Kinetic analysis of glutamine synthetases from various plants

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Glutamine synthetase (GS) was partially purified from the leaves of 7 plant species and the relative contributions of two isoforms, GS1 and GS2, were determined. Only 3 species, *Zea mays, Hordeum vulgare* and *Triticum aesticum,* contained detectable levels of GS1. K_m values for glutamate and ATP of the various GS isolates were all within a narrow range. Both GS isoforms may be inhibited by phosphinothricin, competitively with glutamate. δ-Hydroxylysine also inhibited both isoforms but acted uncompetitively. The kinetic parameters of GS isolated from all 7 species showed a remarkable similarity.

Glutamine synthetase *Phosphinothricin* *δ-Hydroxylysine*

1. INTRODUCTION

Glutamine synthetase (GS) (EC 6.3.1.2) is the first enzyme involved in the assimilation of ammonia by plants. Inhibition of GS might be herbicidal, possibly due to toxicity resulting from increased ammonia levels. DL-Phosphinothricin (DL-PPT) and δ-hydroxylysine have been identified as potent inhibitors of GS [1], but the action of these inhibitors has been studied only with crude preparations of GS. However, it has been shown that GS occurs as two isoforms in plants: GS1 in the cytosol and GS2 in the chloroplast [2,3]. We have therefore examined the inhibition of GS isoforms by DL-PPT and δ-hydroxylysine to see if there is any differential sensitivity of the two enzyme forms. Furthermore, in view of the demonstrated variations in the relative proportions of GS1 and GS2 between species [3], we have examined the effects of DL-PPT on the GS isoforms from a range of plant species. Plants were chosen to represent crop and major attendant weed pairs in the hope that any variation in inhibition sensitivity might offer clues to selective herbicide design. We were encouraged in the search for such selective sensitivity by work in this laboratory on citrate synthase which has revealed striking patterns of inhibition variation between organisms [4].

2. EXPERIMENTAL

All seeds were obtained from ICI, Jealott's Hill Research Station, Bracknell, England. They were planted in Levington potting compost and grown in greenhouses with supplementary lighting giving a day length of 16 h, with a temperature regime of 32°C day/14°C night. The grasses were harvested 10 to 14 days after planting, soya leaves were harvested one month after planting and the leaves of *Cassia obtusifolia, Ipomoea purpurea* and sugar beet were harvested two months after planting.

DL-Phosphinothricin was obtained from Dr S.M. Ridley of ICI Plant Protection Division. δ-Hydroxylysine and glucosamine-6-phosphate were purchased from Sigma.

For enzyme extraction and purification all operations were carried out at 4°C. 7 to 10 g of fresh leaves were used in each experiment. The leaves were homogenized in 20 ml of 100 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 1 mM dithiothreitol using either an Ultra-Turrax tissue emulsifier or a mortar and pestle with sand. After

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filtration through 4 layers of muslin, 1 g of polyvinylpolypyrrolidone was added to the filtrate, which was stirred for 5 min before a second filtration through 4 layers of muslin. This filtrate was centrifuged at 37000 × *g* for 30 min. The supernatant was desalted on a Sephadex G-25 column (Pharmacia PD-10) previously equilibrated with buffer A (25 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 1 mM dithiothreitol). 1 ml fractions were collected and assayed for GS activity in a micro-assay outlined below. Active fractions were pooled and passed through a 0.2 μm filter prior to loading onto a mono-Q column of a Pharmacia fast protein liquid chromatography (FPLC) system. The column was eluted with a linear gradient of NaCl (0–400 mM) over 28 min at a flow rate of 1.5 $ml \cdot min^{-1}$ and 1 ml fractions were collected. Fractions were assayed for GS activity using the micro-assay below. Active fractions were pooled and used for kinetic analysis. Activity could be maintained overnight, without loss, by adding ethylene glycol to a final concentration of 10% (v/v) and freezing.

GS activity was determined using the biosynthetic assay based on γ-glutamylhydroxamate formation as described in [5]. The reaction was terminated after 15 min at 30°C. The amount of γ-glutamylhydroxamate formed was determined colorimetrically at 535 nm using a standard curve prepared with authentic γ-glutamylhydroxamate (Sigma).

A micro-assay was performed in 96-well microtitre plates using the same conditions as the standard assay but in a final volume of 200 μl. The reaction was started by adding 50 μl of enzyme and terminated after 15 min by adding 100 μl of the acidic ferric chloride reagent. Activity was detected visually.

K_m values were determined by the direct linear plot of Eisenthal and Cornish-Bowden [6] using a computer program available at Bath University.

3. RESULTS AND DISCUSSION

GS1 and GS2 were readily separated by FPLC; GS1 was eluted between 0.15 and 0.2 M NaCl, and GS2 between 0.27 and 0.32 M NaCl. These values are similar to those reported by Mann et al. [2] for the elution of GS from DEAE-Sephacel. The two activities were separated by 3–5 fractions which contained no detectable activity. Partial purification in this manner yielded GS1 and GS2 fractions for use in the kinetic analyses within 6 h after homogenisation.

The standard extraction procedure was successful for the isolation of GS from the 7 species in table 1; no GS activity could be isolated from *Ipomoea* possibly due to its high phenolic content. The glutamate dependences of both GS1 and GS2 were found to follow Michaelis-Menten kinetics in all the species examined although both forms exhibited substrate inhibition at ATP concentrations above 7 mM. K_m values for glutamate showed only slight variation, ranging from 4 to 10 mM (table 1). Only two species, *Zea mays* and *Hordeum*

Table 1

Isoform distribution and kinetic parameters of plant glutamine synthetases

Species	% activity		K_m(Glu), mM		K_m(ATP), mM		K_i(DL-PPT), μM	
	GS1	GS2	GS1	GS2	GS1	GS2	GS1	GS2
Barley	9	91	4.9	8.2	0.3	0.6	3.5	6.0
Cassia	–	100	–	7.3	–	0.9	–	8.5
Maize	42	58	5.2	9.8	0.4	0.9	2.0	4.0
Soya	–	100	–	4.1	–	0.3	–	8.0
Sugar beet	–	100	–	8.4	–	1.3	–	6.5
Wheat	4	96	ND	7.7	ND	0.9	ND	9.0
Wild oats	–	100	–	5.5	–	0.6	–	5.0

ND, not determined

vulgare, had sufficient GS1 activity to allow a reliable determination of the glutamate dependence; GS1 had a lower K_m for glutamate than GS2. The K_m values for ATP ranged from 0.3 to 1.26 mM. Again, the K_m values of GS1 for ATP were approximately half those of GS2. All K_m values determined in this study fell within the range compiled by Stewart et al. [7].

The effects of DL-PPT on GS activity were examined. Experiments were performed at 3 concentrations of glutamate: 5, 12.5 and 25 mM, corresponding to 0.5–1 K_m, 1–2 K_m and 2.5–5 K_m, respectively. DL-PPT was varied from 5 to 100 μM, i.e. from 1- to 200-times the K_i value obtained with GS from *Escherichia coli* [8]. When plotted according to Dixon [9] our data yielded straight lines (fig.1) indicating competitive inhibition; this was confirmed by the parallel straight lines obtained by plotting [S]/v against [I]. K_i values (table 1) ranged from 2 to 9 μM. Again, the K_i values for GS1 were half those for GS2 from the same species.

δ-Hydroxylysine [1] and glucosamine-6-phosphate [10] have also been reported to inhibit GS. Glucosamine-6-phosphate was reported to inhibit GS2 but not GS1, though we have been unable to confirm this differential inhibition as no significant inhibition of GS1 or GS2 from maize or barley was observed even at 10 mM concentration.

δ-Hydroxylysine inhibited both GS1 and GS2 from maize with K_i values of 0.49 and 0.85 mM, respectively. On the basis of an [S]/v against [I] plot (fig.2), the inhibition was judged to be uncompetitive for both GS1 and GS2 (results shown only for GS2). δ-Hydroxylysine also proved to be nearly twice as potent an inhibitor of GS1 as of GS2, so that the inhibition of GS1 and GS2 by δ-hydroxylysine follows a similar pattern to the inhibition of GS1 and GS2 by DL-PPT. However, these two inhibitors probably have different modes of action, as one (DL-PPT) is a competitive inhibitor while the other (δ-hydroxylysine) is an uncompetitive inhibitor of GS.

Although GS1 and GS2 have been reported to differ in amino acid composition and secondary structure [11], the present study indicates that the kinetic parameters and inhibitor sensitivities of the GS isoforms from a range of species are very similar. As both GS1 and GS2 are inhibited by DL-PPT and δ-hydroxylysine, any herbicide based on either of these compounds is unlikely to show selectivity towards the two leaf isoforms of GS, and the lack of variation between species suggests that the enzyme does not offer the potential for herbicide selectivity. Any degree of selectivity would be based on differences in uptake by the plant or metabolism to a non-inhibitory compound.

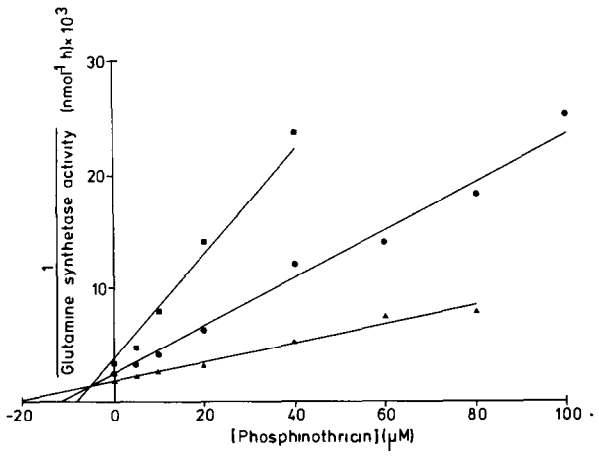

Fig.1. Inhibition of glutamine synthetase, isolated from wild oat, by phosphinothricin at various concentrations of glutamate. (▲—▲) 25 mM glutamate; (●—●) 12.5 mM glutamate; (■—■) 5 mM glutamate. K_i = 5 μM.

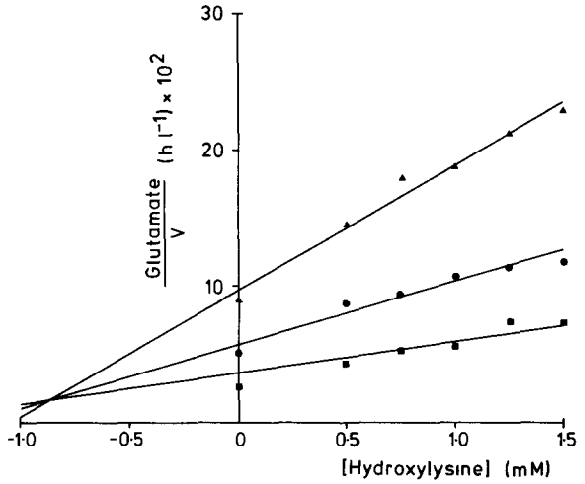

Fig.2. Inhibition of glutamine synthetase 2, isolated from maize, by δ-hydroxylysine at various concentrations of glutamate. (▲—▲) 25 mM glutamate; (●—●) 12.5 mM glutamate; (■—■) 5 mM glutamate. K_i = 0.85 mM.

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