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INHIBITION OF PEA LEAF GLUTAMINE SYNTHETASE BY METHIONINE SULPHOXIMINE, PHOSPHINOTHRICIN AND OTHER GLUTAMATE ANALOGUES

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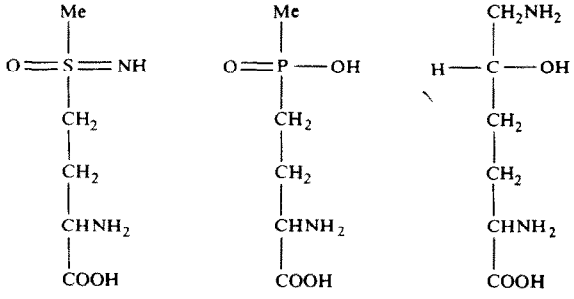
Key Word Index—*Pisum sativum*; Leguminosae; glutamine synthetase inhibition; methionine sulphoximine; phosphinothricin; glutamate analogues.

Abstract—The kinetics of the inhibition of glutamine synthetase from *Pisum sativum* leaves by L-methionine sulphoximine and DL-phosphinothricin were determined. Inhibition by both compounds was mixed-competitive, and apparent K_i values of 0.16 mM and 0.073 mM respectively were determined. DL-5-Hydroxylysine, DL-glutamate-4-tetrazole and L-4-methyleneglutamic acid were also strong inhibitors. Analogues of methionine sulphoximine, DL-ethionine sulphoximine and DL-prothionine sulphoximine were poor inhibitors of glutamine synthetase. Other glutamine and glutamate analogues e.g. azaserine, albizziine, asparagine and kainic acid had no inhibitory action.

INTRODUCTION

Glutamine synthetase (GS) [L-glutamate: ammonia ligase (AMP forming); EC 6.3.1.2] is the first enzyme involved in the assimilation of ammonia in plants [1]. The properties of the enzyme isolated from various plant sources, have recently been reviewed by Stewart *et al.* [2].



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L-Methionine-*S*-sulphoximine (MSO) (**1**) but none of the other three diastereoisomers has been shown by Meister *et al.* to be an irreversible potent inhibitor of sheep brain GS [3–5]. When acting as an inhibitor the sulphoximine nitrogen atom lies in a position normally occupied by the 5-carboxyl oxygen of glutamate. MSO is phosphorylated and the product binds irreversibly to the active site of the enzyme. The GS from *Escherichia coli* is inhibited competitively by MSO [6] with a very low K_i value of 0.0015 mM compared to the K_i values of 0.20 and 0.21 mM obtained for the pea seed and ovine brain enzyme respectively [7].

DL-Phosphinothricin (PPT) (**2**) was initially isolated from a tripeptide antibiotic produced by *Streptomyces viridochromogenes* [8]; the compound was a potent competitive inhibitor of GS from *E. coli* with a K_i of 0.0059 mM but no further investigations have been carried out on the inhibitor with GS from other sources.

MSO has been used by a number of workers (see [1] for review) to demonstrate the evolution of ammonia when it is applied to plant tissue. Liberation of ammonia has been taken as one line of evidence that GS is the first enzyme operating in ammonia assimilation. However, only in a small number of cases have the investigators confirmed that GS has been totally inactivated *in vivo* [9, 10]. The problem is further complicated by the detection of two forms of GS in leaves [11–13] one of which may be present in the chloroplast and the other in the cytoplasm [14].

The aim of this work was to examine the inhibitory action of MSO, phosphinothricin and other glutamate analogues on GS isolated from pea leaves which has been well characterized previously [15, 16]. These experiments thus extend the work previously only carried out on the seed enzyme, and are aimed at identifying new potent inhibitors of glutamine synthetase in higher plants.

RESULTS

The major inhibitory compounds of pea leaf GS are shown in Table 1. A number of potential inhibitors that had no action are also shown; in fact of 110 compounds tested only 13 inhibited greater than 10% at 12.5 mM.

MSO and PPT were selected for further testing at various glutamate and inhibitor concentrations (Figs. 1 and 2). At low concentrations both compounds were competitive inhibitors with respect to glutamate, but

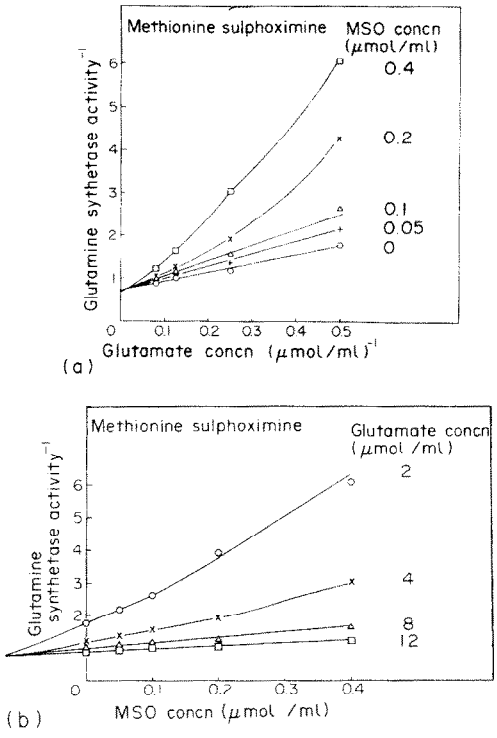

Fig. 1. Inhibitory action of methionine sulphoximine on pea leaf glutamine synthetase. (a) Lineweaver–Burk plot of $1/V$ against 1/S at varying inhibitor conccentrations. (b) Plot of $1/V$ against inhibitor concentration at varying glutamate concentrations.

Table 1. Inhibitory action of compounds on pea leaf glutamine synthetase

Compound	% Inhibition
L-Methionine sulphoximine	100
DL-Phosphinothricin	100
L-5-Hydroxylysine	90
DL-Glutamate-4-tetrazole	65
L-4-Methyleneglutamate	64
DL-Ethionine sulphoximine	15

L-Albizziine, amino-oxyacetic acid, L-asparagine, DL-prothionine sulphoximine, azaserine, glutamate-2, 4-ditetrazole, kainic acid and 4-methylglutamate had no inhibitory action. Compounds (pH 7.5) were tested at equal concentrations to glutamate (12.5 mM). The assay and enzyme isolation were as described in Experimental.

at high concentrations there was evidence of non-competitive inhibition. From the graphs it was possible to calculate K_i values for PPT of 0.073 mM and MSO of 0.161 mM.

DISCUSSION

In barley [12] and rice [13] there is evidence that the two leaf isoenzymes of GS have different properties, in particular their separation on DEAE-Sephacel

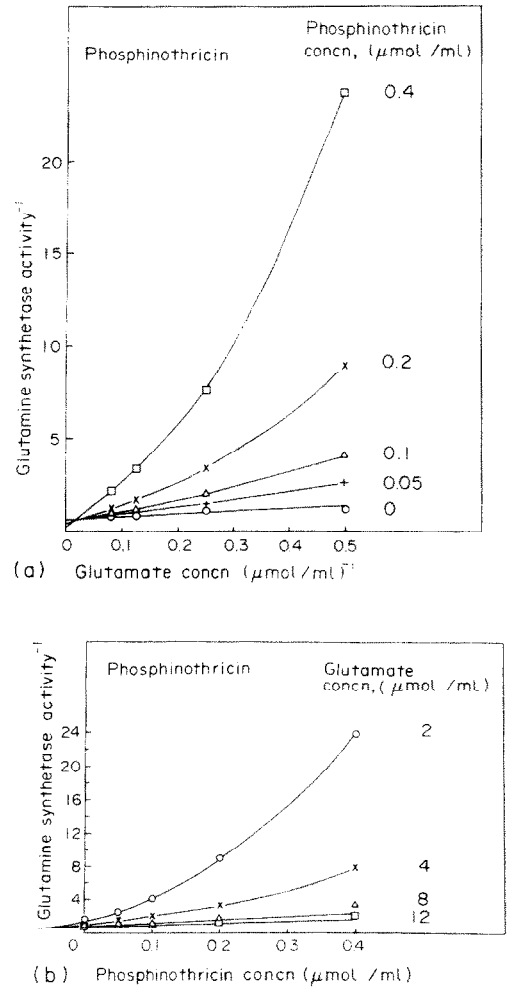

Fig. 2. Inhibitory action of phosphinothricin on pea leaf glutamine synthetase. (a) Lineweaver–Burk plot of $1/V$ againnst $1/S$ at varying inhibitor concentrations. (b) Plot of $1/V$ against inhibitor concentration at varying glutamate concentrations.

and the K_m values for glutamate. Multiple forms of GS have also been detected in a number of leaves, by starch gel electrophoresis [17, 18] in particular. However, two forms of the enzyme have not been separated from pea leaves (Wallsgrove, R. M. and Lea, P. J., unpublished results) and there is no evidence in Figs. 1 and 2 of abnormal kinetics with glutamate as a substrate. It must be concluded that either the enzymes present in the cytoplasm and chloroplast [14] are very similar or that one form is very unstable and is lost early in extraction.

No new potent inhibitor of GS emerged in this survey, although it is clear that PPT is potentially a better inhibitor than MSO particularly as a mixture of all four isomers was used in these tests. 5-Hydroxylysine (3) has been shown to be an inhibitor of GS from blue-green bacteria when ammonia is evolved under nitrogen-fixing conditions [19]. The two less effective inhibitors 4-methyleneglutamate and glutamate-4-tetrazole are not known inhibitors of GS,

although the tetrazole group is an excellent mimic of the carboxyl group in other enzyme reactions [20]. The lack of inhibition detected for ethionine and prothionine sulphoximine is similar to the data obtained for the animal enzyme, although in this case the two compounds were found to be potent inhibitors of γ-glutamycysteine synthetase [21].

The kinetics of the inhibition of pea leaf GS by MSO as shown in Fig. 1, are very similar to the data obtained by Ronzio *et al.* [3] for the inhibition of the sheep brain enzyme. There is a change from competitive inhibition to non-competitive inhibition at high concentrations of MSO; similar results were also obtained for PPT. The K_i value of 0.161 mM obtained for MSO in these experiments is remarkably similar to the values of 0.2 mM and 0.21 mM obtained for the pea seed and sheep brain enzymes by Wedler and Horn [22]. It is however much higher than the value of 0.0015 mM obtained for the *E. coli* enzyme. Similarly there is a large discrepancy between the K_i value of 0.0059 mM obtained for PPT for the *E. coli* enzyme [8] and that of 0.073 mM obtained for pea leaf GS in these experiments. Studies by Wedler *et al.* [7] with phosphorylated derivatives of glutamate have detected major differences between the affinity of the prokaryotic and eukaryotic enzyme for various inhibitors suggesting a possible difference in the action of the active site of the two groups of enzymes. The synthesis of further glutamate analogues may give rise to more information as to the mode of action of higher plant glutamine synthetase.

EXPERIMENTAL

Chemicals. All chemicals were obtained from commercial sources except: phosphinothricin prepared by the method of [8]; glutamate-5-tetrazole and glutamate-1,5-ditetrazole prepared by the method of [20]; 5-methyleneglutamic isolated from *Arachis hypogaea* by the method of [23]; ethionine and prothionine sulphoximines prepared by the method of [21].

Enzyme isolation. 12-day-old light-grown leaves of *Pisum sativum* cv Feltham First were extracted in 50 mM imidazole–acetate pH 7.8 buffer containing 0.5 mM EDTA, 1 mM dithiothreitol, 2 mM $MnCl_2$ and 20% glycerol at 4° in a 'Polytron' blender. The extract was centrifuged at 27 000 *g* for 15 min and brought to 60% satn with $(NH_4)_2SO_4$. The centrifuged precipitate was resuspended in the minimum amount of extraction buffer and desalted over Sephadex G25.

GS assay. GS was measured by the formation of 5-glutamyl hydroxamate in the synthetase reaction [15]. The concn of reactants (mM) in the 1 ml assay mixture was: glutamate, 12.5; ATP, 5; $MgCl_2$, 10; hydroxylamine, 6; EDTA, 2; imidazole–acetate buffer pH 7.8, 100. Assays were linear with respect to time and the protein content of the extract. Care was taken to ensure that compounds under test did not either act as a substrate for the enzyme or react with acidified $FeCl_3$.

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