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PURIFICATION AND PROPERTIES OF 4-METHYLENEGLUTAMINASE FROM THE LEAVES OF PEANUT (*ARACHIS HYPOGAEA*)

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Key Word Index—*Arachis hypogaea*; Leguminosae; peanut; groundnut; 4-methyleneglutaminase; purification and properties.

Abstract—The enzyme 4-methyleneglutaminase has been purified from *Arachis hypogaea* leaves. This enzyme also catalysed the deamidation of glutamine at 20% of the rate of 4-methyleneglutamine, exhibiting the same affinity for both substrates (K_m = 20 mM), but was inactive with asparagine. The hydrolysis of 4-methyleneglutamine was subject to competitive inhibition by glutamine, glutamate-5-hydroxamate and phenol red and non-competitive inhibition by glutamate and 4-methyleneglutamate. The enzyme activity was insensitive to a variety of salts and carboxylic acids.

INTRODUCTION

4(γ)-Methyleneglutamine was isolated initially as the third plant amide from peanut (groundnut, *Arachis hypogaea*) leaves [1]. Subsequently the amide and 4-methyleneglutamate have been isolated from *Tulipa* [2, 3], *Phyllitis* [3] and a variety of legumes [4–7]. Fowden [8] demonstrated that 4-methyleneglutamine was the major nitrogen transport compound from the root to the shoot in peanuts, where it presumably plays a similar role to that of asparagine and the ureides [9]. More recent data [10] has again indicated that 4-methyleneglutamine accounts for up to 95% of the soluble nitrogen in the xylem exudate of both nodulated and non-nodulated peanut plants.

The first step in ammonia assimilation in plants is the conversion of glutamate to glutamine catalysed by glutamine synthetase [11]. Initial attempts to isolate an enzyme catalysing a similar reaction utilising 4-methyleneglutamate in peanut seedlings were unsuccessful [3]. Winter *et al.* [12] isolated two forms of glutamine synthetase from germinating peanut seedlings both of which were capable of converting 4-methyleneglutamate to the amide. However, the K_m value for 4-methyleneglutamate was high (60–90 mM) and the levels of activity were not sufficient to account for the rapid rate of 4-methyleneglutamine synthesis observed in germinating peanuts. Very recently Winter *et al.* [13] have isolated a new enzyme from germinating peanut seedlings that is apparently specific for 4-methyleneglutamate and ammonia and is distinct from both glutamine and asparagine synthetase.

An enzyme capable of converting 4-methyleneglutamine to the free acid and ammonia was demonstrated in extracts of peanuts by Fowden [14]; the same extract hydrolysed glutamine at only 20% of the rate determined for 4-methyleneglutamine. In this paper we report the purification and characterisation of this enzyme, 4-methyleneglutaminase (4-methyleneglutamine deamidase) isolated from peanut leaves. The majority of this work forms the basis of a previously submitted Ph.D. thesis [15].

RESULTS

Enzyme purification

A typical purification of 4-methyleneglutaminase from peanut leaves is shown in Table 1. Attempts to precipitate the enzyme within a narrow range of ammonium sulphate concentrations were unsuccessful, and a final value of 30–80% saturation was employed. The enzyme was precipitated in acetone between 35–50%, and eluted from DEAE-cellulose between 0.07–0.1 M phosphate. 4-Methyleneglutaminase activity eluted as a single peak from Sephadex G-200, separated from a major protein peak, but due to a considerable loss in enzyme activity this method of purification was not used on all occasions. The enzyme also eluted as a single peak after electrophoresis on CF-11 cellulose. The final purified preparation contained only one major protein constituent as determined by polyacrylamide gel electrophoresis and ultracentrifugation using Schlieren optics.

Substrate specificity

Table 1 shows that all stages of purification the enzyme's relative ability to hydrolyse glutamine and 4-methyleneglutamine remained constant. A range of potential glutamine analogues were tested as both substrates and inhibitors (Table 2). Initial studies demonstrated that the enzyme's affinity for 4-methyleneglutamine, glutamine and glutamic acid-5-hydroxamate was very similar, and using standard Lineweaver–Burk plots, K_m values of 20 mM were obtained for all three substrates.

Effect of pH on activity

It was difficult to construct an accurate pH curve for the enzyme as the activity was sensitive to the buffer composition. There was a steep increase in activity up to pH 8.5, followed by a broad plateau up to pH 11.0. The ratio of activity with 4-methyleneglutamine to glutamine (*ca* 5) remained constant throughout the pH range (Table 3).

Table 1. The stepwise purification of 4-methyleneglutaminase from peanut leaves

	Protein (mg)	Total activity (nkat)	Specific activity (nkat/mg protein)	Yield (%)	Purification	Ratio—activities with 4-methylene-glutamine : glutamine
Crude extract	3880	43.9	0.011	100	—	5.2
Ammonium sulphate precipitate	2500	43.3	0.017	98.6	1.54	5.2
Acetone precipitate	347	30.6	0.088	69.7	8.0	5.3
DEAE-cellulose eluate	141	26.1	0.185	59.4	16.8	5.8
Sephadex-G200 eluate	40	14.8	0.37	33.7	33.6	5.4
Heat denaturation	20	14.1	0.70	32.1	63.6	5.6

Table 2. Substrate specificity of 4-methyleneglutaminase from peanut leaves

Compound	Relative substrate activity	Inhibition
4-Methyleneglutamine	100	—
L-Glutamine	20	50
Glutamic acid-5-methyl ester	9	63
Glutamic acid-5-hydroxamate	6.6	35
Glutamic acid-5-ethyl ester	1.6	40
L-N^5-Ethylglutamine (theanine)	0	14
N-Methyl-DL-glutamic acid	0	n.d.
N-CBZ-L-glutamic acid	0	n.d.
L-Glutamic acid-diethyl ester	0	40
N-Acetyl-L-glutamine	0	29
D-Glutamine	0	20
L-Asparagine	0	3
O-Carbamoyl-L-serine	0	n.d.
S-Carbamoyl-L-cysteine	0	78
Albizziine	0	50
N-Acetyl-L-glutamic acid	0	78

Substrate specificity was determined at concentrations of 70 mM for compounds tested. Inhibitor concentrations were 20 mM, with 10 mM 4-methyleneglutamine being used as substrate; n.d., Not determined.

Table 3. Effect of pH on substrate affinity of 4-methyleneglutaminase from peanut leaves

pH	4-Methyleneglutamine K_m (M)	4-Methyleneglutamine V_{max}	Glutamine K_m (M)	Glutamine V_{max}
6.0	0.067	43.3	0.067	8.3
6.4	0.036	55.0	0.036	11.8
6.8	0.029	66.7	0.027	13.5
7.2	0.013	80.0	0.013	15.2
7.6	0.016	108	0.016	21.7
8.0	0.020	152	0.020	31.6
8.4	0.050	257	0.040	45.0

Sodium phosphate buffer (50 mM) was used throughout. K_m values were calculated from Lineweaver plots. V_{max} values are expressed as nkats/mg protein.

The affinity of the enzyme for the two substrates peaked below the pH optimum at around pH 8.2, although again the ratio of the affinities remained constant.

The effect of temperature on activity

The enzyme was stable up to 40°, but above this temperature activity was lost rapidly; at 55°, 50% of the activity was lost in 10 min. There was no evidence of any alteration in the ratio of the rates for the substrates during the inactivation process. Glutamine was able to protect against thermal denaturation, at 55° the protection constant was determined as 24 mM.

V_{max} values in the range 15–40° were calculated from Lineweaver–Burk plots using both 4-methyleneglutamine and glutamine as substrates. No changes in the ratio of enzyme activity for the two substrates or in the K_m values were detected over the temperature range. The Arrhenius plot of log V_{max} against the reciprocal of absolute temperature (Fig. 1) did not deviate from linearity in either case. The activation energy E_a given by slope $= E_a/2.3R$

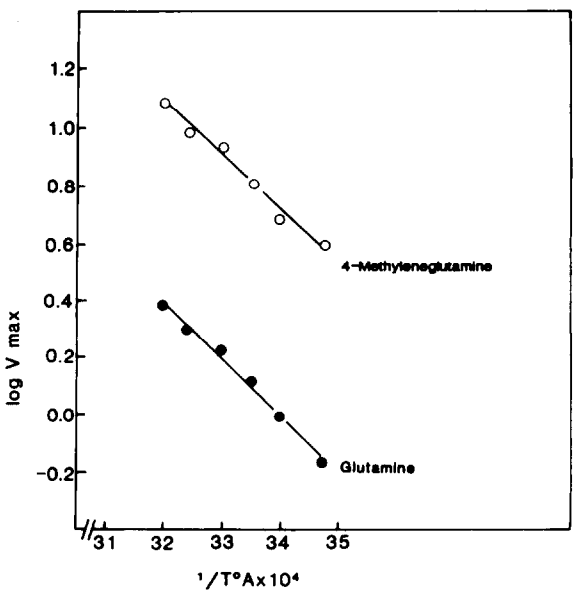

Fig. 1. The Arrhenius plot for 4-methyleneglutaminase for the two substrates 4-methyleneglutamine and glutamine.

was calculated from both plots and was found to be 9.1 kcal/mol in each case.

The effect of salts on enzyme activity

Table 4 shows the action of various divalent and monovalent anions on the activity of the enzyme with the two substrates. Phosphate and sulphate had no effect, even when tested at 0.5 M. Iodide was a weak inhibitor with a K_i value of 0.15 M for 4-methyleneglutamine as a substrate and 0.1 M for glutamine. Borate was a strong non-competitive inhibitor with a K_i of 1.25 mM for either substrate.

A wide range of monovalent and divalent cations had no action on the activity with either substrate at 1 mM, only Cu^+ showing 20% inhibition. EDTA and a range of carboxylic acids, in particular pyruvate and 2-oxoglutarate, had no effect on enzyme activity with either substrate when tested at up to 5 mM. There was no evidence of any reaction with 2-oxoglutarate in the presence of reduced methylviolgen or NAD(P)H.

Kinetics of inhibition of enzyme activity

A number of compounds related to 4-methyleneglutamine inhibited the enzyme as shown in Table 2. The hydrolysis of 4-methyleneglutamine was competitively inhibited by glutamine, glutamate-5-hydroxamate and phenol red (K_i values 20, 20 and 3.8 mM respectively) and non-competitively by 4-methyleneglutamate and glutamate (K_i values 5.4 mM for both compounds). Inhibition by *N*-acetylglutamate was somewhat unusual as plots of $1/v$ against $1/s$ at various inhibitor concentrations were linear and indicated competitive inhibition. However, when $1/v$ was plotted against [i] at various concentrations of 4-methyleneglutamine, a series of parabolic curves were obtained (data not shown).

The enzyme activity utilizing 4-methyleneglutamine was measured in the presence of various combined concentrations of two inhibitors; glutamine and phenol red. The data are plotted in Fig. 2 showing a family of parallel lines when the concentration of either inhibitor was varied.

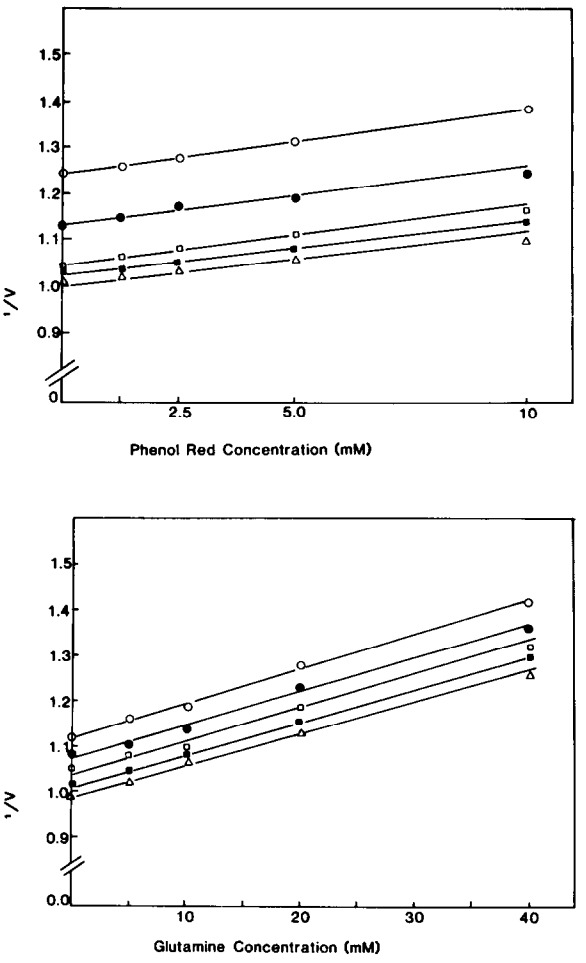

Fig. 2. Kinetics of multiple inhibition. The initial velocity (nkat) was measured at a fixed 4-methyleneglutamine concentration of 0.04 M in 50 mM Na phosphate pH 8. In Fig. 2a the reciprocal of the velocity was plotted against phenol red concentration at fixed concentrations of glutamine. (△), No glutamine added; (■), 0.005 M glutamine; (□), 0.01 M glutamine; (●), 0.02 M glutamine; (○), 0.04 M glutamine. In Fig. 2b the reciprocal of the velocity (nkat) was plotted against glutamine concentration at fixed concentrations of phenol red. (△), No phenol red added; (■), 0.00125 M phenol red; (□), 0.0025 M phenol red; (●), 0.05 M phenol red; (○), 0.01 M phenol red.

DISCUSSION

It is clear from the purification detailed in Table 1 that the activity of the 4-methyleneglutaminase enzyme from peanut leaves co-purifies with the ability to hydrolyse glutamine at *ca* 20% of the rate of deamidation of 4-methyleneglutamine.

The enzyme is also able to hydrolyse compounds with substituents on the amide group, e.g. esters and the hydroxamic acid derivative. Similar results have been obtained with a higher plant asparaginase [16], but 4-methyleneglutaminase is unable to utilize asparagine as a substrate. This agrees with the suggestion made by Iceland and Joy [17], that the major route of asparagine breakdown in leaves is via a transamination reaction. The enzyme is somewhat different from those of bacterial origin in that it requires both a free 1-carboxyl and a free 2-amino group and will not hydrolyse D-glutamine [18, 19]. The enzyme was subject to inhibition by a wide

Table 4. The effect of anions on 4-methyleneglutaminase activity

Addition	Concentration (M)	Relative activity (%) 4-methylene-glutamine	glutamine
Na_2HPO_4	0.15	110	100
Na_2SO_4	0.15	110	100
Na arsenate	0.15	76	60
Na borate	0.15	0	0
NaF	0.1	98	95
NaCl	0.1	81	61
NaBr	0.1	84	60
NaI	0.1	20	14
NaCN	0.02	84	90

range of glutamine analogues similar to other glutamine-dependent enzymes isolated from higher plants [20–22]. The affinity of the enzyme for glutamine and 4-methyleneglutamine was low; K_m values of 20 mM are much higher than those reported for the bacterial enzymes [23].

The pH optimum of the enzyme was similar to that originally reported by Fowden [14] and is close to that of similar enzymes present in most animals and bacteria, although *Escherichia coli* and *Clostridium welchii* have glutaminases with acidic pH optima [18]. An examination of Table 3 shows that the enzyme did not show maximum affinity for the substrate at the V_{max}. A plot of log V_{max}/K_m against pH (figure not shown, but data in Table 3) in fact revealed the possibility of two pKa's at pH 6 and 8.4 [24]. As the shape of the curves of log V_{max}/K_m against the pH was the same for 4-methyleneglutamine and glutamine it must be assumed that the two substrates bind to the same group on the enzyme active site.

The activity versus temperature curves for the two substrates were very similar, as were the Arrhenius plots (Fig. 1). The activation energy of 9.1 kcal/mol is similar to that of 8.4 for *E. coli* and 8.3 for pig brain glutaminases [25]. There was no indication of variation of the K_m values for either glutamine or 4-methyleneglutamine at different temperatures, a result also obtained with *E. coli* [18] and pig brain glutaminases [25].

4-Methyleneglutaminase from peanut does not seem to be a member of the group of glutaminases activated by anions or carboxylic acids [18, 25]; the enzyme seems more akin to glutaminases A and B from *Pseudomonas aeruginosa* [26]. The inhibition by borate was unusual as the ion has been reported to activate glutaminase from *Pseudomonas* [27]. The lack of effect of cations and EDTA suggests that metal ions are not involved in the catalytic reaction, Hartman [18] reached a similar conclusion with the *E. coli* glutaminase.

As there were no changes in the intercepts of the double reciprocal plots in the presence of glutamine and glutamic acid-5-hydroxamate it must be assumed that they bind to the same active site as 4-methyleneglutamine. Phenol red also acted as a competitive inhibitor and the dual inhibition studies indicated that it was also bound at the same point as glutamine and 4-methyleneglutamine. However the differences in the K_i value for glutamine (20 mM) and phenol red (3.8 mM) suggest that the enzyme-inhibitor complexes may be different.

It is obvious from the purification data and the comparative effects of pH, temperature, salts and other inhibitors that an integral part of the 4-methyleneglutaminase activity is the ability to use glutamine as a substrate. At no time was there any evidence of a differential effect on the two substrates. If glutamine is deamidated by the enzyme in the leaf a futile cycle would be set up as the ammonia would have to be reassimilated in the leaf by the enzyme glutamine synthetase [11], which would waste ATP in the process. It must be assumed that under normal physiological conditions the ratio of 4-methyleneglutamine to glutamine at the site of the enzyme is such that only 4-methyleneglutamine is deamidated; measurements of amino acid levels in peanut leaves suggest a 5:1 ratio [8] although these may vary with the nitrogen status of the plant [10]. The 2-amino group of 4-methyleneglutamate is probably transaminated to protein amino acids, but the metabolism of the 2-oxo derivative has not been examined. There was no suggestion that 4-methyleneglutamine could take part in a glutamate synthase type reaction, which has also been shown for asparagine [9].

EXPERIMENTAL

Plant material. Seeds of *Arachis hypogaea* were purchased from the English Peanut Roasting Co. Ltd. Healthy seeds were germinated in sterilized soil and grown for 3 weeks in a glasshouse at 30° until 4–5 leaves were fully developed. The shoots, excluding the partially withered cotyledons, were stored at −30° until required.

Substrates and analogues. L-4-Methyleneglutamine, L-theanine and L-albizziine were natural materials isolated in our laboratories. All other glutamine analogues were obtained as described previously [20, 21].

Enzyme assay. The standard assay contained 0.1 ml of 0.1 M substrate and 0.04 ml enzyme preparation in NaPi (50 mM) buffer pH 8. The reactions were carried out for various times at 37° and terminated by boiling for 2 min. The substrate and products were separated by PC in *n*-BuOH–H_2O–HOAc (90:29:10 by vol.). Amino acids on the chromatograms were visualized by a cadmium acetate/ninhydrin reagent [28] and the amount of 4-methyleneglutamate formed quantified by reading the *A* of the eluted spots at 510 nm [29]. The *A* was linear up to 1 μmol per assay tube.

Enzyme purification. The frozen shoots were extracted twice in an Atomix blender in cold buffer (10 mM NaPi pH 7.4) at a final ratio of 4 vols: g of tissue and centrifuged at 10000 *g* for 15 min. The protein that precipitated between 35–80% saturated $(NH_4)_2SO_4$ was collected by centrifugation and redissolved in extraction buffer, and Me_2CO at −30° was added to produce a 10% mixture. The ppt was removed and more Me_2CO added to give a 50% mixture. The resulting ppt was extracted extensively in two batches of extraction buffer and dialysed overnight. The sample was applied to 3 × 30 cm column of DEAE-cellulose and the column washed with extraction buffer. The chromatogram was developed by a 10–200 mM linear NaPi gradient, with the enzyme eluting just under 100 mM buffer concentration. The enzyme was further purified on a 2 × 36 cm Sephadex G-200 column equilibrated in extraction buffer. Glutamine was added to the pooled fractions to 50 mM in order to protect the enzyme and the pH was adjusted to 5 with 0.2 M HOAc. The soln was then heated at 50° for 2 min, rapidly cooled to room temp. and the pH readjusted to 7.4. The precipitated protein was removed by centrifugation, and supernatant used as the enzyme source.

Other methods. Analytical PAGE with a 5% resolving gel was carried out as described in ref. [30]. Electrophoresis on CF-11 cellulose was carried out as described in ref. [31]. Ultracentrifugation was carried out in a Beckman fitted with a Schlieren optical system at 10° in extraction buffer containing 0.1 M KCl. Photographs were taken at intervals of 16 min. Protein was determined by the method of ref. [32] with BSA as a standard.

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NOTE ADDED IN PROOF

After submission of this paper, a report of a more highly purified enzyme from the same source has appeared [Powell, G. K. and Dekker, E. E. (1983) *J. Biol. Chem.* **258**, 8677]. The properties of the two enzymes are essentially similar.