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SHORT COMMUNICATION

L-CYSTATHIONINE AND ITS SELENIUM ANALOGUE IN *NEPTUNIA AMPLEXICAULIS*

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Abstract—Both L-cystathionine and L-selenocystathionine have been isolated from the selenium-accumulating legume *Neptunia amplexicaulis*.

INTRODUCTION

ALTHOUGH L-cystathionine is a well known intermediate in transsulfuration in fungi, bacteria and mammalian liver its occurrence in higher plants is in doubt.[1] In fungi *O*-acetylhomoserine is involved in cystathionine biosynthesis,[2] while in bacteria *O*-succinylhomoserine is utilized.[3] Spinach leaves on the other hand are reported to synthesize cystathionine from both derivatives of homoserine.[4] However, the product was not characterized and could have arisen, as the authors point out, by an exchange reaction and not by net synthesis. More recently these authors have purified a β-cystathionase enzyme from spinach and shown that cystathionine is not synthesized at a significant rate from homocysteine and serine.[5]

In contrast to the lack of information on the occurrence of cystathionine in plants, the occurrence of the seleno-analogue is well established. It has been isolated and characterized from the selenium-accumulating plants *Neptunia amplexicaulis*,[6] *Morinda reticulata*,[7] *Lecythis ollaria*,[8] *Astragalus pectinatus*[9] and shown to be present in *Stanleya pinnata* var. *bipinnata*.[9] Cystathionine on the other hand was not detected in a chromatographic survey of the *Astragalus* genus[10] nor could it be detected on amino acid analyser traces of *A.*

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pectinatus[11] or *A. osterhouti*[12] using methods specifically developed to resolve the selenium and sulphur analogues. In such plants it would seem that there is an absolute discrimination between sulphur and selenium. To check whether this hypothesis is likely, or whether there is a marked difference of enzyme selectivity and rate of reaction, we have looked to see if trace amounts of cystathionine occur along with selenocystathionine. During the isolation of this latter compound from *N. amplexicaulis* seeds, we found that our isolates from different batches of seed had a purity of up to 95 per cent based on selenium analysis, although they were homogeneous as judged by high voltage paper electrophoresis at various pH's and by thin layer and paper chromatography. In this communication we report the separation of an impurity from a bulk preparation of selenocystathionine, and record its characterization as cystathionine.

RESULTS AND DISCUSSIONS

Normal amino acid extraction and ion exchange procedures were employed to separate and isolate crystalline selenocystathionine from *N. amplexicaulis* seeds. Such methods however, did not reveal a contaminant (I) in the selenocystathionine. Following the modification of Martin and Gerlach[11] designed to separate sulphur and selenium amino acids, an amino acid analyser tracing revealed the presence of a small contaminant eluting at 213 min followed by selenocystathionine eluting at 220 min. The analyser procedure was scaled up and 6 mg of I was eventually isolated from 300 mg of selenocystathionine.

Assignment of a structure to I was based on the following consideration: (*a*) Its behaviour on high voltage paper electrophoresis at three pH values and paper chromatography in three solvents was identical to cystathionine. (*b*) Its elution time on an amino acid analyser was identical to cystathionine and the two could not be resolved from a mixture of both. (*c*) Hydrogenolysis with Raney nickel[13] gave rise to two neutral amino acids in approximately equimolar amounts corresponding to alanine and α-aminobutyric acid on high voltage paper electrophoresis and chromatography. Cystathionine behaves similarly. (*d*) The NMR spectra of I in acidified D_2O was identical with that for cystathionine and was closely similar to selenocystathionine. The 60 NMR spectrum of cystathionine was complicated by the coupling of non-equivalent geminal protons. The signals at δ3·27 (2H) and 4·42 (1H) were consistant with an uncompletely resolved ABX pattern and were assigned to the C_1' and C_2' protons respectively. Multiplets centered at δ2·30 (2H) and 2·87 (2H) were assigned to C_3 and C_4 protons respectively while doublets at δ4·26 (1H, $J = 1$ Hz, $J' = 7$ Hz) were assigned to the C_2 proton. In the case of selenocystathionine, a closely similar spectra was obtained. The C_1' and C_2' protons gave signals at δ3·15 and 4·30 while C_3 and C_4 protons gave signals at δ2·26 and 2·77. Doublets at δ4·10 (1H, $J = 6$ Hz, $J' = 1$ Hz) were assigned to the C_2 proton. (*e*) The optical rotation recorded for selenocystathionine was $[\alpha]_D^{20}$ +24·8 in N HCl (cf. L-cystathionine $[\alpha]_D^{20}$ +23·7[14]) which indicates that the material is of the L-configuration. Insufficient compound I was available for an optical rotation measurement so tests for configuration were carried out using L-amino acid oxidase from snake venom and D-amino acid oxidase from hog kidney. When a sample of I was incubated with the latter enzyme, there was no detectable uptake of oxygen. By contrast, treatment of I with the L-amino acid oxidase preparation led to a rapid uptake of oxygen and the disappearance of I as assayed by ninhydrin reactions on paper. This evidence supports

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the adoption of the L-configuration for I. (*f*) IR spectra (KBr disc) of I and L-cystathionine were identical in all respects but in marked contrast to L-*allo*-cystathionine. This evidence has led us to conclude that I is L-cystathionine.

To check whether cystathionine and selenocystathionine occur together in other biological systems, we have assayed a sample of cystathionine from yeast on the amino acid analyser. No selenocystathionine was detected but selenium analyses allowed us to calculate a sulphur:selenium ratio of 8500:1 which is below the analyser detection limit. Since the selenium accumulating plant *N. amplexicaulis* produces predominantly selenocystathionine with trace amounts of cystathionine, while other organisms not accumulating selenium produce cystathionine with only traces of the selenium analogue, comparative studies of trans-sulfuration in such organisms now seem to be of greater interest than previously considered.

EXPERIMENTAL

Isolation of selenocystathionine + I. N. amplexicaulis seed (1 kg, Se content 6430 ppm) was ground and extracted at room temp. for 24 hr with light petroleum, ether and methanol. The residue from the methanol extract was then extracted with water (25 l., 60°). Repeated concentration and centrifugation of the aqueous extract, followed by dialysis yielded 25 g amino acids. The dialysate was adjusted to pH 11 with NaOH, divided into 8 equal portions and after neutralization with HCl absorbed on to columns of Amberlite IRA 400 (Cl^- form, 100–200 mesh, 30 × 2 cm dia.) and eluted with 0·1 N NH_4OH. The appropriate fractions were combined, concentrated *in vacuo* and selenocystathionine precipitated by the addition of ethanol. A yield of 3·5 g of crude selenocystathionine was obtained.

Separation of I from selenocystathionine. The amino acids (300 mg) were dissolved in buffer (pH 2·2, 0·1 M, citrate-HCl) and applied to a Dowex 50 (×8) column (Na^+ form, 200–400 mesh, 130 × 2 cm dia.). The column was washed with pH 3·2 buffer and the amino acids eluted with pH 4·25 buffer. Compound I eluted first and was closely followed by selenocystathionine. Fractions 45–48 were pooled after checking with an amino acid analyser that none of the seleno-analogue was present. Fractions 49–51 contained both amino acids. Selenocystathionine in fractions 52–60 were also pooled. Both amino acids were separately passed through Dowex 1 columns (Cl^- form, 200–400 mesh, 30 × 3 cm dia.) to remove the citrate and after concentrating, were desalted on Sephadex G-10 columns (30 × 3 cm dia.). Selenocystathionine did not completely separate from NaCl, so the appropriate fractions were again passed through Sephadex G-10. Both amino acids were crystallized from ethanol–water to yield 6 mg compound I and 200 mg selenocystathionine.

Chromatographic and electrophoretic methods. Descending paper chromatography was performed on Whatman No. 3MM filter paper using 75% (w/w) PhOH in the presence of NH_3 vapour, *n*-BuOH–HOAc–H_2O (12:3:5) and *n*-BuOH–pyridine–H_2O (1:1:1). High voltage paper electrophoresis was performed on Whatman No. 3MM filter paper using a Miles HiVolt (Shoreham-by-Sea, Sussex) apparatus at pH 2 (formic–acetic buffer[6]), pH 3·4 or pH 5·3 (pyridine–acetic buffer[15]). Boric acid buffer pH 9·2 was also used but Whatman No. 1 filter paper was necessary.[6] Hydrogenolysis of I and selenocystathionine was carried out according to the method of Mozingo *et al.*[13] and the remaining nickel removed with 8-hydroxyquinoline in chloroform. The amino acids were separated by chromatography and electrophoresis and quantitated following the method of Blackburn.[16] For resolution of the sulphur and seleno-analogues, a Beckman 120C amino acid analyser was used with a 12 hr procedure as for 'physiological fluids' with the modification of procedure recommended by Martin and Gerlach.[11]

Additional methods. NMR measurements were made on a Varian T60 spectrometer at 60 MHz, while optical rotations of selenocystathionine were measured in a Bendix NPL polarimeter. To check that I was an L-amino acid, *Crotalus adamanteus* L-amino acid oxidase was used following the manometric method of Wellner and Meister.[17] Hog kidney D-amino acid oxidase was also used in a manometric procedure, following the method of Burton.[18] A Perkin-Elmer NaCl spectrophotometer was used for the IR studies of amino acids in KBr discs.

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Key Word Index—Neptunia amplexicaulis; Leguminosae; L-cystathionine; L-selenocystathionine.