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Short Communication

Multiple Subunit Composition of Chloroplastic Glutamine Synthetase of *Nicotiana tabacum* L.

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ABSTRACT

Chloroplastic glutamine synthetase from tobacco leaves (*Nicotiana tabacum* L. var Xanthi) was purified to homogeneity. By using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and high performance liquid chromatography, a single subunit was identified with a molecular weight of 45,000 daltons. However the native protein seems to be composed of four different subunits which can be separated by isoelectrofocusing. It is suggested that different genes with eventual posttranslational and/or posttranscriptional modifications may control the synthesis of the chloroplastic glutamine synthetase.

The molecular structure of GS[1] (EC 6.3.1.2) appears to be conserved in a wide range of higher plants. Although multiple forms of the enzyme have been identified in an increasing number of angiosperms, a mol wt for the native protein of around 360,000 D with eight identical subunits of 45,000 D is frequently reported in the literature (1, 4, 10, 11, 13, 14).

Recently, Groat and Schrader (3) identified polypeptides of 40,000 and 45,000 D for the enzyme in alfalfa root nodules. The fungal enzyme of *Neurospora crassa* also possesses two nonidentical subunits (15). In this organism, the relative proportions of the two subunits in the native protein may vary between the different strains and under different growth conditions (15).

In this study, the chloroplastic GS from tobacco leaves has been purified to homogeneity and the subunit composition of the protein analyzed by polyacrylamide gel electrofocusing in the presence of 8 M urea.

MATERIALS AND METHODS

Plant Culture. Tobacco plants (*Nicotiana tabacum* L. var Xanthi) were grown in plastic containers filled with vermiculite and watered daily for 3 months with a complete Hoagland solution (8) in a greenhouse. RH was 70% saturation and the temperature was 15°C during the night and 24°C during the day. Daylength was 16 h.

Enzyme Extraction and Purification: Preparation of Specific Antibodies. Purification of the chloroplastic GS from tobacco leaves (1 kg) was carried out as previously described for the cytosolic GS from barley leaves (7). Specific antibodies were raised against the purified protein essentially as described in a previous study (6).

Determination of Subunit Mol Wt. Electrophoresis in the presence of SDS was conducted according to Weber and Osborn (16) by using 10% polyacrylamide cylindrical gels to determine subunit mol wt. HPLC has also been used in order to estimate the subunit mol wt. A model 6000 A solvent delivery system with a U6K injector (Waters Associates, Milford, MA) was used in this experiment. Chromatographic separations were performed using a Spherogel TSK 4000 SW exclusion column (Beckman). The absorbance of the column eluate was monitored at 280 nm using a model 450 variable wavelength absorbance detector (Waters) and recorded on a linear chart recorder (Omniscribe B 5000, Houston Instruments, Austin, Tx). All separations were carried out in 10 mM Na-phosphate buffer (pH 7) containing 0.1% SDS with a flow rate of 0.5 ml/min. Samples containing 10 to 20 μg of dissociated proteins (boiled 5 min in the presence of 1% β-mercaptoethanol and 1% SDS) were injected in a volume of 100 μl. In both experiments, standard proteins were used as mol wt markers: BSA (66,000), egg albumin (45,000), pepsin (34,500), and β-lactoglobulin (18,400) (Sigma Chemical Company).

Electrofocusing. Isoelectrofocusing of purified GS from tobacco leaves was performed as described by Kung *et al.* (9) except that ampholines (pH range 4–6) (LKB Instruments) and 8 M urea were used in this experiment.

RESULTS AND DISCUSSION

At the end of the purification, 6.5 mg of purified GS_2 from tobacco leaves were obtained with a specific activity of 43 μmol/min· mg protein, which is comparable to that found for several other leaf cytosolic or chloroplastic GS (4, 6, 7, 11). The purity of the preparation was checked by SDS-PAGE. A single protein band with a mol wt of 45,000 D was detected after Coomassie blue staining (Fig. 1A). Similar results were also obtained by SDS gel filtration using HPLC. Figure 2 shows that a single peak of protein corresponding to a mol wt of about 45,000 D was eluted from the column. This new method gives a reliable estimation of the subunit mol wt as well as a 100% recovery of the purified subunit from the effluent. In a previous study, it has been shown that many higher plants possess only a GS_2 (12).

In tobacco, a single peak of GS activity corresponding to the chloroplastic GS was detected after ion exchange chromatography on DEAE-Sephacel. This peak eluted at the same salt con-

[1] Abbreviations: GS, glutamine synthetase; GS_1, cytosolic glutamine synthetase; GS_2, chloroplastic glutamine synthetase.

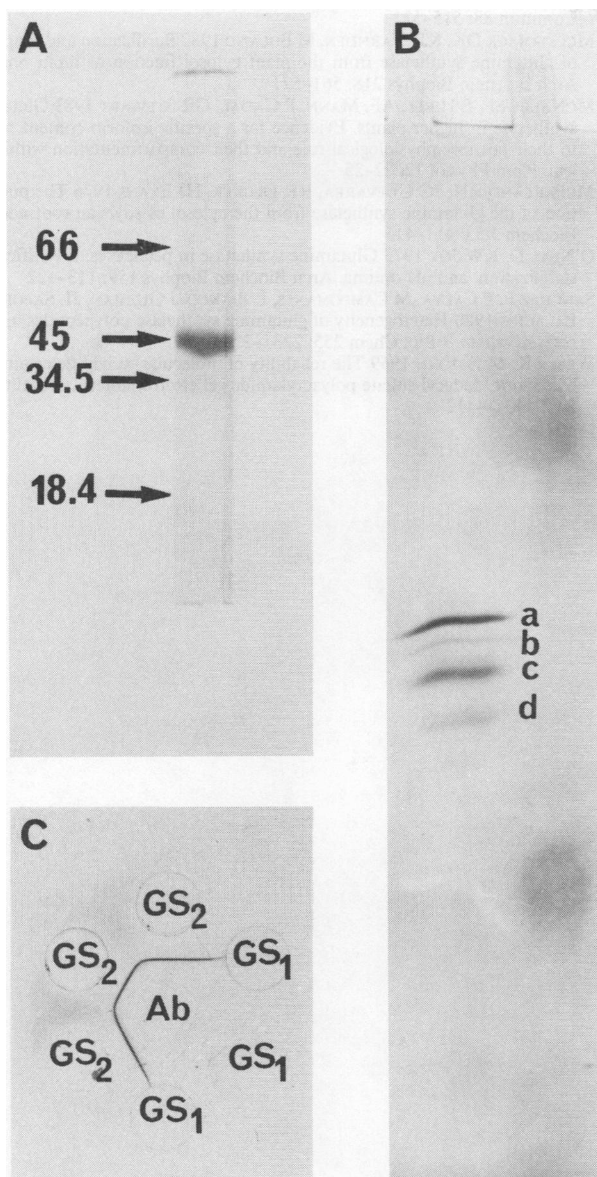

FIG. 1. A, PAGE in the presence of SDS of purified GS_2 (30 μg) from tobacco leaves. Mol wt of the markers (kD) are indicated by arrows. B, Polyacrylamide slab gel isoelectrofocusing (pH range 4–6) in the presence of 8 M urea of purified GS_2 from tobacco leaves. C, Immunochemical characterization of GS from tobacco leaves. Three wells on the left contained 5 μg of purified GS from tobacco leaves (GS_2) and three walls on the right contained 5 μg of purified cytosolic GS from rice leaves (GS_1). The central well contained 8 μl of crude antiserum raised against GS from tobacco leaves (Ab).

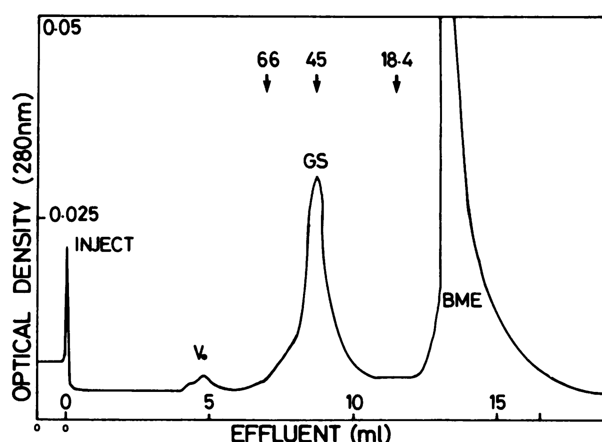

FIG. 2. Chromatography of purified and dissociated GS from tobacco leaves on a gel filtration column by using HPLC. V_0 is the void volume with undissociated proteins. GS represents glutamine synthetase subunit and BME the peak of β-mercaptoethanol. Calibration of the column was performed in the same conditions using marker probes. Position of the markers eluted from the column is indicated by arrows with their respective mol wt expressed in kD.

centration (0.22 M NaCl) as that found for spinach GS_2 (6).

In order to check that the GS_2 peak is not contaminated by some GS_1 due to inefficient separation on the ion exchange resin, immunodiffusion using tobacco GS antibodies was performed with the purified GS from tobacco leaves and a cytosolic GS isolated from rice leaves (4). GS from tobacco leaves is specifically recognized by the antibodies whereas no recognition of the cytosolic GS was observed, indicating that there is no contamination of GS_2 by GS_1 (Fig. 2C). This test based on the fact that higher plants GS_1 possess very similar antigenic sites (7) once again demonstrates that certain C_3 plants are characterized in having only a chloroplastic GS (12).

This was also confirmed using antibodies raised against GS_1 from barley leaves which recognize several cytosolic GS isolated from various higher plants (7). No cross-reaction was observed with the tobacco GS.

Purified GS_2 from tobacco leaves was subjected to polyacrylamide gel isoelectrofusing in 8 M urea. Four major bands (a, b, c, and d) were found in the pH 5 region of the gel (Fig. 1B). Staining with Coomassie brillant blue R 250 also shows that the different subunits are present in different relative proportions. The presence of several subunits has also been reported for another chloroplastic enzyme, ribulose-bisphosphate carboxylase containing at least three different peptides as components of the large subunits and two for the small subunits (9). Large subunits are coded by the chloroplastic genome and small subunits by the nuclear genome (2). We have been able to show recently that a neosynthesis of GS_2 occurs during greening of etiolated leaves and that the protein is probably of a cytosolic origin (5).

However, a participation of the chloroplastic genome in certain plants of the four groups recently described (12) like for ribulose-bisphosphate carboxylase cannot be completely excluded. The presence of several subunits differing by their isoelectric point and their relative proportions in the native enzyme might suggest that several genes chloroplastic and/or nuclear are involved in the synthesis of the different peptides (9).

Proteolytic cleavages occurring during the purification have also to be considered, but it is unlikely that an active protein purified electrophoretically (6) is contamined by proteases. Moreover, the absence of smaller peptides or contaminants after SDS-PAGE even with a large amount of purified protein (Fig. 1A) shows that the preparation is homogenous.

Studies are now in progress to elucidate the gene expression of GS_2 in different groups of higher plants (12). This will determine whether or not the heterogeneity of the subunits is the result of the expression of different genes and also if posttranscriptional and/or posttranslational modifications occur during their neosynthesis.

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