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QUARTERLY

Purification and partial characterization of glutamine synthetase isoforms from *Triticale* seedlings

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Two isoforms of glutamine synthetase (EC 6.3.1.2), cytoplasmic (GS_1) and chloroplastic (GS_2) were isolated from shoots of 14-day-old *Triticale* seedlings, and purified 260-fold and 248-fold, respectively. Specific activities of the two preparations were 35.1 and 33.5 μmol × min^{-1} per mg of protein, respectively. Both crude extracts and homogeneous GS_1 and GS_2 preparations required divalent metal ions (Mg^{2+}, Mn^{2+}, Co^{2+}) for their activities. Mg^{2+} was the most effective activator, the highest activity of GS_1 being reached at 5 mM, and that of GS_2 at 20 mM $MgCl_2$. The optimum pH for the two isoforms showed large differences, dependent also on the kind of divalent ion. Molecular masses of GS_1 and GS_2 were 305000 Da and 385200 Da, respectively. It seems that native protein of GS_1 is built from eight identical subunits of M_m 38000 Da and that of GS_2 of the same number of subunits but of M_m of about 48000 Da. Proteins of GS isoforms differed significantly in their amino-acid composition.

The leaves of vascular plants contain two forms of glutamine synthetase, cytoplasmic GS_1 and chloroplastic GS_2 (EC 6.3.1.2) [1–4]. The activity of either form and their mutual relationship are dependent, among others, on the plant species, type and age of individual plant organ, availability of light, and the form of inorganic nitrogen [2, 5, 6]. The cytoplasmic form differs often from the chloroplastic one in kinetic properties, regulation of the activity and molecular mass. Molecular masses of GS_1 and GS_2 range from 320 kDa to 480 kDa depending on plant species and intracellular localization [7–10]. Generally, the chloroplastic isoform showed a higher molecular mass than the cytoplasmic form [8, 9]. Both GS isoforms are as a rule composed of eight subunits either identical or different types [8–12]. However, the sugar beet leaf isoforms were active both in an octameric and in a tetrameric form. The tetramer was even more active than the octamer but needed 2-mercaptoethanol for stabilization of the activity [13]. Subunits of cytoplasmic and chloroplastic forms of GS from the same plant species are encoded by highly homologous but different nuclear genes [14–17].

The aim of the present work was to obtain more information on the subunit structure of the chloroplastic and cytoplasmic forms of glutamine synthetase, and to compare the amino-acid composition of proteins of both enzyme forms, purified to homogeneity. It is expected that the results would make possible to answer the question whether GS_1 and GS_2 are proteins differing in their secondary or primary structure.

MATERIALS AND METHODS

Shoots of 14-day-old seedlings of *Triticale* (variety Malno) were used for the experiments. The seedlings were grown in hydroponic cultures, with aeration of the root system. Proce-

dures for sterilization of seeds, composition of the medium, and growth conditions were as described previously [18].

Separation and purification of glutamine synthetase isoforms. All steps of the purification procedure were performed at 0–4°C. Shoots of the seedlings (350 g) were cut into 2-cm long pieces and homogenized in 0.1 M Tris/HCl buffer, (1:5, w/v), pH 7.9, containing 1 mM EDTA and 10 mM $MgCl_2$, filtered through 3 layers of cheese-cloth and centrifuged at 20000 × *g* for 20 min. Ammonium sulphate was added to the supernatant, under constant stirring, up to 0.4 saturation. After another 10 min of stirring, the mixture was recentrifuged and the sediment was discarded. $(NH_4)_2SO_4$ was added to the supernatant to 0.55 saturation, and the precipitate was centrifuged and dissolved in a small volume of 0.05 M Tris/HCl buffer, pH 7.9, containing 1 mM EDTA and 20 mM $MgCl_2$. Column chromatography on Sephacryl S-300 gel (2.6 cm × 93 cm) constituted the next step of purification. The protein (460 mg) was applied onto the column and eluted with 0.05 M Tris/HCl buffer, pH 7.9, containing 1 mM EDTA and 20 mM $MgCl_2$, at a rate of 25 ml/h. Fractions of 4 ml were collected. The fractions showing the highest activity were pooled and applied onto DEAE Sephacel column (2 cm × 40 cm) equilibrated with 0.025 M Tris/HCl buffer, pH 7.9, containing 1 mM EDTA and 10 mM $MgCl_2$. For elution and simultaneous separation of GS_1 and GS_2, a linear NaCl concentration gradient (0 → 4 M) was applied; the elution rate was 15 ml/h. The separated GS_1 and GS_2 were salted out with $(NH_4)_2SO_4$ at 0.8 saturation, centrifuged at 20000 × *g* for 20 min, and dissolved in 5 ml of 0.050 M Tris/HCl buffer, pH 7.9, containing 1 mM EDTA and 10 mM $MgCl_2$. The two forms were separately purified by affinity chromatography on Blue Sepharose CL-6B gel columns (1 cm × 5 cm), equilibrated and then eluted with the same buffer. The collected GS_1 or GS_2-containing fractions were further purified on hydroxyapatite columns (1 cm × 10 cm) equilibrated with 0.05 M phosphate buffer, pH 7.0. Each enzymic protein was applied onto the column, and washed with 15 ml of phosphate buffer. The hydroxyapatite-bound proteins were eluted with a linear phosphate buffer concentration gradient (0.05 → 0.2 M) at a rate of 5 ml/h. The highly purified GS_1 and GS_2 preparations were further purified by preparative electrophoresis as described by Hirel *et al.* [19]. Analytical electrophoresis was performed on 7% polyacrylamide gel by the method of Davis [20], in 0.05 M Tris/glycine buffer of pH 8.3 or 9.1. The protein bands were stained for 12 h with 0.1% Coomassie Blue R-250 solution in 7% acetic acid and 20% methanol. The gels were extensively washed with 7% acetic acid-20% methanol mixture and transferred to 7% acetic acid. The activity of the electrophoretically separated enzyme isoforms was estimated by the method of Hirel & Gadal [7], based on the formation of calcium phosphates from $CaCl_2$ added and the release of phosphate from ATP.

Determination of molecular masses of GS_1 and GS_2 and their subunits. Molecular mass of GS_1 and GS_2 was determined by chromatography on a Sephacryl S-300 column (2.6 cm × 100 cm) equilibrated with 0.05 M Tris/HCl buffer, pH 7.9, containing 1 mM EDTA, 10 mM $MgCl_2$ and 0.5 M NaCl. Fractions of 4 ml were collected at a rate of 18 ml/h. The column was calibrated with Blue Dextran and four standard proteins. Molecular mass of subunits of GS_1 and GS_2 was determined by electrophoresis on 10% polyacrylamide gel containing 0.1% SDS, as described by Weber & Osborn [21].

The enzyme assay. The activity of the enzyme was determined colorimetrically according to O'Neal & Joy [22], with some modifications [18] and protein after Lowry *et al.* [23] adapted to plant proteins [18].

Determination of amino-acid composition of GS subunits. Homogenous proteins GS_1 and GS_2 were hydrolysed for 22 h in 6 M HCl at 130°C in sealed tubes washed previously with nitrogen. The liberated amino acids were determined by HPLC in the Gold System with ninhydrin.

All the results presented are mean values from the experiments made at least in triplicates.

RESULTS

Purification

The purification procedure applied permitted a 260-fold purification of GS_1, and 240-fold purification of GS_2; their respective activities were 35.1 and 33.5 μmol × min^{-1} per mg of

Table 1

Purification of cytoplasmic (GS_1) and chloroplastic (GS_2) isoforms of glutamine synthetase from Malno Triticale seedling leaves.

Protein content and activity of the enzyme isoforms were determined as described in Materials and Methods.

Purification step	Total activity ($\mu m \times min^{-1}$)	Total protein (mg)	Specific activity ($\mu m \times min^{-1}$ per mg of protein)	Purification factor	Recovery (%)
Crude extract	749.1	5549.10	0.14	1	100.0
0.4–0.5 saturated $(NH_4)_2SO_4$	563.3	920.00	0.61	4	75.0
Sephacryl S-300	290.2	143.20	2.03	15	39.0
GS_1					
DEAE-Sephacel	20.9	9.10	2.30	17	2.8
Blue Sepharose CL-6B	20.1	1.38	14.56	108	2.7
Hydroxyapatite	17.4	0.50	34.80	258	2.3
Preparative electrophoresis	12.3	0.35	35.14	260	1.6
GS_2					
DEAE-Sephacel	192.0	20.9	9.19	68	25.6
Blue Sepharose CL-6B	170.0	10.40	16.35	121	22.7
Hydroxyapatite	82.0	2.57	31.91	236	10.9
Preparative electrophoresis	63.0	1.88	33.51	248	8.4

Fig. 1. Electrophoretic pattern of cytoplasmic (1) and chloroplastic (2) glutamine synthetase from Triticale seedlings in 7% polyacrylamide gel: A, at pH 9.1; B, at pH 8.2.

The gels were stained for protein with Coomassie Blue.

protein (Table 1). The purification step on Blue-Sepharose gel proved to be the most effective, especially for GS_1: the losses of total activity were only 4–11%, with a several fold increase in specific activity at the same time. Homogeneity of the purified final preparations was checked by electrophoresis on 7% polyacrylamide gel. As shown in Fig. 1, both at pH 8.2 and 9.1 the GS_1 and GS_2 preparations gave each a single protein band.

Activation by divalent metal ions. Both enzymic isoforms require for their activity the presence of divalent ions. The effect of Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, Cu^{2+} and Ca^{2+} ions on GS_1 and GS_2 activity is presented in Table 2. Only Mg^{2+}, Mn^{2+} and Co^{2+} activated the enzyme, and the highest activity was obtained with Mg^{2+} in the incubation mixture; the other two ions, although showing high affinity to the enzyme isoforms, resulted in an activity lower by a half than in the presence of Mg^{2+}.

Activation by Mg^{2+} of GS_1 and GS_2 was observed also over a wide range of ion concentration (Fig. 2). GS_1 activity was the highest at Mg^{2+} concentration of 5 mM, whereas for the maximum activity of GS_2 a 4 times higher concentration of Mg^{2+} was required. It should be

noted also that at 5 mM Mg^{2+} concentration GS_2 exhibited only 10–12% of its maximum activity, and that GS_1 activity decreased at $MgCl_2$ concentration exceeding 10 mM.

Effect of pH

The optimum pH for GS_1 and GS_2 was strictly dependent on the kind of divalent ion, and was the highest with Mg^{2+} and the lowest with Mn^{2+} (Table 2). In all the experiments the optimum pH for the cytoplasmic enzyme form was slightly lower than that for the chloroplastic form.

Molecular masses of GS_1 and GS_2 and their subunits

As established by Sephacryl S-300 gel filtration, molecular mass of cytoplasmic GS_1 was 305500 ± 10000 Da and that of chloroplastic GS_2 was 385200 ± 11000 Da (Fig. 3). SDS-10% polyacrylamide gel electrophoresis at pH 7.2 showed that GS_1 and GS_2 were built each of a single type of subunits, of 38000 ± 1200 and 48000 ± 1500 Da, respectively (Fig. 4). The molecular masses of the enzyme isoforms and their subunits indicate that either form of glu-

Table 2

Effect of divalent metal ions on the activity and pH optimum of glutamine synthetase isoforms GS_1 and GS_2.

The activities of 10.8 and 10.2 units × min^{-1} per ml were taken as 100 for GS_1 and GS_2, respectively. The effect of ions was determined under the optimum conditions (pH, ion and substrate concentration) for either enzyme isoform. For determination of the pH optimum, 0.1 M Tris/maleate buffer was used over the pH range 5.1–9.0. The results are mean values from three determinations.

Divalent metal ions	Ion concentration (mM)		Activity (%)		pH optimum	
	GS_1	GS_2	GS_1	GS2	GS_1	GS2
Mg^{2+}	5.0	20.0	100 ± 2.5	100 ± 2.6	7.70	8.15
Co^{2+}	3.0	3.0	43 ± 1.8	44 ± 1.7	6.80	7.00
Mn^{2+}	2.0	2.0	50 ± 2.0	53 ± 2.0	5.30	5.40

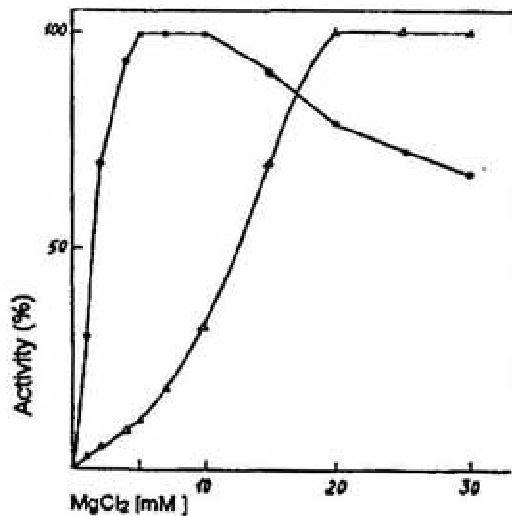

Fig. 2. Effect of $MgCl_2$ concentration on the activity of GS_1 (O) and GS_2 (Δ).

The activity was determined in 0.1 M Tris/HCl buffer of pH 7.70 and 8.15 for GS_1 and GS_2, respectively. The activities referred to as 100% were: 10.8 and 10.2 units × min^{-1} per ml for GS_1 and GS_2, respectively.

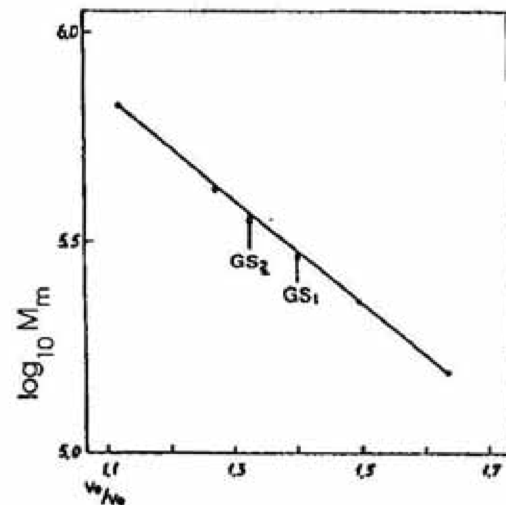

Fig. 3. Plot of log molecular mass of standard proteins and glutamine synthetase isoforms against the V_e/V_o value determined by molecular gel filtration on Sephacryl S-300.

Protein standards: thyreoglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa).

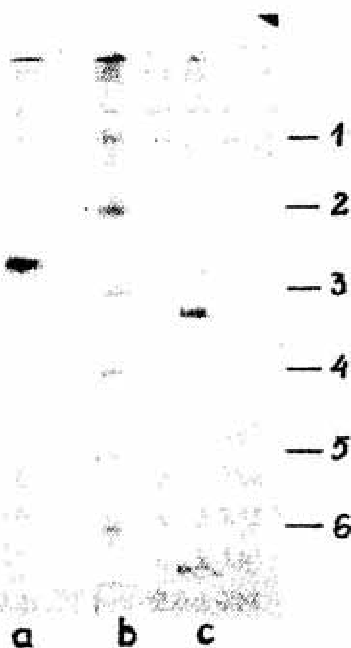

Fig. 4. Electrophoregrams of GS_1 (c) and GS_2 (a) preparations.
Standard proteins (b): 1, phosphorylase *b* (94 kDa); 2, bovine serum albumin (67 kDa); 3, chicken egg albumin (43 kDa); 4, carbonic anhydrase (30 kDa); 5, soybean trypsin inhibitor (20.1 kDa); 6, α-lactalbumin (14.4 kDa).

tamine synthetase from Malno *Triticale* leaves is composed of eight subunits.

Amino-acid composition of GS_1 and GS_2 proteins

Proteins of the two enzyme isoforms differed markedly in their amino-acid composition (Table 3). In the chloroplastic enzyme, the contents of alanine, tyrosine and serine were higher, and those of glycine and proline were lower, than in the cytoplasmic form. Besides, GS_2 contained more basic amino acids, which constituted almost 20% of the total amino-acid pool of the protein. On the other hand, in GS_1 protein the content of acidic amino acids was higher than in GS_2.

DISCUSSION

The procedure for fractionation and purification of glutamine synthetase isoforms applied in the present work, permitted to obtain homogeneous preparations showing high specific activity. A similar, or slightly higher, specific activity of GS_1 and GS_2 from cereals was reported earlier by Hirel & Gadal [7] and Hirel *et al.* [24]. It should be noted that GS_2 was less stable during purification than GS_1. This might indicate differences in their physico-chemical properties. A similar phenomenon was observed by others who attempted to purify both enzyme isoforms [25, 26].

Mg^{2+} present in large amounts in the leaves of many vascular plant species activated most effectively the enzyme isoforms from Malno *Triticale* leaves. However, full saturation of GS_1 with magnesium ion was achieved at its concentration 1/4 that necessary for saturation of GS_2. This distinctly higher affinity for Mg^{2+} of GS_1 as compared to GS_2 corresponds well with the concentration of Mg^{2+} in chloroplasts. It is known that Mg^{2+} concentration in chloroplast stroma in day-light reaches a level as high as 10–15 mM, whereas in cytoplasm the content of magnesium is much lower [27]. Moreover, with a marked accumulation of cations in stroma, mainly of Mg^{2+}, after illumination of plants, pH increased from 7.0 to 8.0 which additionally improved conditions for the activity of the chloroplastic form of glutamine synthetase [28]. It should be remembered that the optimum pH for the chloroplastic enzyme of numerous plant species, including Malno *Triticale*, is by almost 0.5 higher than that for the cytoplasmic enzyme [7, 26]. The mechanisms of activation of glutamine synthetase and its isoforms by divalent metal ions so far have not been precisely defined. Knight & Langston-Unkefer [29] have provided the evidence that Mg^{2+} binds to the allosteric site of each subunit of the enzyme to form an Mg-ATP^{2-}-complex. On the other hand, other authors suggest that the Mg-ATP^{2-} complex is formed in the active site of the enzyme [30]. Eads *et al.* [31], in describing the mechanism of the glutamine synthetase-catalyzed reaction, postulate that divalent metal ions stabilize formation of a tetrahedric derivative from ammonia and γ-glutamylphosphate. Another difficulty in elucidation of the mechanism of glutamine synthetase activation by Mg^{2+} or other divalent ions comes from the results reported by Mäck & Tischner [13] who demonstrated that the enzyme from sugar beet showed no requirement for divalent ions. In our experiments, in addition to Mg^{2+}, also Co^{2+} and Mn^{2+} enhanced the activity of GS_1 and GS_2 in the Malno *Triticale* leaves, although in their presence the activation was much lower than

Table 3
Amino-acid composition of the cytoplasmic and chloroplastic isoforms of glutamine synthetase.
For details see Material and Methods. The results are mean values from three determinations.

Amino acid	Content (mol %)		Amino-acid residues per monomer	
	GS_1 ·	GS_2	GS_1	GS2
Aspartic acid + Asparagine	8.5 ± 0.5	7.4 ± 0.5	26.0 ± 1.5	26.0 ± 1.7
Threonine	3.5 ± 0.2	3.6 ± 0.2	12.0 ± 0.7	14.0 ± 0.8
Serine	8.8 ± 0.4	9.5 ± 0.5	34.0 ± 1.6	42.0 ± 2.2
Glutamic acid + Glutamine	18.5 ± 0.6	11.4 ± 0.5	51.0 ± 1.6	36.0 ± 1.5
Proline	3.4 ± 0.1	2.5 ± 0.1	12.0 ± 0.4	10.0 ± 0.3
Glycine	10.2 ± 0.4	7.1 ± 0.3	55.0 ± 2.1	44.0 ± 1.9
Alanine	9.2 ± 0.3	10.2 ± 0.3	42.0 ± 1.4	53.0 ± 1.6
Valine	5.2 ± 0.2	4.0 ± 0.2	18.0 ± 0.7	16.0 ± 0.6
Cysteic acid	1.2 ± 0.1	1.8 ± 0.1	4.0 ± 0.3	7.0 ± 0.4
Methionine	5.1 ± 0.2	5.1 ± 0.2	14.0 ± 0.5	16.0 ± 0.5
Leucine	4.2 ± 0.1	5.6 ± 0.2	13.0 ± 0.3	20.0 ± 0.7
Isoleucine	1.3 ± 0.1	2.3 ± 0.1	4.0 ± 0.3	8.0 ± 0.5
Tyrosine	3.6 ± 0.2	7.0 ± 0.3	8.0 ± 0.5	18.0 ± 0.8
Phenylalanine	1.6 ± 0.1	2.5 ± 0.1	4.0 ± 0.3	7.0 ± 0.4
Histidine	5.0 ± 0.2	5.4 ± 0.2	13.0 ± 0.5	16.0 ± 0.6
Lysine	5.4 ± 0.2	8.5 ± 0.4	15.0 ± 0.6	27.0 ± 1.3
Arginine	5.2 ± 0.2	6.0 ± 0.3	12.0 ± 0.5	16.0 ± 0.8

with Mg^{2+}. Much higher concentration of Co^{2+} and Mn^{2+} than of Mg^{2+} required for the maximum reaction rate, indicates much lower affinity of the enzyme isoforms to these ions than to Mg^{2+}. Similar results have been reported for soybean leaves [26].

The cytosolic and chloroplastic isoforms of GS differed largely with respect to molecular masses which were 305000 Da and 385200 Da, respectively. These values were in general agreement with those reported for other plant species namely, 320000 Da and 360000 Da for rice [9], 304000 Da and 360000 Da for pea [17]. Similarly as in many other species of vascular plants and in algae [8, 9], molecular mass of the chloroplastic form of glutamine synthetase was higher than that of the cytoplasmic form. However, in some plant species molecular masess of GS_1 and GS_2 are similar, or even that of the cytoplasmic enzyme is higher [7, 10].

The electrophoregrams of the GS_1 and GS_2 preparations obtained in the presence of SDS, and comparison of their molecular masses with those of their respective subunits indicate that either of the glutamine synthetase isoforms is built from eight identical polypeptides of molecular mass 38000 Da and 48800 Da, respectively. These results are in agreement with the opinion that glutamine synthetase from vascular plants (or its isoforms) is built of eight identical monomers arranged into two flattened, closely stacked tetramer structures [11]. A somewhat different suggestion has been made by Mäck & Tischner [13]. According to these authors in the sugar beet photosynthesizing organs two oligomer forms, tetrameric and octameric, were consistently present, but mutual proportions of their activity differed, depending on the plant age, organ, and the form of inorganic nitrogen supplied. The octamer was an only active enzyme form in old, yellowed leaves and in roots. The studies on subunit structure of glutamine synthetase performed on root nodules of some *Papillionaceae* species have demonstrated that the enzyme can occur in multiple isoenzymatic forms, always built from eight subunits composed in different proportions of four different polypeptides [12]. On

the other hand, Höpfner *et al.* [32] have demonstrated by isoelectrofocusing that glutamine synthetase of *Sinapis alba*, although it is built from eight subunits but that, this octamer is composed of six different types of polypeptide chains. Moreover, the native enzyme, when deprived of Mg^{2+}, dissociates into two active tetramers of molecular mass half that of the octamer. This diversity of polypeptides which form the octamer makes possible the functioning of numerous isoforms of glutamine synthetase. This problem requires further studies because there are no comprehensive data on the role of the various enzyme isoforms in cellular metabolism.

The different amino-acid composition of the two glutamine synthetase forms provides further evidence that GS_1 and GS_2 proteins differ in primary structure. Comparison of amino-acid composition of glutamine synthetase isoforms from different plant species showed that lupin [30], soybean [30], curcubita pepo [10] and *Triticale* have different content of basic and acidic amino acids, alanine and cysteine. The higher content of cysteine in the chloroplastic isoform and lower stability of this form than of the cytosolic during purification could indicate that cysteine plays an important role in enzyme activity regulation.

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