Chloroplastic glutamine synthetase from tobacco leaves: a glycosylated protein

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The amino acid and carbohydrate content of chloroplastic glutamine synthetase from tobacco leaves has been analysed. The enzyme subunit contanins 5% carbohydrate, mainly represented by glucosamine, galactosamine, glucose, galactose and mannose residues. The enzyme subunit displayed a single band of molecular mass 44000 Da after sodium dodecyl sulphate (SDS) electrophoresis. However, when isoelectrofocussing electrophoresis was performed, four subunits were evident differing by their charge. Furthermore, the four different subunits stained positively when tested with periodic acid Shiff reagent, showing that sugars and amino sugars were present within all the subunits.

Glutamine synthetase *Isoform* *Amino acid* *Carbohydrate* *Glycosylation*

1. INTRODUCTION

Most work on the glycosylation of plant proteins has been focussed on lectins and storage glycoproteins (see review, [1]) and very little attention has been directed toward the study of plant enzyme glycoproteins. The presence of sugars or amino sugars, however, has been reported in peroxidase [2], β-fructosidase from horseradish [1,3,4], glycosidases from pea leaves [5] and α-amylase from cereal seeds [6]. Different forms of each enzyme were characterized and shown to differ in their carbohydrate moieties [2,3,5]. Another plant enzyme which also exists in multiple molecular forms is glutamine synthetase (GS) [7]. In green leaves of higher plants one or two GS isoenzymes can be present. One form is localized in the cytoplasm (GS 1) and the other in the chloroplast (GS 2). Each isoenzyme displays specific kinetic and regulatory properties, such as differences in their heat stability, pH optima and K_m for glutamate [8,9]. Further, they also possess different antigenic determinants [10–12]. The finding that GS 1 and GS 2 are compartmentalized in the cytoplasm and chloroplast, respectively, and also that the chloroplastic isoform is synthesized de novo in the cytosol during greening [13,14] suggests that the plastidial isoenzyme must be transported from the cytosol into the chloroplast. It has been reported that many plant proteins transported from one subcellular compartment to another are glycosylated [4,15]. It was therefore considered worthwhile to investigate the possible glycosylation of chloroplastic glutamine synthetase.

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2. MATERIALS AND METHODS

2.1. *Enzyme extraction and purification*

Chloroplastic glutamine synthetase from tobacco (*Nicotiana tabacum* L. var. Xanthi) was purified to electrophoretic homogeneity as described previously [12] using sequentially, DEAE Sepharose CL 6 B (Pharmacia) ion exchange chromatography, Bio Gel HTP (BioRad) absorption chromatography, Sepharose CL 6 B (Phar-

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macia) gel filtration and preparative acrylamide gel electrophoresis on a native 5% cylindrical gel followed by an electroelution of the protein [9]. The final enzyme preparation was extensively dialysed against sterile bidistilled water in sterile dialysis bags before amino acid and carbohydrate analyses.

2.2. *Gel electrophoresis, protein and carbohydrate staining*

Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) was performed according to [16] on 10% acrylamide cylindrical or slab gels using a BioRad apparatus. All the chemicals, of the highest purity available, were from BDH or Sigma.

Standard proteins were used as M_r markers: BSA (66000); egg albumin (45000); β lactoglobulin (18400) (Sigma). Proteins were stained for 1 h with 0.25% Coomassie brilliant blue R 250 in methanol:acetic acid:water (45:10:45, v/v) and carbohydrates by the periodic acid Schiff (PAS) method of [17].

Isoelectrofocussing was conducted as described previously [18].

2.3. *Chemical analyses*

Protein content was determined according to [19]. Carbohydrate content was determined as described in [20]. Trimethyl eluted carbohydrates were separated by gas liquid chromatography (Hewlett-Packard model 5710 A equipped with a column of 3% OV-225 using a temperature gradient of 160–230°C. Mannitol was used as the internal reference and a mixture of free carbohydrates was used as standard for quantification. Amino acid and hexosamine analyses were performed by ion exchange chromatography on an automatic analyser following hydrolysis of the sample in 6 N hydrochloric acid under vacuum at 105°C for 24 and 6 h, respectively [21].

3. RESULTS

The purity and subunit molecular mass of chloroplastic GS from tobacco leaves was checked by PAGE in the presence of SDS using either slab or cylindrical gels according to the method of [16]. As stated previously [18], chloroplastic GS from tobacco leaves possesses a single subunit with a molecular mass estimated to be of 44000 Da (fig.1A). This molecular mass estimation, using marker probes, was confirmed after amino acid analysis of the purified protein and calculation from the number of amino acid and carbohydrate residues (table 1). Table 1 also shows that glucosamine and galactosamine were detected after a short hydrolysis in 6 N HCl. Carbohydrate content of GS from tobacco chloroplasts was further analysed by gas liquid chromatography in order to identify and quantify the carbohydrate content of the enzyme molecule. Following these various analyses mannose, galactose and glucose residues were found and their total contribution estimated to be about 5% of the enzyme subunit (table 1).

The presence of carbohydrates in tobacco GS subunit was also confirmed after PAS reagent staining in polyacrylamide gels. The subunit localized by Coomassie blue R 250 staining was coincident with a red coloured band which appeared after PAS staining (fig.1A). Similar results were also obtained when the four polypeptides were separated isoelectrofocussing [18], and were stained by the PAS reagent (fig.1B).

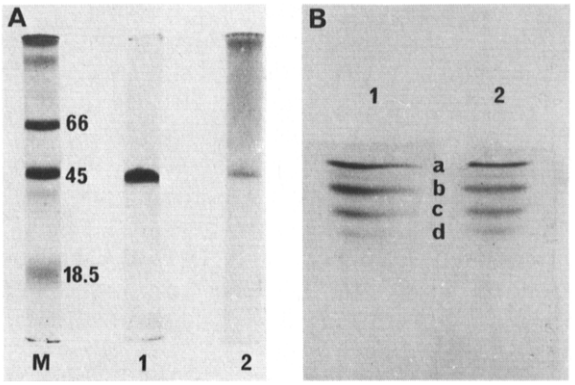

Fig.1. Coomassie brilliant blue and carbohydrate PAS staining of purified GS 2 from tobacco leaves. (A) 10% PAGE in the presence of SDS. (M) Molecular mass markers with their respective molecular masses indicated in (1) Coomassie blue staining of purified GS 2 (30 μg). (2) periodic acid Schiff reagent staining of purified GS 2 (100 μg). (B) Polyacrylamide slab gel electrofocussing in the presence of 8 M urea of purified GS 2 from tobacco leaves. (1) Coomassie blue staining (30 μg protein). (2) Periodic acid Schiff reagent staining (200 μg protein). a,b,c and d are the four enzyme subunits which can be separated by their charge differences.

Table 1

Amino acid and carbohydrate composition of glutamine synthetase from tobacco leaves

Amino acid/ carbohydrate	Content (mol %)	Amino acid and carbohydrate content [mol/mol of subunit (M_r 44000)]
Aspartic acid	11.9	39
Threonine	4.5	17
Serine	8.0	33
Glutamic acid	13.1	39
Proline	5.6	21
Glycine	8.2	48
Alanine	10.2	50
Valine	3.4	13
Cysteine	2.2	4
Methionine	1.2	4
Leucine	5.6	19
Isoleucine	3.3	11
Tyrosine	3.4	8
Phenylalanine	2.6	7
Histidine	2.0	6
Lysine	5.5	16
Arginine	3.5	9
Mannose	0.4	1
Galactose	1.3	3
Glucose	1.6	4
Glucosamine	1.2	3
Galactosamine	1.3	3

The values represent the average of 3 separate determinations

4. DISCUSSION

Many plant proteins and only a few enzymes have been shown to contain carbohydrate moieties [1]. Furthermore, glycosylation of enzymes of primary metabolism has not been studied previously. Chloroplastic glutamine synthetase, which is involved in primary and secondary ammonia assimilation [22], has been shown in the present study to contain sugars (mannose, galactose and glucose) and amino sugars (glucosamine and galactosamine). Glycopeptide linkages containing such compounds have been identified in a number of plant proteins [1]. Further analysis will determine the exact nature of the glycopeptide linkages in the enzyme and also if the linkages differ in the four peptide components of the enzyme molecule. Although cytosolic GS isoenzymes from other plants have a similar amino acid composition but with a slightly different molecular mass (single subunit of 41000 Da) they do not contain any carbohydrate components (not shown). The presence of carbohydrates detected in the chloroplastic GS (5%) might explain the slight difference observed in the subunit molecular mass and the specific immunological behaviour of the various cytosolic and chloroplastic isoforms from higher plants [11,12]. It has been previously demonstrated, using specific antibodies prepared against either chloroplastic or cytosolic GS, that each isoenzyme possesses very different antigenic determinants, and it was therefore concluded that they were probably different proteins.

Like many other C_3 plants, tobacco contains only a single form of the enzyme located in the chloroplast [11,18]. However, the chloroplastic GS of tobacco is light regulated [14] which is similar to the situation found in rice, another C_3 plant but which possesses both cytosolic and chloroplastic GS [13]. In both tobacco and rice, there is an increase in the activity of chloroplastic GS during the greening process. It has also been shown that this is due to protein neosynthesis occurring in the cytosol [13]. Therefore, a selective transfer of the newly synthesized protein into the chloroplast must occur. The glycosylation of chloroplastic GS found in tobacco leaf cells may facilitate the transport from the cytosol to another intracellular compartment, a situation frequently reported for mammalian cells [23], and reported more recently to occur in plant cells [4,15].

The process of glycosylation and transport of chloroplastic GS is being further investigated. This will determine whether or not the plastidial enzyme is post-translationally glycosylated. Such a modification could be used as a specific signal for the transfer of GS into the chloroplast.

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