Characterization of Glutamine Synthetase of Roots, Etiolated Cotyledons and Green Leaves from *Sinapis alba* (L.)

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Glutamine synthetase of roots, etiolated cotyledons and green leaves from mustard plants cannot all clearly be separated by DEAE-Sephacel chromatography. However, the enzyme of the roots, etiolated cotyledons and green leaves, respectively, differed in the kinetic properties determined in the crude extract. The root enzyme showed a pH-optimum of about 6.9, a K_m value of 3 mM for glutamate and a temperature optimum at 48 °C. Glutamine synthetase of etiolated cotyledons possessed a K_m for glutamate of 6 or 12 mM, depending on the dithioerythritol concentration in the homogenisation buffer and a temperature optimum at 46 °C. The enzyme of green leaves was characterized by a temperature optimum at 40 °C, a pH-optimum at about 7.4 and a low glutamate affinity with positive cooperative substrate binding. Based on isolation of chloroplasts and identification of glutamine synthetase the enzyme of green leaves seems to be the chloroplastic form. This enzyme was purified by DEAE-Sephacel, hydroxylapatite and Sephacryl S-300 chromatography. Affinity for glutamate and $MgSO_4$ of the purified enzyme differed from that found in the crude extract. The function of the different isoenzymes is discussed.

Introduction

Three different isoenzymes of GS (L-glutamate: ammonia ligase, ADP forming, EC 6.3.1.2) were first found in rice plants: a root enzyme (GS_R), a cytosolic leaf enzyme (GS_1) and an enzyme, which is located inside the chloroplasts (GS_2) [1, 2]. The isoenzymes of GS can be separated by ion exchange chromatography [3, 4]. They differ especially in their heat stability, pH-optimum and affinity for glutamate [5–8]. The isoenzyme pattern depends on the plant species [9] as well as the state of development [10–12]. In this paper we report on the isoenzymes of GS of mustard plants and their kinetic properties and on the purification of GS of green leaves.

Abbreviations: DTE, 1,4-dithioerythritol; Glu, glutamate; GS, glutamine synthetase; GS_1, cytosolic glutamine synthetase; GS_2, chloroplastic glutamine synthetase; GS_R, root glutamine synthetase; GS_{et}, glutamine synthetase of etiolated cotyledons; ME, 2-mercapto-ethanol.

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Materials and Methods

Plant material

Plants of *Sinapis alba* were cultured as described [13]. Etiolated plants were grown in the dark at 20 °C and 75% relative humidity for 6–7 days.

Enzyme extraction

All steps were carried out at 4 °C. The plant material was ground in a mortar in 0.1 M Tris-HCl, pH 7.8, 0.5 mM EDTA, 1 mM $MgSO_4$, 5 mM DTE (roots) or 10 mM DTE (green leaves and etiolated cotyledons) unless otherwise stated. The resulting pulp was centrifuged for 10 min at $20000 \times g$ and the supernatant used to study the enzyme.

Chloroplasts of mustard leaves were isolated as described [14]. They were broken by the addition of 10 mM Tris HCl, pH 7.8, 0.5 mM EDTA, 1 mM $MgSO_4$ and 25 mM ME. The soluble stroma proteins obtained by centrifugation (10 min at $20000 \times g$) were applied on a DEAE-Sephacel column (2×10 cm).

DEAE-Sephacel chromatography

The crude extract was desalted on a Sephadex G-25 column (1.5×30 cm) in 10 mM Tris-HCl, pH 7.8, 0.5 mM EDTA, 1 mM $MgSO_4$ and 1 mM DTE (root extract) or 25 mM ME (leaf extract). Desalted ex-

tract was applied on a DEAE-Sephacel column (2 × 10 cm) previously equilibrated with the above buffer. Proteins were eluted with 0–0.4 M KCl or NaCl in a total volume of 200 ml buffer. Four-ml fractions were collected and the flow rate was 25 ml × h^{-1}.

Purification of GS of green leaves

Green mustard leaves (95 g fresh weight) were homogenized in 360 ml 0.05 M Tris-HCl, pH 8.3, 0.5 mM EDTA, 1 mM $MgSO_4$ and 30 mM ME in a Starmix MX (Braun, Inc.). The pulp was filtered through four layers of cheesecloth and centrifuged at 30000 × *g* for 40 min. The supernatant was applied on a DEAE-Sephacel column (3 × 15 cm) previously equilibrated with the same buffer. The column was washed with a linear KCl gradient (0–0.6 M) in a total volume of 600 ml buffer.

Flow rate was 48 ml × h^{-1} and 8-ml fractions were collected. Fractions with GS activity were pooled and mixed with 0.025 volume of 2 mM imidazol, pH 7.0. pH was adjusted to 7.0 with 1 M HCl. The protein solution was applied on a hydroxylapatite (Bio Gel HTP, Bio Rad) column (3 × 10 cm) previously equilibrated with 0.05 M imidazol, pH 7.0, 0.5 mM EDTA, 1 mM $MgSO_4$, and 30 mM ME. After washing the column with 100 ml of buffer, also containing 0.1 M potassium phosphate, proteins were eluted with a linear K-phosphate gradient (0.1–0.4 M) in a total volume of 500 ml buffer. Fractions of 7.5 ml were collected at a flow rate of 24 ml × h^{-1}. Fractions with GS activity were pooled and precipitated with ammonium sulfate (70% saturation). After centrifugation (10 min at 20000 × *g*) the pellet was dissolved in 0.05 M Tris-HCl, pH 7.8, 50 mM $MgSO_4$, 1 mM EDTA and 30 mM ME, and layered on a Sephacryl S-300 column (3 × 60 cm) previously equilibrated with this buffer. Elution was carried out with the same buffer at a flow rate of 24 ml × h^{-1}. Fractions of 7.5 ml were collected. Fractions with GS activity were pooled and concentrated on a small DEAE-Sephacel column (2.5 ml gel volume) and eluted with the buffer also containing 0.3 M KCl. Fractions with GS activity higher than 2.5 units × ml^{-1} were pooled. At this stage the kinetic properties of the purified enzyme were checked.

Enzyme assay

GS activity was measured at 37 °C by the formation of γ-glutamylhydroxamate in the synthetase reaction [15]. The reaction mixture contained in 2 ml 100 mM imidazol, pH 7.2, 10 mM EDTA, 50 mM $MgSO_4$, 10 mM NH_2OH, 15 mM ATP, 50 mM sodium glutamate with the root enzyme, 100 mM with the enzyme of etiolated cotyledons and 150 mM with the enzyme of green leaves. One unit of activity corresponds to 1 μmol γ-glutamylhydroxamate × min^{-1}.

Protein determination

Protein concentration was assayed by the Bio-Rad protein assay.

Results

Chromatographic properties of GS from roots, etiolated cotyledons and green leaves of mustard plants

DEAE-Sephacel chromatography of desalted extracts of roots, etiolated cotyledons and green leaves all yielded one peak of GS activity eluting at about 0.25 M KCl or NaCl (Fig. 1A). The extract of etiolated cotyledons showed an additional smaller peak

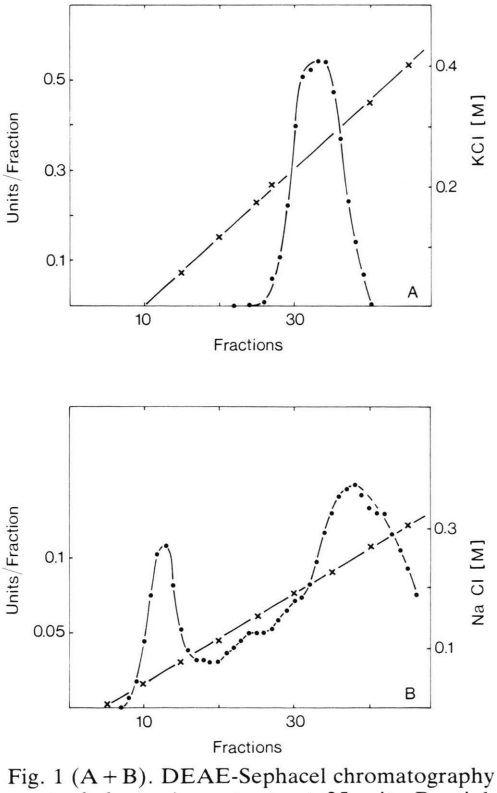

Fig. 1 (A + B). DEAE-Sephacel chromatography of GS of mustard plants. A: root extract, 25 units. B: etiolated cotyledons, 5 units. GS activity (●), salt gradient (x).

at about 0.05 M NaCl (Fig. 1B). Ion exchange chromatography of the soluble chloroplastic proteins also resulted in one peak of GS activity eluting at 0.25 M KCl (data not shown). Consequently GS of roots and green leaves of mustard plants cannot be separated by ion exchange chromatography at least under the conditions employed, whereas in reports on other plant species both isoenzymes are always found to differ in their elution patterns using ion exchange chromatography [1, 2, 16].

Kinetic properties of GS

The kinetic properties of GS from roots, etiolated cotyledons and green leaves are summarized in Table I. The data indicate that mustard plants have three different isoenzymes of GS, which differ in their temperature optimum, pH-optimum, and affinity for glutamate. The temperature optimum and the affinity of GS for glutamate of green leaves depend on the DTE concentration in the homogenization buffer [17]. Further detailed examination demonstrated that the glutamate affinity of GS of etiolated cotyledons is dependent on the thiol concentration in the homogenization buffer, too. Low DTE concentration (10 mM) caused high glutamate affinity ($K_m = 6$ mM) and high DTE concentration (30 mM) low glutamate affinity ($K_m = 12$ mM) (Fig. 2).

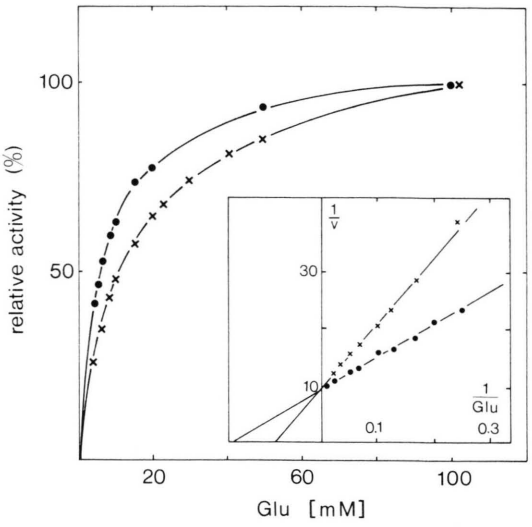

Fig. 2. The effect of different DTE concentrations in the homogenization buffer on the affinity of GS of etiolated cotyledons for glutamate in crude extract. $K_m = 5.6$ and 12.4 mM with 10 mM DTE (●) and 30 mM DTE (x), respectively. 100% activity corresponds to 110 μmol/h·g FG.

Table I. Kinetic properties of GS from crude extract of roots (GS_R), etiolated cotyledons (GS_{et}) and green leaves (GS_L) of mustard plants.

	GS_R	GS_{et}		GS_L	
Concentration of DTE (mM) in homogenization buffer	5	10	30	5	30
pH-optimum	6.9	n.d.		7.4	
Temperature optimum (°C)	48	46	n.d.	37	40
Activation energy (kJ/mol)	56	49	n.d.	53	61
K_m (ATP) (mM)	1.0	n.d.		1.0	
Glutamate: K_m (mM)	3.4	5.6	12.4	–	–
$S_{0.5}$ (mM)	–	–	–	50	13
$MgSO_4$: $S_{0.5}$ (mM)	22	n.d.		26	25

n.d., not determined.

Purification of GS of green leaves

The purification steps are summarized in Table II. The elution patterns from a DEAE-Sephacel column and a hydroxylapatite column showed always one peak of GS activity (Fig. 3 + 4) and thus give evidence that green mustard leaves contain only one isoenzyme of GS. The purification procedure yielded a 97-fold purification of GS and the final specific activity is comparable with values found with GS of rice leaves (1) and tomato leaves [18]. However, the saturation

Table II. Purification of GS from green mustard leaves.

Purification step	Total protein [mg]	Total activity [Units]	Specific activity [Units/mg protein]	Recovery [%]	Purification -fold
Crude extract	1401	819	0.58	100	1
DEAE-Sephacel	128	419	3.27	51	5.6
Hydroxylapatite	8.5	351	41.3	43	71
70% $(NH_4)_2SO_4$ precipitation	4.4	191	43.4	23	75
Sephacryl S-300	3.7	144	38.9	18	67
DEAE-Sephacel	2.5	140	56	17	97

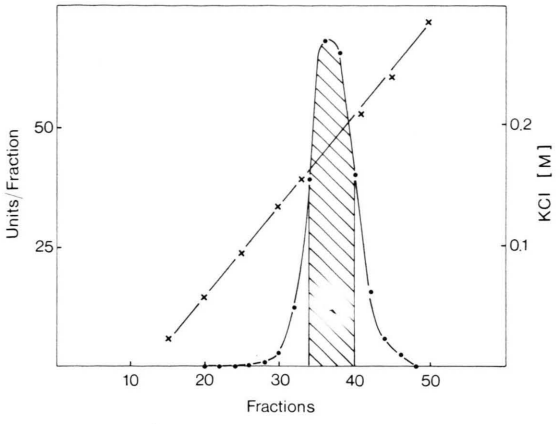

Fig. 3. Elution profile of GS of green mustard leaves from a DEAE-Sephacel column (3 × 15 cm). 819 units were loaded onto the column and eluted with 600 ml 0–0.6 M KCl gradient. GS activity (●), salt gradient (x); the hatched area indicate the fractions used in further purification steps.

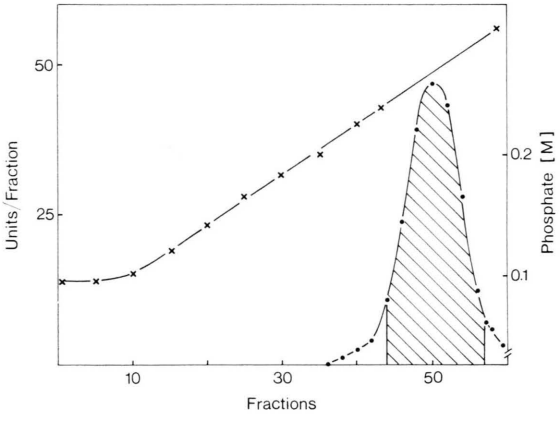

Fig. 4. Elution profile of GS of green mustard leaves from a hydroxylapatite column (3 × 10 cm) previously purified on DEAE-Sephacel. 351 units were loaded onto the column and eluted with 500 ml 0.1–0.4 M K-phosphate gradient. GS activity (●), salt gradient (x); the hatched area indicates the fractions used in the further purification procedure.

curves of the purified enzyme for Mg^{2+} and glutamate were quite different from the curves found with the crude extract (Fig. 5), which indicates that the enzyme is changed during the purification process and activity is decreased due to diminished affinity of the enzyme.

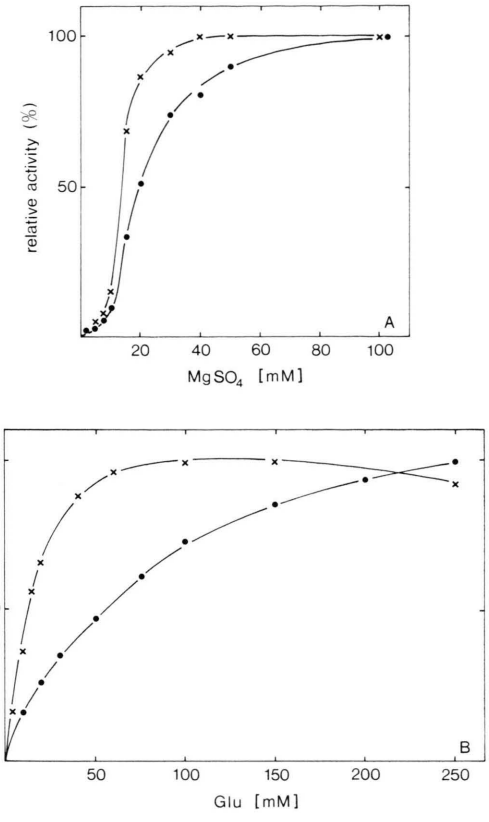

Fig. 5 (A + B). Affinity of GS from green leaves of mustard towards Mg^{2+} (A) and glutamate (B). Affinity of GS of crude extract (x). Affinity of GS purified as described under "Materials and Methods" (●). The enzyme assay for Mg^{2+} saturation curve contained only 0.5 mM EDTA.

Discussion

According to the kinetic properties, e.g. temperature optimum, glutamate affinity and pH-optimum, it seems that mustard plants possess three different isoenzymes of GS. However, the root enzyme and the enzyme of green leaves cannot be separated by DEAE-Sephacel chromatography at least under the conditions used.

The root enzyme, GS_R, is characterized by high glutamate affinity and a high temperature optimum, which also has been demonstrated for GS_R of rice plants [1].

As isolated chloroplasts contain high GS activity and purification of GS of green leaves yielded only one peak of GS activity throughout the purification procedure, we assume that only the chloroplastic GS

(GS_2) is present in green leaves. Further evidence comes from the low glutamate affinity, the pH-optimum and the low temperature optimum of this isoenzyme, which has also been confirmed for GS_2 of other plants [1, 3, 8]. Thus *Sinapis alba* belongs probably to that group of plant species, which contain only a chloroplastic GS in green leaves [9].

The third isoenzyme we called GS_{et}, because we exclusively could find it in etiolated cotyledons. Its time of presence and its kinetic properties (*i.e.* glutamate affinity and temperature optimum) suggest that it is the cytosolic isoenzyme of the leaves, GS_1.

The purification of GS of green leaves has demonstrated that kinetic properties of the enzyme are changed during the purification process. This could be the result of oxidation of SH-groups of the enzyme, which have been discussed to be involved in glutamate binding [17]. Therefore we conclude that it is better to determine kinetic properties of the enzyme in the crude extract than with the purified enzyme.

Between the three isoenzymes of GS of mustard plants exists a clear difference in the glutamate affinity, which has also been described for other plant species [1, 3, 8]. GS_1 and GS_R have a high affinity and GS_2 a low affinity for glutamate. According to Walker *et al.* [19], the glutamate concentration of wheat leaves increases in the light. Therefore we suppose that GS_2 having low glutamate affinity functions in the light during photorespiration. This assumption is supported by evidence found by other authors [6, 9]. However, GS_R and GS_1 might function at low glutamate concentrations, *i.e.* in cotyledons during germination, in green leaves during the dark and in roots all the time. Both isoenzymes are responsible for NH_4^+ assimilation derived from nitrate assimilation and protein breakdown. If that is true, other plant species, which contain only GS_2 in green leaves, should also possess another isoenzyme of GS with high glutamate affinity in the leaf during germination, as we have found with mustard plants.

Further investigations are now in progress in order to clarify, whether other plant species contain an isoenzyme of GS, which is only present in the etiolated state, and whether there are other plants having isoenzymes not separable by ion exchange chromatography.

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