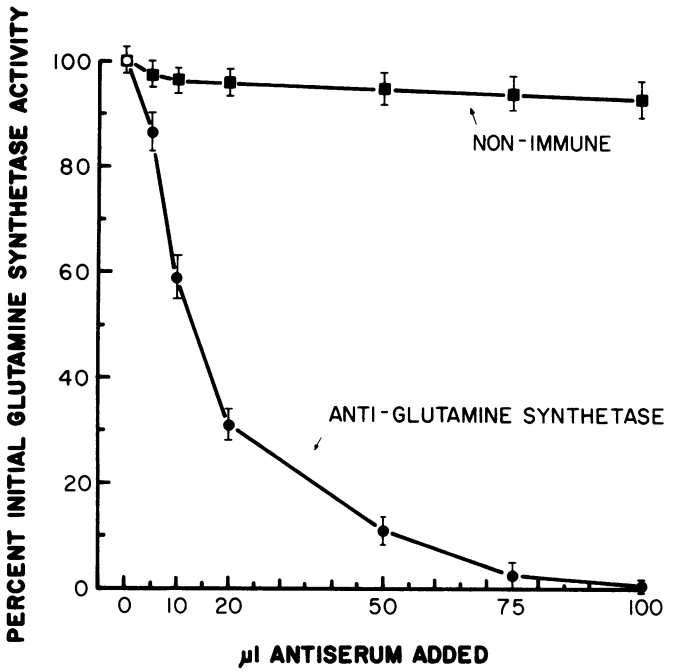

FIG. 5. Immunotitration of GS activity in a crude extract of the plant fraction of alfalfa nodules. Constant amounts of nodule extract (500 μg protein) were incubated with increasing amounts of either anti-GS (●) or non-immune (■) IgGs (at 0.5 mg/ml) in a final volume of 1.0 ml. Samples were incubated for 1 h at 37°C, then for 5 h at 4°C. Immune complexes were removed by adding 50 μl of a 10% (w/v) suspension of heat-killed formalin-fixed *Staphylococcus aureus* cells, incubating for 1 h at 30°C, followed by centrifugation (5 min, Beckman microfuge). The supernatant was assayed for GS activity.

Crossed Immunoelectrophoresis. Crude extracts of soluble protein in the plant fraction of alfalfa nodules were resolved by SDS-PAGE (10% acrylamide [6]). Slab gel lanes were cut out and then subjected to electrophoresis in the second dimension into antibody-containing 1% agarose gel (200–500 μg IgG/ml). Free SDS was trapped ahead of the antibody-containing gel by an intermediate agarose gel containing 1% (v/v) Triton X-100 as described previously (1, 2). A schematic of the crossed immunoelectropho-

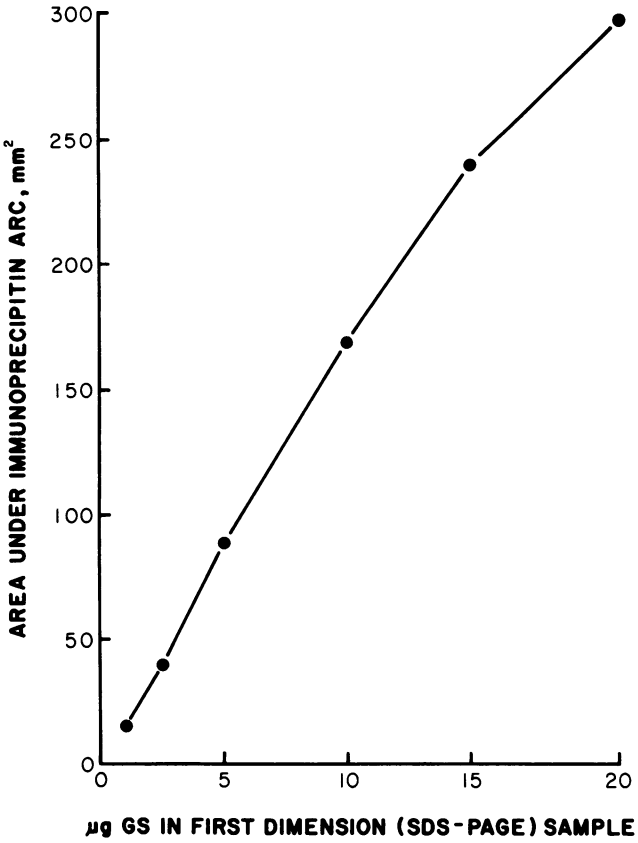

FIG. 6. Standard curve for immunochemical quantitation of GS. Known amounts of purified GS protein were subjected to SDS-PAGE (10% acrylamide) followed by electrophoresis in the second dimension into 1% agarose gel containing 250 g anti-GS IgGs/ml.

resis system is shown in Figure 1. Agarose gels for immunoelectrophoresis contained 80 mM Tris-acetate (pH 8.5 at 25°C), 40 mM sodium acetate, and 2 mM EDTA. Plant GS in crude nodule extracts was quantitated immunochemically by comparison with areas of immunoprecipitin arcs generated by known amounts of pure antigen.

RESULTS AND DISCUSSION

The specific activity of GS from the plant fraction of N_2-fixing alfalfa nodules was increased 100-fold through purification, suggesting GS comprises about 1% of total protein. Our purification procedure is simpler and more rapid than a previously reported procedure for purification of plant GS from soybean nodules (8). Purified alfalfa nodule GS appeared as two major polypeptides of 40,000 to 45,000 mol wt following SDS-PAGE (Fig. 2). The subunit size of GS appears to be highly conserved in this mol wt range among eukaryotes. The apparent heterogeneity of the 40,000 to 45,000 mol wt preparative SDS-PAGE fraction could have been generated by extraction or electrophoresis, but may indicate that alfalfa nodule plant GS consists of more than one type of subunit, as in *Neurospora* (11).

Purified mouse IgGs raised against this alfalfa nodule GS SDS-polypeptide fraction were monospecific as judged by double immunodiffusion (Fig. 3) and crossed immunoelectrophoresis (Fig. 4). Immunotitration (Fig. 5) specifically inhibited 100% of the GS activity in crude extracts. The results of Figures 3 to 5 collectively indicate that a single immunochemically active GS species was present in alfalfa nodule extracts. This suggests that only one of the two major GS SDS-polypeptides was immunochemically active or that both polypeptides may associate to form a single immunochemically active native protein in the absence of SDS. Soybean nodule plant GS has been reported to consist of eight identical polypeptides of about 43,000 mol wt which associate to form a native enzyme of about 350,000 mol wt (8).

By comparison with areas under immunoprecipitin arcs generated by known amounts of pure antigen (Fig. 6), plant GS was determined to be present at 150 µg/g fresh weight or 12 µg/mg soluble protein (1.2% of total soluble protein) in N_2-fixing alfalfa nodules. Our standard curve was essentially linear in the range of 1 to 20 µg antigen. The working range for this and other types of immunochemical methods are variable, however, depending upon specific antibody titer. This (two-dimensional) crossed immunoelectrophoresis technique should be generally applicable for immunochemical quantitation of specific proteins when standard (one-dimensional) rocket immunoelectrophoresis techniques are not of sufficient resolution, due to low antigen abundance or low specific antibody titer. We intend to use immunochemical methods to further study the biosynthesis of this plant protein.

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