

Molecular Composition of Glutamine Synthetase of *Sinapis alba* L.

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Chloroplastic glutamine synthetase of *Sinapis alba*, purified to homogeneity by a simple three step procedure, revealed a molecular weight of about 395 kDa.

The native enzyme is composed of eight subunits of identical molecular weight (about 50 kDa (each), although isoelectrofocusing yielded six distinct bands in the pH 5.6 region of the gel. Labelling of the enzyme with the glutamate analogue herbicide [^{14}C]phosphinothricin and with [$\gamma\text{-}^{32}\text{P}$]ATP indicated that glutamine synthetase has eight reactive centers per molecule. The native enzyme dissociated into two enzymatically active subaggregates of about 195 kDa after Mg^{2+} deprivation.

Introduction

Two different isoenzymes of GS (L-glutamate ammonia ligase, ADP forming, EC 6.3.1.2.), namely a plastidic type and a cytosolic type, are normally found in green leaves of higher plants [1]. These two isoforms are clearly distinct with regard to immunological and physiological properties [1–3]. Nevertheless, they show similarities in their molecular structure (e.g. [4]).

The molecular weight (MW) is about 350–400 kDa. The holoenzyme consists of eight subunits with identical MWs, but there are a number of reports demonstrating differences in the isoelectric points (IEPs) of the subunits [5–7].

Though there are some studies on the structure of the reactive centers (e.g. [8]), little is known about the total number of reactive centers in a GS-molecule.

In this paper we report on the structure and subunit composition of the plastidic GS of mustard plants. We present evidence that there are eight reactive centers per GS-molecule.

Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; GS, glutamine synthetase; IEP, isoelectric point; MSO, methionine sulfoximine; MW, molecular weight; PPT, phosphinothricin; PAGE, polyacrylamide gel-electrophoresis; SDS, sodium dodecyl sulfate.

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Materials and Methods

Plant material

Plants of white mustard (*Sinapis alba* L.) were cultivated as described previously [9].

Enzyme extraction and purification

All experiments were carried out at 4 °C unless otherwise stated.

GS was extracted from green leaves of 20 days old plants. Typically 170 g of leaves were homogenized in two volumes of TMME-buffer (50 mM Tris/HCl, 1 mM MgSO_4 , 0.5 mM EDTA, 30 mM β -Mercaptoethanol) pH 8.3 in a Braun Starmix.

The homogenate was filtered through four layers of cheesecloth and the filtrate was centrifuged ($20000 \times g$, 40 min, 0 °C). The supernatant (RE) was applied to a 30 ml DEAE-Sephacel column (equilibrated with TMME pH 7.8) at a flow rate of $70 \text{ ml} \cdot \text{h}^{-1}$. The column was rinsed with the same buffer until the non-binding proteins were removed. GS was eluted with a linear KCl-gradient (0–0.6 M in TMME pH 7.8) at a flow rate of $50 \text{ ml} \cdot \text{h}^{-1}$.

Fractions containing GS-activity were pooled (E1), applied at $35 \text{ ml} \cdot \text{h}^{-1}$ to a 30 ml hydroxyapatite column previously equilibrated with TMME, rinsed, and eluted using a linear K-phosphate-gradient (0.1–0.5 M in TMME pH 7.8, flow rate $25 \text{ ml} \cdot \text{h}^{-1}$).

Fractions with GS-activity were pooled (E2), diluted with three volumes of TMME pH 7.8 and concentrated on a small (3 ml) DEAE-Sephacel column. Application, rinsing, and elution was performed at a flow rate of $20 \text{ ml} \cdot \text{h}^{-1}$. GS was eluted with elution buffer (50 mM Tris/HCl, 50 mM MgSO_4 , 1 mM



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EDTA, 0.3 M KCl, 30 mM β -Mercapto-ethanol) at pH 8.3. The fractions containing the highest GS-activity were pooled (E3) and used in the subsequent experiments.

Assay methods

GS-activity was measured at 37 °C by the formation of γ -glutamyl-hydroxamate in the synthetase reaction [10]. The reaction mix contained 100 mM imidazole/HCl pH 7.2, 50 mM MgSO_4 , 1 mM EDTA, 10 mM NH_2OH , 150 mM sodium glutamate, and 15 mM ATP in a total volume of 2 ml.

One unit of activity (U) corresponds to the formation of 1 μmol γ -glutamylhydroxamate per minute.

Protein concentrations were determined with the BIO-RAD protein assay.

Determination of holoenzyme MW

The MW was determined on a Knauer HPLC system equipped with a BioSil TSK 250 column (300 \times 7.5 mm, Bio-Rad Laboratories) and a pre-column (75 \times 7.5 mm).

The purified GS was applied to the column in 75 mM K-phosphate-buffer pH 7.0, with or without 1 mM MgSO_4 .

Standard-proteins were: thyroglobulin (669 kDa), ferritin (443 kDa), BSA (67 kDa), ovalbumin (45 kDa), and myoglobin (17.8 kDa).

Determination of the reactive centers

We used 1) the [^{14}C]labelled glutamate-analogue herbicide phosphinothricin ([^{14}C]PPT) and 2) [γ - ^{32}P]ATP as tracers, similar to the method of Ronzio *et al.* [11]. [^{14}C]PPT was a kind gift from Hoechst Inc., Frankfurt.

Labelling took place in the reaction mix as described for the enzyme assay, except that in 1) glutamate was substituted by 100 mM PPT. For the experimental line 2) we used 5 mM ATP and 1 M PPT.

One ml of the mix was gelfiltrated on a Sephadex G-50 column after 15 min incubation at 37 °C and chilling to 0 °C. The protein-containing fractions were pooled (4 ml).

Bound radioactivity was counted in a Beckman LS1801 Liquid Scintillation Counter; protein determination in the pooled fractions was performed using the Bio-Rad microassay.

Gel-electrophoresis of the subunits

Electrophoresis on 10% SDS-polyacrylamide slab-gels (SDS-PAGE) was performed as described by Laemmli [12]. Standard proteins for MW-determination were: phosphorylase B (92.5 kDa), BSA (67 kDa), ovalbumin (45 kDa), carboanhydrase (29 kDa), and myoglobin (17.8 kDa).

Isoelectrofocusing was made in the presence of 8 M urea in cylindrical polyacrylamide-gels according to the method of O'Farrell [13]. The respective IEP's were calculated from the pH values of the corresponding control gel regions.

Protein bands were visualized by Coomassie blue staining.

Results and Discussion

GS of *Sinapis alba* was purified to homogeneity by a simple three step procedure (see Table I). The elution profiles of DEAE-Sepacel and hydroxyapatite chromatography always showed only one single peak of GS-activity, eluting at a high ionic strength (about 220 mM KCl and 280 mM K-phosphate, respectively).

There are several further indications that there is exclusively a chloroplastic GS in the green leaves of *Sinapis alba* [2].

Table I. Purification of GS from 170 g fresh mustard leaves. The abbreviations are the following: RE = crude extract, E1 and E2 are the pooled GS-containing fractions after DEAE-Sepacel and hydroxyapatite chromatography, E3 indicates pure GS.

Source	Protein [mg/ml]	Total [mg]	Recovery [%]	Enzyme activity [U/ml]	Total [U]	Recovery [%]	Spec. act. [U/mg protein]	Purification
RE	1.13	437.3	100	0.47	181.4	100	0.42	1
E1	1.03	49.0	11.2	2.07	99.4	54.8	2.03	4.9
E2	0.04	1.2	0.27	1.68	47.0	25.9	39.07	94.1
E3	0.13	0.7	0.16	6.73	33.6	18.6	50.2	121

HPLC size exclusion chromatography of the purified enzyme also reveals only one protein peak showing GS-activity (Fig. 1) thus confirming the homogeneity of the GS preparation.

The calculation yielded a MW of about 395 kDa in the presence (Fig. 2A) and 195 kDa in the absence of Mg^{2+} (Fig. 2B).

The MW of 395 kDa is within the range of data already reported [4, 6, 18] for the native enzyme. Mg^{2+} -deprivation obviously caused the enzyme to dissociate into two subaggregates of identical MW.

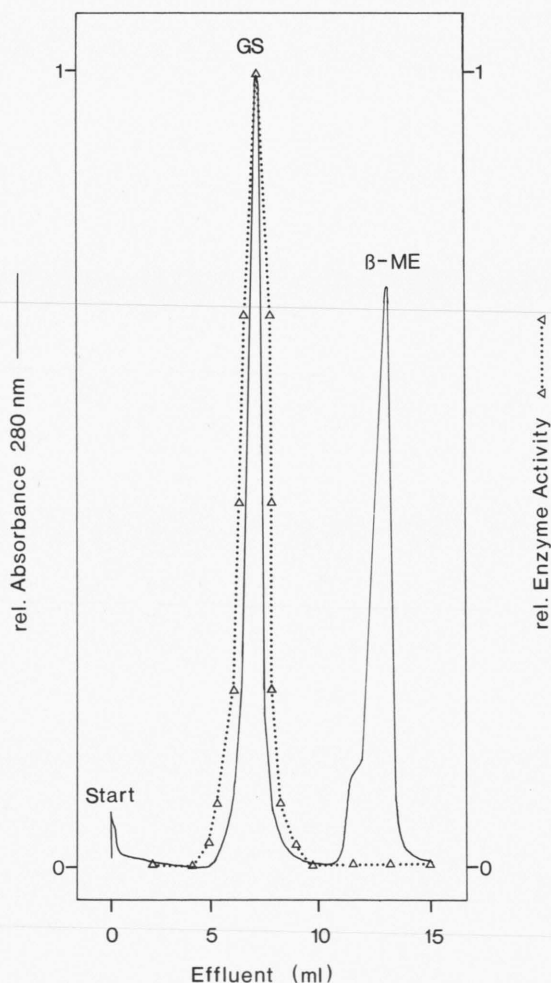


Fig. 1. HPLC elution profile of the purified GS and distribution of the enzyme activity. The adjuncts GS and β -ME indicate the peaks of glutamine synthetase and β -Mercaptoethanol, respectively. Distribution of GS-activity is represented by the dotted line.

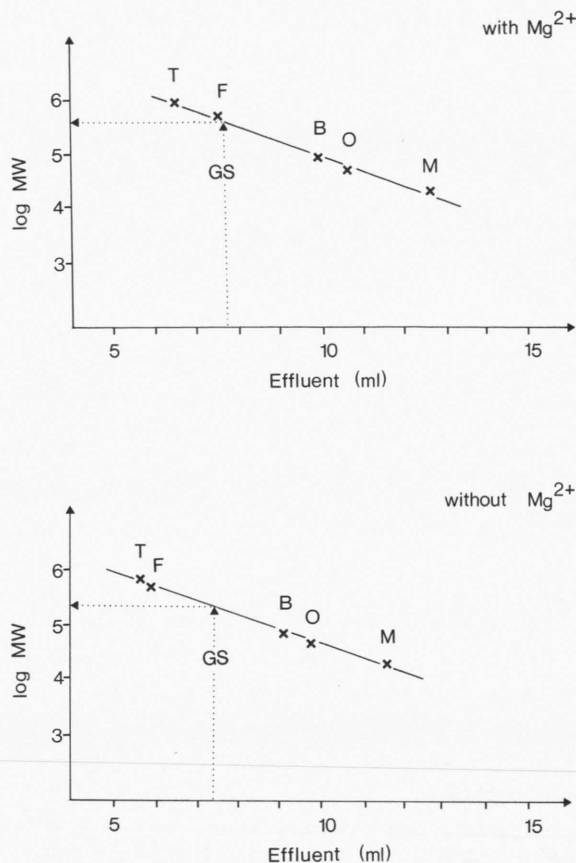


Fig. 2 (A + B): MW-determination based on the HPLC elution profile of purified GS using a Mg^{2+} -containing (A) and a Mg^{2+} -deficient elution buffer (B). The position of the respective standard proteins is indicated: T = thyroglobulin, F = ferritin, B = BSA, O = ovalbumin, and M = myoglobin.

These aggregates are nonetheless enzymatically active.

One single protein band migrating at a MW of about 50 kDa can be detected on SDS-PAGE (Fig. 3). This is in good agreement with the results of other groups [5–7].

Nevertheless, isoelectrofocusing yields two major and four minor bands in the pH 5.4–5.8 region of the gel (Fig. 4), one of these bands is very faint.

Usually the separation of up to four polypeptide bands by isoelectrofocusing is described (*e.g.* [5, 7]).

The possibility of proteolytic damage to the protein during the purification procedure and the subsequent electrophoresis cannot be excluded.

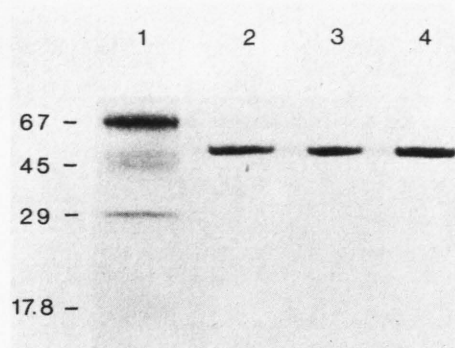


Fig. 3. SDS-PAGE of purified GS. The respective MWs of the marker proteins are indicated on the left margin of the photograph. Lane 1: standard proteins, lanes 2–4: purified GS from three different preparations.

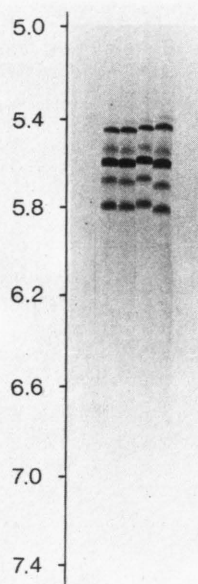


Fig. 4. Isoelectrofocusing of purified GS from different preparations in the presence of 8 M urea. The pH-range is indicated at the left margin of the photograph.

However, this explanation seems to be unlikely because of preliminary results of two-dimensional gelelectrophoresis of purified GS. Furthermore, the relative proportions of the different polypeptide bands seem to be very constant.

We suggest that the GS of mustard plants is composed of up to six slightly different types of catalytically active subunits.

Those differences might be a result of the presence of carbohydrates in the plastidic GS as described by Nato *et al.* [15] or to other posttranslational or post-transcriptional processes. However, it has to be considered that no significant glycosylation was found in plastidic GS of *Pisum sativum* and *Nicotiana plumbaginifolia* [16, 17].

We assume that eventually multiple genes might be responsible for the synthesis of the different subunits. This problem is under further investigation.

The holoenzyme seems to be organized in two aggregates of four subunits each. This finding is in agreement with electron microscopy data as presented by Pushkin *et al.* [4].

Both, labelling of the purified enzyme with [^{14}C]PPT and with [$\gamma\text{-}^{32}\text{P}$]ATP confirms the existence of eight reactive centers per GS-molecule as shown in Table II. It appears as if each subunit has its own active center.

As far as we know, this is the first report on this topic concerning the plastidic GS and the results are in good agreement to the studies of Ronzio *et al.* [11] on the GS from sheep brain.

Obviously the binding of phosphate and PPT occurs in a 1:1 stoichiometry while PPT does not bind to GS without addition of ATP (Fig. 5, see also Table II). Further indications that irreversible binding of PPT occurs as a PPT-phosphate were reported previously [18]. A similar situation has already been shown in the case of MSO [11, 19], another irreversible inhibitor of GS activity.

Table II. Determination of active centers of purified GS. Protein containing fractions were pooled for measurement of radioactivity and protein. The presented data are obtained from this pool. Calculations are performed assuming a MW of 400 kDa for the GS.

Exp. No.	Marker substance	Spec. act. [Ci/mol]	Protein [$\mu\text{g}/\text{ml}$]	[nmol/ml]	Bound act. [total dpm]	[nmol/pool]	Molar ratios	
							PPT/GS	ATP/GS
1	[^{14}C]PPT	2.16	5.5	0.014	2080	0.438	7.82	–
2	[^{32}P]ATP	8.68	5.0	0.013	8720	0.456	–	8.77

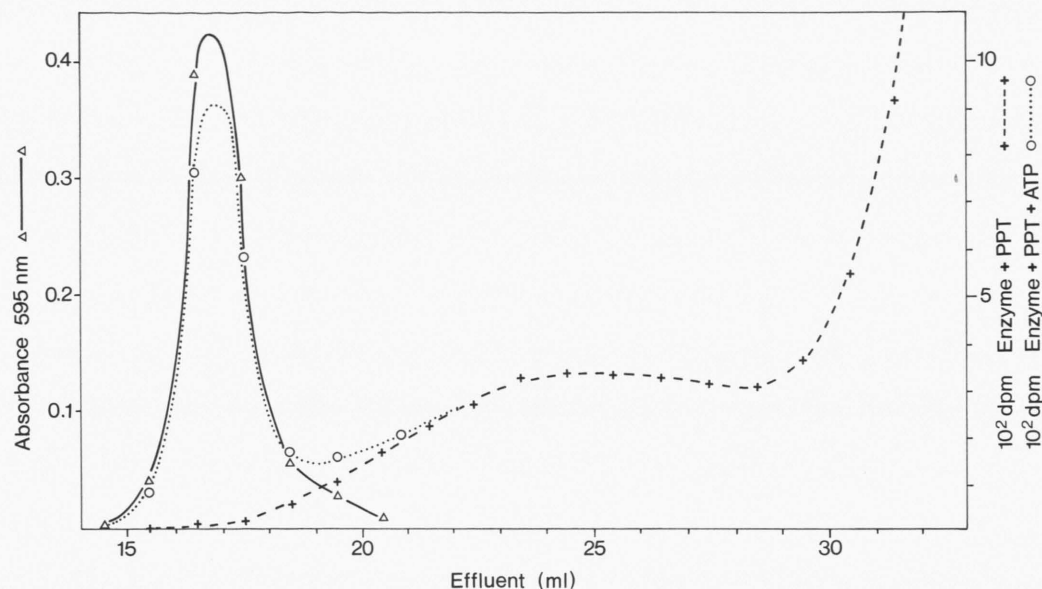


Fig. 5. Gelfiltration profiles of the labelling assays of purified GS under different conditions. Elution of protein (—), distribution of radioactivity using the reaction mix with PPT but without ATP (+ - +), and eluted radioactivity using ATP and PPT in the labelling assay (O ··· O).

Further studies are in progress in order to clarify, whether the GS from green leaves of *Sinapis alba* is glycosylated and whether the heterogeneity of the subunits is the result of different expression of multiple genes.

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