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Identification of two forms of Glutamine synthetase in barley (Hordeum vulgare)

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Summary. Two forms (designated GS_I and GS_{II}) of glutamine synthetase (E.C.6.3.1.2) have been found in barley (Hordeum vulgare) which can be separated by ion exchange chromatography. Light grown shoots contain both GS_I and GS_{II}, roots, seeds and etiolated shoots exhibit only GS_I activity. In light grown shoots GS_{II} appears to be chloroplastic and GS_I cytosolic. Sedimentation coefficients of both forms are similar (GS_I,14.04; GS_{II},14.13 S_{20w}) as are their Stokes radii (GS_I,6.13; GS_{II}, 6.35 nm) and molecular weights (GS_I 349,000; GS_{II} 363,000 daltons). The pH optimum for GS_{II} (pH 7.5) is somewhat more alkaline than that of GS_I (pH 7.0). GS_{II} is less stable than GS_I; in the absence of protective ligands it loses activity at 30°C and is inactivated by thiol reactive agents. GS_I is stable at 30°C and its activity is unaffected by thiol reactive agents.

Introduction

The discovery of glutamate synthase activity in higher plants has had important consequences as regards the pathways of ammonia assimilation (1) and has led to the recognition that glutamine synthetase (L-glutamate:ammonia ligase (ADP) E.C.6.3.1.2) plays a key role in the assimilation and control of nitrogen metabolism (see 2,3). The enzyme has been characterised in only a few higher plants (4,5,6,7,8) and it appears that while only a single molecular form has been found in plant cells, activity is found in both chloroplasts (9,10,11) and cytosol (11). The chloroplast activity is suggested to function primarily in nitrogen assimilation while that in the cytosol plays a role in the re-assimilation of ammonia released in the photorespiratory nitrogen cycle (12).

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Here we report preliminary investigations on the glutamine synthetase of barley which demonstrate the existence of two distinct forms of the enzyme which although similar in molecular weight, differ in their tissue and subcellular localization and stability.

Methods.

Plant Material: Hordeum vulgare L. (var. Golden Promise) was grown in water culture using $\frac{1}{4}$ strength Long Ashton solution (13) with 5 mM nitrate either in a growth chamber at 22°C day 15°C night 12 hr cycle or under continuous illumination at 23°C. Material for protoplast isolation was grown in vermiculite and watered with distilled water.

Extraction and ion exchange chromatography: Fresh material was ground in a mortar and pestle with 50 mM Tris HCl buffer pH 8.0 containing 1 mM EDTA, 1 mM dithiothreitol, 1 mM mercaptoethanol, 1 mM reduced glutathione, 10 mM magnesium sulphate, 5 mM glutamate, 10% ethylene glycol. The homogenate was filtered through muslin and centrifuged at 20,000 g for 20 min. The supernatents were applied to a DEAE-Sephacel (Pharmacia Ltd.) column equilibrated with extraction buffer. Elution was carried out with a 0-0.6 M KCl linear gradient in extraction buffer.

Sucrose density and gel filtration: Sedimentation coefficients were obtained from 5-20% sucrose gradients on an MSE superspeed 65 centrifuge with standard proteins of known $S_{20}w$ according to the general procedure of Martin and Ames (14). Gel filtration was carried out using a 1.5 x 15 cm column of sepharose CL6B and standard proteins of known Stokes radii. Molecular weight and frictional ratio were calculated according to the method of Siegel and Monty (15).

Protoplast isolation: Protoplasts were prepared, purified, ruptured and chloroplasts isolated on sucrose density gradients using a modification of the procedures of Wallsgrove et al (11).

Enzyme assays: The transferase and synthetase activities were determined as described by Rhodes et al (16).

Results and Discussion.

Ion exchange chromatography on DEAE-Sephacel of light grown barley leaf extracts revealed the presence of two peaks of glutamine synthetase activity. These have been designated GS_I and GS_{II} and were eluted at 0.15 M and 0.33 M KCl respectively (Fig. 1a). Re-chromatography of the fractions containing GS_I and GS_{II} after dialysis yielded single peaks of GS_I and GS_{II}

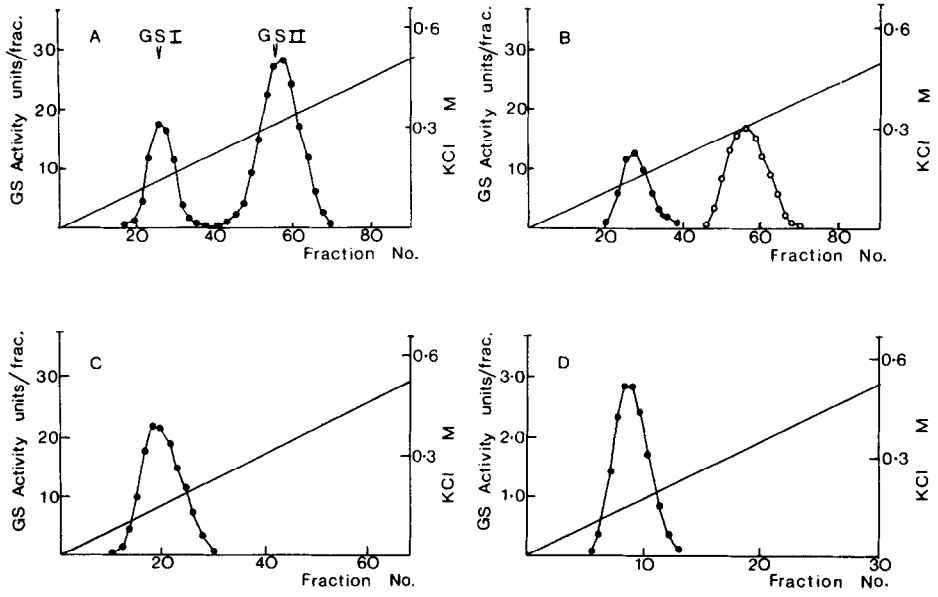

Figure 1: Elution profiles of barley glutamine synthetase on DEAE-Sephacel. Glutamine synthetase activity determined as transferase, 1 unit = 1 μmole γ-glutamyl hydroxamate/min at 30°C.
a) Light grown leaf extract, 550 units loaded onto 2.5 x 6 cm column, eluted with 400 ml 0-0.6 M KCl gradient.
b) Rechromatography of GS_I and GS_{II} after dialysis reapplied to 1.5 x 6 cm column, eluted 200 ml 0-0.6 M KCl gradient ●—● GS_I o—o GS_{II}.
c) Root extract, 183 units loaded onto 6 x 1.5 cm column eluted with 300 ml 0-1 M KCl gradient.
d) Seed extract, 17 units loaded onto 6 x 1.5 m column eluted with 60 ml 0-0.6 M KCl gradient.

respectively (Fig. 1b). This suggests that the two forms are not an artefact of the chromatographic procedure and that they are not under these conditions interconvertible. When extracts of root, seed (Fig. 1c,d) and etiolated leaf tissue (Fig. 2a) were applied to DEAE-Sephacel only a single peak of glutamine synthetase activity, which corresponded to GS_I of light grown leaves, was present. After 17 h exposure to light, GS_{II} was detectable in etiolated leaf tissue (Fig. 2b).

Subcellular localization studies of GS_I and GS_{II} were carried out using protoplasts prepared from light grown leaf tissue and the results in Fig. 3a show the presence of gluta-

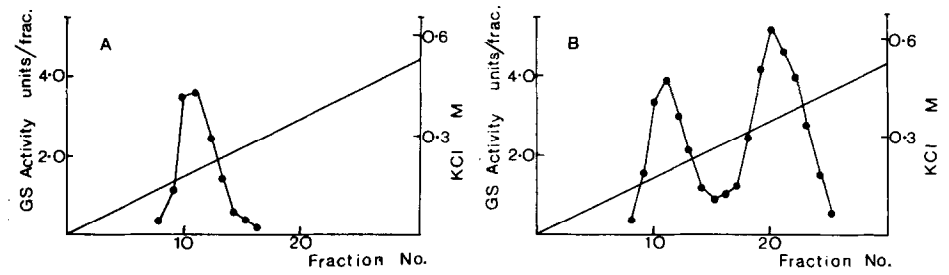

Figure 2: Elution profile of barley shoot glutamine synthetase.
A Etiolated leaf extract 18.0 units loaded onto 4 x 1.5 cm column eluted 60 ml 0-0.6 M KCl gradient. Total activity 5.5 units/gfwt.
B Same material as A after 17 h light exposure 50 units loaded onto 4 x 1.5 cm column, eluted 60 ml 0-0.6 M KCl gradient. Total activity 16 units/gfwt.

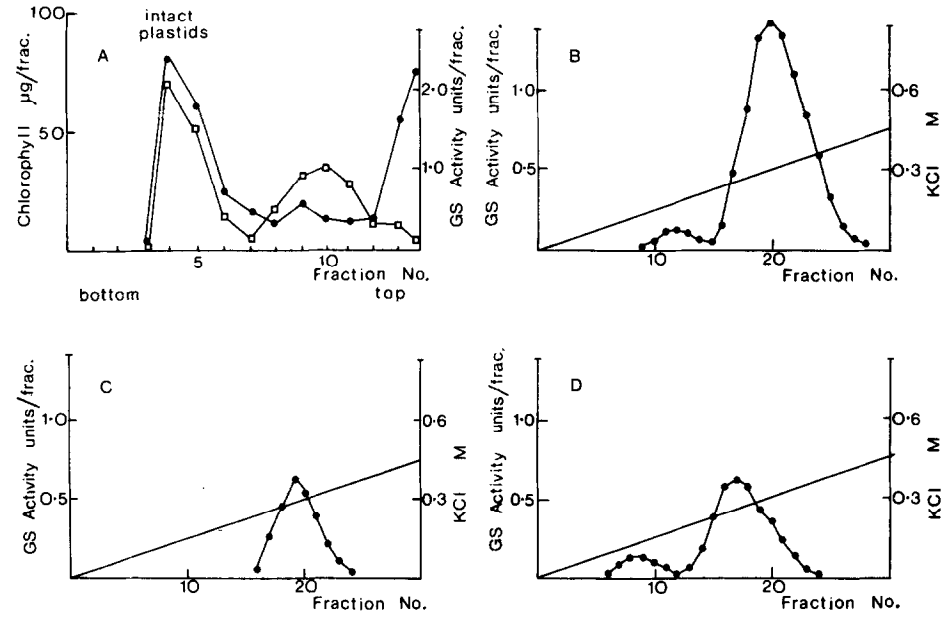

Figure 3: a) Sucrose density fractionation of ruptured protoplast preparation, 11.6 units glutamine synthetase loaded, 500 ug chlorophyll.
□—□ chlorophyll ●—● glutamine synthetase activity.
b) DEAE-Sephacel elution profile of protoplast glutamine synthetase, 11.6 units loaded onto 1.5 x 5 cm column, eluted 40 ml 0-0.6 M KCl gradient.
c) DEAE-Sephacel elution profile of intact chloroplast fraction, 3.7 units loaded, column conditions as (b).
d) DEAE-Sephacel elution profile of supernatant fraction, 4.0 units loaded, column conditions as (b).

mine synthetase activity in intact chloroplasts and in supernatent fractions (representing material from ruptured chloroplasts and cytosol) from sucrose density gradients of ruptured protoplasts. 50% of the chlorophyll and 47% of the glutamine synthetase activity were recovered in the intact chloroplast fractions. DEAE-Sephacel chromatography of protoplast extracts indicated the presence of 5-10% GS_I and 90-95% GS_{II} (Fig. 3b). Chromatography of the intact chloroplast fraction showed however the presence of only one peak, corresponding to GS_{II} (Fig. 3c). Chromatography of the supernatent fractions (Fig. 3d) showed an apparent enrichment in GS_I activity (15% GS_I, 85% GS_{II}) compared with protoplast extracts. These results suggest then that GS_{II} is located in the chloroplasts while GS_I is located in the cytosol.

The sedimentation coefficients, Stokes radii, frictional coefficients and calculated molecular weights of GS_I and GS_{II} are similar (see Table 1) indicating that differences in the state of polymerization is not the molecular basis for two forms of barley glutamine synthetase. The pH optimum for the magnesium dependent synthetase activity of GS_I was 7.0 while that of GS_{II} was 7.5

During purification studies GS_{II} was found to be more unstable than GS_I and the results in Table 2 show that magnesium, glutamate and a thiol such as dithiothreitol are necessary to stabilize the activity of GS_{II} at 30°C. These results also show that thiol reactive reagents such as N-ethyl maleimide (NEM) and 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB) rapidly inactivate GS_{II} but have relatively little effect on GS_I. These results suggest that there may be conformational differences between GS_I and GS_{II} which affect the exposure of sulphydryl groups.

Table 1.

Physical Properties of GS_I and GS_{II}

	Sedimentation Coefficient $S_{20}{}^w$	Stokes Radius nm	Molecular Weight daltons	Frictional Ratio f/fo
GS_I	14.04	6.125	349,000	1.33
GS_{II}	14.125	6.3	363,000	1.35

Table 2.

Comparison of Stability of GS_I and GS_{II}

Treatment	% Activity after 1 h incubation	
	GS_I	GS_{II}
Tris buffer pH 8.0 30°C	92	42
+ Mg^{2+} (10mM) 30°C	100	68
+ Dithiothreitol (3mM) 30°C	95	73
+ Glutamate (5mM) 30°C	94	42
+ Mg^{2+} + Dithiothreitol + Glutamate 30°C	100	100
+ Mg^{2+} + Glutamate + NEM (0.1mM) (0°C)	92	28
+ Mg^{2+} + Glutamate + DTNB (0.1mM) (0°C)	100	60

Enzyme desalted on Sephadex G75 column into 50 mM Tris Cl pH 8.0. Synthetase activity determined.

Similar differences in accessibility of sulphydryl groups to titration by thiol reagents have been reported for the "taut and relaxed" forms of *E. coli* glutamine synthetase (17,18) and for the active and deactivated forms of the *Lemna minor* enzyme (3).

While more detailed studies are necessary to establish the precise metabolic roles of GS_I and GS_{II} it is unlikely that GS_I functions exclusively in the photorespiratory nitrogen cycle since it is the only form present in roots and seeds. Differences in the stability of the two forms could relate to the control of GS_{II} in the chloroplast. Several chloroplastic enzymes of photosynthetic carbon metabolism are light activated by mechanisms

involving changes in thiol groups (19,20) and there is the possibility that GS_I and GS_{II} could be chemically modified forms of the same protein and that light mediates a conversion of GS_I to GS_{II}.

Acknowledgements

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