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TWO FORMS OF GLUTAMINE SYNTHETASE IN LEAVES OF *CUCURBITA PEPO*

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Key Word Index *Cucurbita pepo*; Cucurbitaceae; pumpkin; glutamine synthetase; isoenzymes; properties; chloroplasts; cytosol.

Abstract—It has been shown that the leaves of pumpkin (*Cucurbita pepo*) contain two molecular forms of glutamine synthetase (GS), one occurring in the cytosol (GS_1) and the other in the chloroplasts (GS_2). The activities of both forms were greater when ammonium ion was infiltrated into the leaves and this was shown to be due to *de novo* synthesis. The two synthetases were purified by ammonium sulphate fractionation, ion exchange chromatography on DEAE-cellulose, selective adsorption on calcium phosphate gel, and preparative polyacrylamide gel electrophoresis. The MWs of GS_1 and GS_2, estimated by gel filtration on Sephacryl S-200, were 480000 and 370000 respectively. During polyacrylamide gel electrophoresis in the presence of SDS both GS_1 and GS_2 were dissociated into polypeptide chains with MWs of 58000 and 50000 respectively, suggesting that GS_1 and GS_2 are octamers consisting of identical monomers. The synthetases showed noticeable differences in their amino acid composition. In GS_1 and GS_2 the proportions of α-helical segments were 34 and 17% respectively. In the presence of Mg^{2+}, the pH optima for GS_1 and GS_2 were 7.25 and 7.75 respectively, and K_m values toward L-glutamate were 13 and 46 mM respectively. From the experimental data it is inferred that GS_1 and GS_2 are isoenzymes.

INTRODUCTION

Glutamine, whose synthesis is catalysed by glutamine synthetase (GS, EC 6.3.1.2) [1], occupies a key position in cellular nitrogen metabolism. The structure and properties of GSs from microorganisms [1], animals [2] and *Chlorella* [3] have been thoroughly investigated and the enzymes shown to be oligomers (a dodecamer, octamer and hexamer, respectively) composed of identical monomers with a MW of *ca* 50000. O'Neal and Joy [4] detected GS in the chloroplasts and cytosol of spinach leaf cells and they purified GS from intact pea leaves, measured its MW and determined some of its properties [5].

Using polyacrylamide gel electrophoresis, we demonstrated [6,7] that pea leaf cells in fact contain two molecular forms of GS, one of which occurs in the cytosol (GS_1) and the other in the chloroplast (GS_2). We isolated GS_2 in homogeneous form and showed that it had a MW of 480000, $s_{20,w}$ 16.3 and consisted of eight identical monomers [8].

More recently the two molecular forms of GS were also detected in barley seedlings [9], rice seedlings [10] and soybean hypocotyls [11]. However, these enzymes were not isolated in a homogeneous state and the intracellular localization of each GS form was not determined.

The purpose of the present investigation was to study the effect of ammonium ion on GS activity in the chloroplasts and cytosol of pumpkin leaves, to purify GS_1 and GS_2 from this source and to examine the structure and properties of the two molecular forms of the enzyme.

RESULTS AND DISCUSSION

The zymograms of GS from intact leaves and chloroplasts of the pumpkin showed that the chloroplasts contained one form of the enzyme (GS_2) whereas the intact leaves contain two forms (GS_2 and GS_1, which has a lower electrophoretic mobility). The method we used to isolate the chloroplasts maintained *ca* 50% of them in the native state, as demonstrated microscopically. Calculations showed that 30–50% of the total GS activity occurred in the chloroplasts and 50–70% in cytosol. Similar findings were reported by O'Neal and Joy [4] for spinach leaves and by Evstigneeva *et al.* [6] for pea leaves. GS activity was not observed in the mitochondria. It can be concluded, therefore, that the enzyme with the lower electrophoretic mobility corresponded to the cytosol form (GS_1).

When ammonium ions were infiltrated into intact leaves there was an increase in GS activity, both in the chloroplasts and the cytosol. Inhibitors of protein synthesis in chloroplasts [12] and cytosol [13] (i.e. chloramphenicol and cycloheximide respectively) completely reversed this ammonium ion effect, indicative of the *de novo* synthesis of both GS_1 and GS_2.

To clarify whether GS_1 and GS_2 were isoenzymes, they were isolated and their structure and properties were studied on a comparative basis. Since pumpkin leaf chloroplasts are easily degraded during isolation we used extracts from intact leaves that contained both forms of GS which were then separated during purification.

To prepare extracts, pumpkin leaves frozen in dry ice were ground in a mortar to a powder. This was extracted for 1 hr with 2 vol. of 50 mM Tris–HCl buffer (pH 7.8) containing 2 mM EDTA, 2 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. Fragments of disrupted cells were separated by centrifugation at 6000 *g* for 20 min. Other organelles and chlorophyll-containing chloroplast segments were sedimented by centrifugation at 144000 *g* for 20 min. Nucleic acids were then removed by adding

Table 1. GS activities in chloroplasts and cytosol of pumpkin leaves after infiltration with ammonium ions and protein synthesis inhibitors

	GS activity (U/ml extract)	(%)	GS specific activity (U/mg protein)	Protein (mg/ml extract)	Ammonia (μg/ml extract)
Cytosol (GS_1)					
H_2O	7.5	100	13.4		4.2
NH_4^+	11.1	149	15.4		32.1
NH_4^+ + chloramphenicol	9.2	123	15.8		33.6
NH_4^+ + cycloheximide	7.5	100	20.8		30.3
Chloroplasts (GS_2)					
H_2O	3.1	100	6.2	0.50	0.08
NH_4^+	4.3	138	7.4	0.58	3.2
NH_4^+ + chloramphenicol	3.4	110	10.6	0.32	4.4
NH_4^+ + cycloheximide	4.5	145	5.0	0.92	19.0

Table 2. Purification of GS from pumpkin leaves

Stage of purification	Protein in the fraction (mg)	GS activities		Yield (%)	Degree of purification
		Total (U)	Specific (U/mg protein)		
Extract after 144000 g	1920	1496	0.80	100	1
Precipitation of nucleic acids	1690	1465	0.86	98	1.1
Fractionation with $(NH_4)_2SO_4$	741	1343	1.80	90	2.25
Chromatography on DE-32	77	948	12.3	64	13.1
Adsorption on calcium phosphate gel	3.75	627	167.1	42	209

Table 3. Amino acid composition of GS from chloroplasts and cytosol of pumpkin leaves

Amino acid	No. of amino acid residues per monomer	
	Chloroplast GS	Cytosol GS
Lysine	33	23
Histidine	8	12
Arginine	20	31
Aspartate/asparagine	39	34
Threonine	19	27
Serine	26	24
Glutamate/glutamine	27	55
Proline	30	35
Glycine	41	49
Alanine	30	44
Cysteine	17	23
Valine	24	34
Methionine	8	12
Isoleucine	18	21
Leucine	34	51
Tyrosine	11	22
Phenylalanine	13	23
Sum of amino acid residues per monomer	398	529
Calculated MW	45770	59800

10% streptomycin sulphate dropwise to bring the final concentration to 1%. After 20 min the residue was centrifuged at 18000g for 20 min and crystalline ammonium sulphate was added to the supernatant to give 30% saturation. Thirty minutes later the residue was centrifuged off (18000g for 20 min), and ammonium sulphate was added to the supernatant to reach 50% saturation. After 1 hr the residue was separated (18000g for 30 min), dissolved in the extracting buffer and desalted on a Sephadex G-25 column (2.5cm × 40cm). The resultant preparation was chromatographed on a column (3cm × 8cm) of DEAE-cellulose DE-32. Proteins were eluted from the column by a linear gradient of $MgCl_2$ (0–0.35 M) in 50mM Tris–HCl buffer, pH 7.5, containing 2mM EDTA, 2mM $MgSO_4$ and 10mM 2-mercaptoethanol (Fig. 1). GS_1 and GS_2 were eluted in one peak and all fractions having GS activity were combined. Calcium phosphate gel was added to the resultant solution, the required quantity of the gel being determined in a pilot test. A step-by-step elution of the protein from the gel was carried out by using the initial buffer containing increasing concentrations of magnesium sulphate. The bulk of the GS was eluted with 0.8 M magnesium sulphate. The eluate with GS activity was combined and desalted on a Sephadex G-25 column. At that stage the enzyme was purified 209 times and had a specific activity of 167 U/mg protein (Table 2). A gel electrophoretic study of the resultant preparation revealed that both GS_1 and GS_2 were present. In order to separate these two forms, a number of procedures were tried: only polyacrylamide gel electrophoresis proved successful. The preparation was first concentrated using an Amicon XM-300 membrane and then subjected to preparative polyacrylamide (6%) gel electrophoresis. The enzyme position in the gel was determined and the bands were cut from the gel and homogenized. The enzyme was eluted with the extracting buffer at 4° for 5–8 hr. The gel was separated by centrifugation at 15000g for 10 min. The specific activities of GS_1 and GS_2 were 38 and 98 U/mg protein respectively. These activities were lower than the activity of the total enzyme preparation before separation, presumably due to partial enzyme inactivation during electrophoresis. Electrophoregrams of the resultant GS_1 and GS_2 indicated that the two molecular forms of GS were separated and obtained in a homogeneous state.

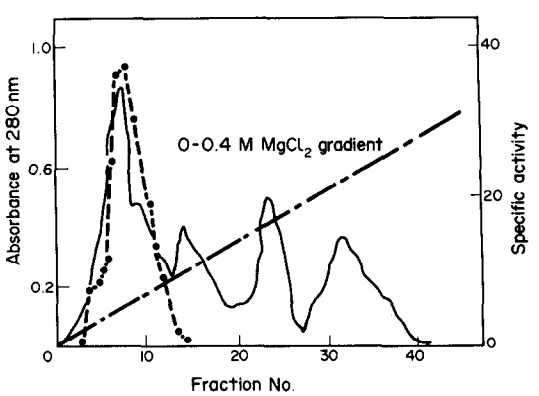

Fig. 1. Curve of protein elution from a column of DEAE-cellulose DE-32: ——— absorption at 280nm, ●——● GS-specific activity.

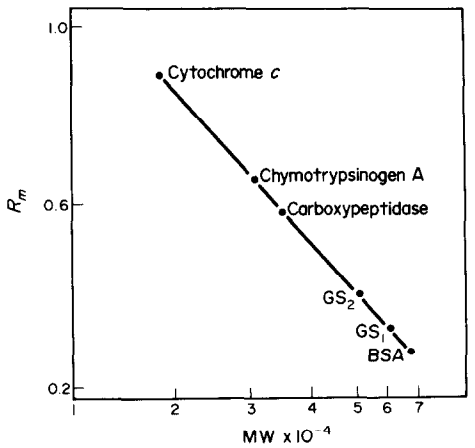

Fig. 2. Measurement of MWs of GS_1 and GS_2 treated with SDS by polyacrylamide gel electrophoresis.

We measured the MW of the above GS forms by gel filtration through Sephacryl S-200 using suitable markers (see Experimental). The MW of GS_1 was estimated to be 480000 and that of GS_2 370000. Thus, GS_1 and GS_2 differ not only in their electrophoretic mobilities but also in their MWs.

During polyacrylamide gel electrophoresis in the presence of SDS, GS_1 and GS_2 were dissociated into polypeptide chains with MWs of 58000 and 50000, respectively (Fig. 2). From the MWs of oligomers and monomers of GS_1 and GS_2 of pumpkin leaves it can be inferred that each of the above enzymes are octomers containing different sets of identical monomers. This was previously demonstrated for soybean root nodule cytosol GS [14] and pea chloroplast GS [8].

A study of the amino acid composition of GS_1 and GS_2 from pumpkin leaves (Table 3) also shows differences between the two molecular forms. GS_1 contained significant amounts of glutamic acid, leucine, glycine and alanine whereas GS_2 contained appreciable quantities of glycine, aspartic acid, leucine and lysine. A comparative examination of the amino acid composition of GS_1 and GS_2 according to the method of Marchalonis and Weltman [15] indicated important differences between these proteins (SΔQ being 81). We also measured the portion of α-helical segments in the enzymes. This parameter was 34% for GS_1 and 17% for GS_2. Therefore, the two enzymes also differ in their secondary structure. In addition, GS_1 and GS_2 showed differences in pH optima in the presence of Mg^{2+} (7.25 and 7.75 respectively) and in their affinity for glutamate (Fig. 3).

Therefore, it can be concluded that pumpkin leaf cells contain two molecular forms of GS, presumably isoenzymes, one of which occurs in chloroplasts and the other in the cytoplasm.

EXPERIMENTAL

The experiments were carried out on leaves of 3-week-old pumpkin plants, Vitaminnaya variety, grown in soil in a greenhouse.

The ammonium ion effect on GS synthesis in pumpkin leaves was studied by infiltrating a 25mM soln of $(NH_4)_2HPO_4$ into intact leaves that were then kept in the light for 4 hr at 25°. In

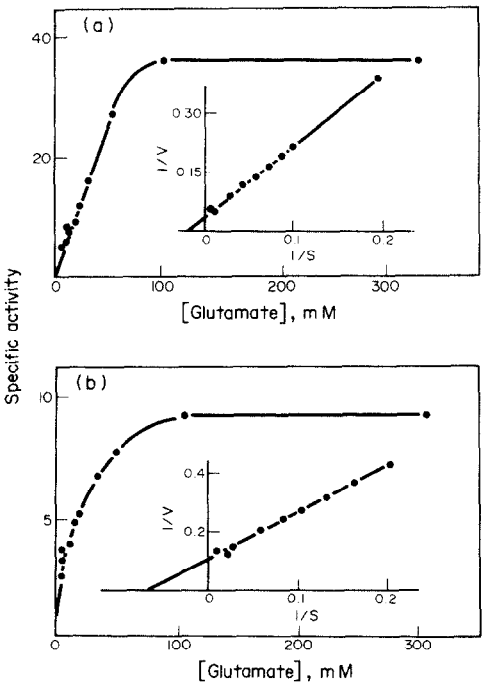

Fig. 3. Curves of GS_2 (a) and GS_1 (b) after saturation with L-glutamate.

some expts, to examine GS synthesis separately in chloroplasts and the cytoplasm, ammonium ion was infiltrated simultaneously with the protein synthesis inhibitors chloramphenicol (for chloroplasts) and cycloheximide (for cytosol).

Chloroplasts were isolated from pumpkin leaves according to ref. [16]. Leaves were cut into small pieces with scissors and then ground in 50 mM Tris–HCl buffer (pH 8.0) containing 5 mM 2-mercaptoethanol, 2 mM EDTA, 2 mM $MgSO_4$, 10 mM NaCl and 0.4 M sucrose, for 10 sec at 8000 rpm in a tissue grinder RT-1. Chloroplasts were disrupted by osmotic shock in the initial buffer devoid of sucrose and NaCl.

GS activity was measured by the method of ref. [17]. The experimental mixture with a final vol. of 2.25 ml contained 260 mM Tris–HCl buffer, 60 mM NH_2OH–HCl, 50 mM $MgSO_4$, 6.25 mM ATP, 250 mM Na monoglutamate, and 0.5–1.0 μg of enzyme, pH 7.5. Incubation was carried out at 37° for 15 min. The reaction was stopped by addition of 0.75 ml of a soln containing aliquots of 10% $FeCl_3 \cdot 6H_2O$ in 0.2 N HCl, 24% TCA and 18.5% HCl. The quantity of γ-glutamyl hydroxamate formed in the reaction:

$$\text{Glutamate} + NH_2OH + ATP \xrightarrow{Mg^{2+}} \gamma\text{-Glutamyl hydroxamate} + ADP + Pi$$

was estimated by measuring the soln at A_{540nm} against a suitable control. The quantity of GS catalysing the synthesis of 1 μmol γ-glutamyl hydroxamate per min in the above reaction mixture was the unit of enzyme activity.

Protein was measured according to the method of ref. [18]. During column elutions, protein was recorded by A_{280nm}.

Analytical polyacrylamide gel electrophoresis was performed by the method of ref. [19] using 6.0 and 7.5% gels and 25 mM Tris–glycine buffer (pH 8.5) containing 5 mM 2-mercaptoethanol. Electrophoresis was carried out with a current of 2 mA/tube. Gels were stained with 0.2% amido black in a mixture of HOAc, MeOH and H_2O (1:4:10) for 30 min at 37°. Excess stain was washed out with 7.5% HOAc.

Preparative electrophoresis was in 6% polyacrylamide gel in a device built according to the description and drawings of ref. [20] at 4° with a current of 50 mA at 500–600 V.

To obtain zymograms, gels were placed into the reaction mixture containing 0.2 M Tris–HCl buffer, 0.2 M Na monoglutamate, 0.1 M NH_4Cl, 0.1 M $MgSO_4$ and 0.02 M ATP, pH 7.5. Incubation was carried out for 30 min at 37°. The reaction was stopped by addition of a soln of 10 parts of 1.8% $FeSO_4 \cdot 7H_2O$ in 0.3 N H_2SO_4 and 1 part of 6.6% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 7.5 N H_2SO_4. After 10–20 min the gel areas containing H_3PO_4 were stained blue. To detect phosphatases we used a reaction mixture that contained no sodium glutamate.

The minimum MW of GS was measured electrophoretically in the presence of SDS according to the method of ref. [21]. The MW of GS was estimated in a column (1 × 100 cm) of Sephacryl S-200 pre-equilibrated with 50 mM Tris–HCl buffer, pH 7.5, containing 2 mM EDTA, 0.3 M $MgCl_2$ and 10 mM mercaptoethanol. GS and standard proteins (ferritin, phycocyanin and phycoerythrin) dissolved in the same buffer were passed through the column at a rate of 6 ml/hr.

The portion of α-helical segments in the enzyme molecules was determined with respect to their circular dichroism [22]. Amino acid composition of protein hydrolysates was assayed in an amino acid analyser according to ref. [23].

The following reagents were used in the study: Na L-monoglutamate obtained from Ajinomoto (Japan); ATP, EDTA, Tris from Reanal (Hungary); Sephadex G-25, Sephacryl S-200 from Pharmacia (Sweden); DEAE-cellulose DE-32 from Whatman (U.K.); amino black from Ferak (West Berlin), SDS, ferritin, chymotrypsinogen from Serva (FRG); egg albumin, BSA, cytochrome *c* from Kahlbaum (FRG); $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ from Laborchemie Apolda (GDR).

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