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ENZYME CATALYSED α,β-ELIMINATION OF SELENOCYSTATHIONINE AND SELENOCYSTINE AND THEIR SULPHUR ISOLOGUES BY PLANT EXTRACTS

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Key Word Index—*Pisum sativum*; *Astragalus*; *Neptunia*; Leguminosae; *Spinacia oleracea*; Chenopodiaceae; spinach; cystathionine; selenocystathionine; selenocystine; β-cystathionase; cystine lyase; selenium.

Abstract—Extracts of peas (*Pisum sativum*), spinach (*Spinacia oleracea*) and *Astragalus sinicus* were resolved into two activities which catalysed α,β-elimination of djenkolate to form pyruvate. The major activity (enzyme I) had properties similar to β-cystathionase; it had highest activity and affinity towards djenkolate and cystathionine (K_m *ca* 0.2–0.5 mM) and was competitively inhibited by β-cyanoalanine. Enzyme I also supported α,β-elimination of selenocystathionine. This is consistent with the possibility that selenomethionine is synthesized via the trans-sulphurylation pathway in species which do not accumulate selenium. Enzyme I also catalysed the cleavage of cystine and selenocystine at low rates. The minor activity (enzyme II) exhibited relatively greater activity with cystine and selenocystine than djenkolate and cystathionine. Extracts of the selenium accumulator species *Astragalus bisulcatus*, *A. racemosus* and *Neptunia amplexicaulis* contained very low enzyme I activity but nevertheless supported α,β-cleavage of selenocystathionine albeit at low rates.

INTRODUCTION

Cystathionine is an intermediate of the trans-sulphurylation pathway, the major route for the synthesis of methionine from cysteine [1–3]. In plants, cystathionine is subject to α,β-elimination by the enzyme β-cystathionase (EC 4.4.1.9) according to the reaction:

$$\text{Cystathionine} + H_2O \rightleftharpoons \text{homocysteine} + \text{pyruvate} + NH_3.$$

β-Cystathionase activity has been detected in crude extracts of leaves from many plants [3, 4] and the enzyme from spinach leaf tissue has been purified and characterized [4]. In barley leaf protoplasts, *ca* 50% of the activity is associated with chloroplasts, the remainder being associated with the cytosol and mitochondrial fractions [5]. The purified spinach leaf enzyme supports the α,β-elimination of djenkolate and also shows low activity towards L-cystine [4]. Conversely, the enzyme cystine lyase (EC 4.4.1.—) catalyses the α,β-elimination of cystine and reportedly exhibits low activity towards various other substrates including cystathionine [6]. The substrate specificity of cystine lyase and β-cystathionase and the distinction between these two enzymes in various cruciferous plants has been the subject of some debate [6–8].

The elimination reactions catalysed by β-cystathionase and cystine lyase are of interest with respect to selenium metabolism especially in those plants, known as selenium accumulators, which under appropriate conditions synthesize large amounts of selenocystathionine and other selenium isologues of intermediates (and their derivatives) of the sulphate assimilation pathway [9, 10].

In this paper we report a study of the α,β-elimination of cystathionine and cystine and their selenium isologues by partially purified extracts from various plants. We found that extracts from both selenium-accumulator and non-accumulator species are active towards the sulphur and selenium isologues of these substrates.

RESULTS

Properties of α,β-cleavage activity of peas

Crude extracts of acetone powders of pea leaf tissue catalysed the production of pyruvate from L-djenkolate at rates of 1.1–1.5 nmol/mg protein/min. The simple purification procedure (see Experimental) enhanced the specific activity *ca* 6–8 fold. However, the purified enzyme was unstable (*ca* 10–15% decrease in activity per day at 2°). Thus by the time experiments with the partially purified enzyme were undertaken, the activity with djenkolate as substrate was sometimes as low as 4–6 nmol/mg protein/min (Table 1). Nevertheless, purification was essential to decrease the fluorescence values associated with enzyme incubations of crude extracts in the absence of substrate; presumably this involved removal of other NADH oxidizing enzymes and various endogenous substrates. A further feature of the purification procedure was that the α,β-cleavage activity associated with the 25–40% ammonium sulphate fraction was resolved during gel filtration on Sephacryl S200 into a major fraction (referred to as enzyme I) eluting near the void volume and a minor fraction of lower molecular weight (referred to as enzyme II). With djenkolate as substrate, enzyme II was usually *ca* 15% of the activity of enzyme I (Fig. 1).

Enzyme I exhibited highest activity towards djenkolate and L-cystathionine. The mean activity with cystathionine was *ca* 80% of the activity with djenkolate (Fig. 2) and the K_m values for the two substrates were also similar (Table 2). Importantly, enzyme I from pea, a species which

Table 1. Typical purification of enzyme I α,β-cleavage activity* from extracts of acetone powders of pea

Treatment	Protein (mg)	Specific activity (nmol/min/mg protein)
Crude extract	611	1.2
$(NH_4)_2SO_4$ (25–40%)	205	7.6
Sephacryl S200†	116	2.2
DEAE-cellulose	16.6	9.1

*Activity determined with 9 mM djenkolate as substrate.
†This treatment resolved enzyme I from enzyme II activity (see Fig. 2).

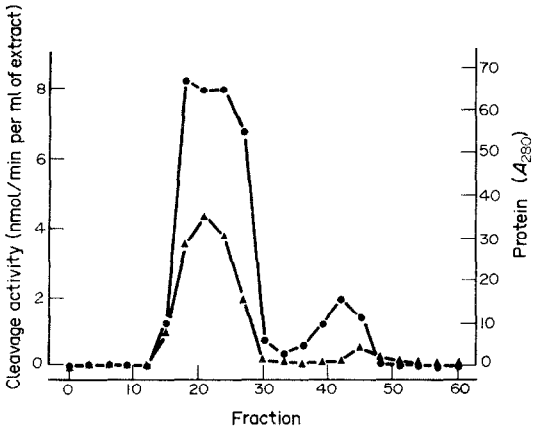

Fig. 1. Elution profile of α,β-elimination activity (●) and protein (▲) from a Sephacryl S200 column. Pea extract from an acetone powder was subjected to fractionation with $(NH_4)_2SO_4$ (see Experimental) prior to application to the column. α,β-Elimination activity was determined with djenkolate as substrate. The major and minor activities eluting from the column are referred to as enzyme I and enzyme II respectively.

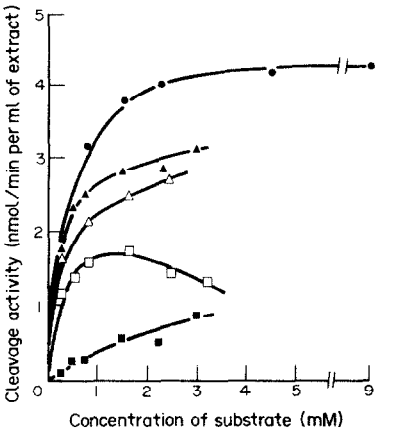

Fig. 2. Effect of substrate concentration on the α,β-elimination activity of pea enzyme I. All activities are expressed as nmol of pyruvate formed as determined by the oxidation of NADH in the presence of lactate dehydrogenase. (●) L-Djenkolate; (▲) L-cystathionine; (Δ) L-selenocystathionine; (■) L-cystine; (□) DL-selenocystine.

does not accumulate selenium, supported the production of pyruvate from L-selenocystathionine at *ca* 80–90% of the rate for L-cystathionine (Fig. 2) and exhibited similar affinity for the two substrates (Table 2). The fraction containing enzyme I also cleaved L-cystine to pyruvate but, at 3 mM, the maximum rate with this substrate was only *ca* 20% of the rate with djenkolate and the K_m value for cystine was considerably greater. Pyruvate was also produced from DL-selenocystine by fractions containing enzyme I at rates slightly greater than for cystine. However, whereas the kinetic constants for different enzyme preparations of enzyme I towards djenkolate, cystathionine, selenocystathionine and cystine were reproducible, the activity towards selenocystine relative to cystine was rather variable. For different enzyme preparations, the activity at 1.5 mM DL-selenocystine was 10–150% greater than for 3 mM L-cystine. Concentrations greater than 2 mM were inhibitory (Fig. 2) and the activity with selenocystine did not comply with Michaelis–Menten kinetics. With djenkolate (9 mM) as substrate, enzyme I from peas was inhibited by the thiol group reagents *N*-ethylmaleimide (85% inhibition at 5 mM) and iodoacetate (41% at 5 mM). β-Cyanoalanine also inhibited enzyme I activity; the kinetics of inhibition by this reagent (Fig. 3) were typical of competitive inhibition (K_i < 0.1 mM). DL-Propargylglycine, an inhibitor of cystathionine-γ-synthase in *Lemna* [11], caused relatively little inhibition (8% at 5 mM). The thiols dithiothreitol (5 mM) and mercaptoethanol (5 mM) enhanced activity by *ca* 15%. EDTA (3 mM) did not significantly affect enzyme activity.

Enzyme II from pea exhibited low but measurable activity towards djenkolate and was routinely detected during gel filtration by this activity (Fig. 1). Relative to activity on djenkolate, enzyme II exhibited higher activity towards selenocystine and cystine but the absolute rate of pyruvate production from selenocystine by enzyme II was considerably less than that from djenkolate by enzyme I. Enzyme II exhibited higher activity towards selenocystine than cystine but showed very little activity towards cystathionine though pyruvate was produced from selenocystathionine at low rates (Fig. 4). With selenocystine as substrate, enzyme II activity was not strongly inhibited by *N*-ethylmaleimide (15% inhibition at 5 mM) or β-cyanoalanine (2.6 mM).

α,β-*Cleavage activity of spinach*

Crude extracts prepared from acetone powders of spinach also catalysed the production of pyruvate from djenkolate. The purification procedure enhanced the specific activity *ca* 5-fold and gel filtration on Sephacryl S200 resolved the activity from spinach into two components. Most of the activity (*ca* 85%) eluted near the void volume and exhibited similar properties to enzyme I from peas. Spinach enzyme I showed greatest activity towards djenkolate but was also active towards cystathionine, selenocystathionine and selenocystine. The kinetic constants for these substrates were similar to those for pea enzyme I (Table 2). Spinach enzyme I also catalysed the production of pyruvate from cystine at slow rates but, as for peas, the K_m for this substrate was much greater than

Table 2. K_m values for various substrates which are subject to α,β-cleavage activity by enzyme I from pea, spinach and *A. sinicus*

Source	K_m(mM)			
	L-Djenkolate	L-Cystathionine	L-Seleno-cystathionine	L-Cystine
Pea, Expt 1	0.35	0.50	0.37	1.5
Expt 2	0.27	0.22	0.42	1.9
Expt 3	0.30	0.20	0.27	3.0
Spinach	0.20	0.48	0.35	5.4
A. sinicus	0.36	0.47	0.21	2.3

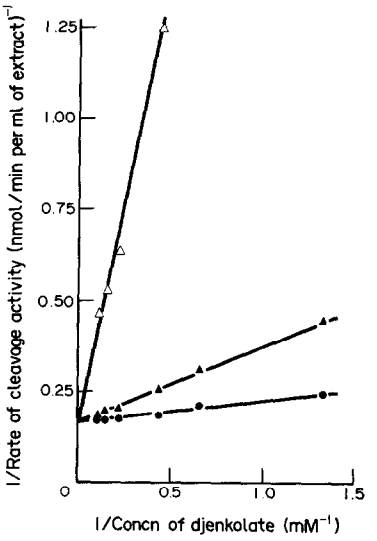

Fig. 3. Effect of β-cyanoalanine on the kinetics of pea enzyme I with respect to concentration of L-djenkolate. (●) Without β-cyanoalanine; (▲) 0.26 mM β-cyanoalanine; (△) 2.6 mM β-cyanoalanine.

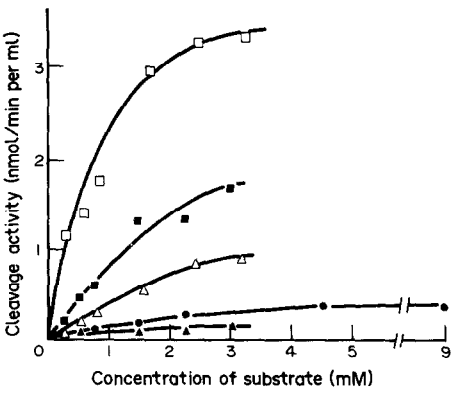

Fig. 4. Effect of substrate concentration on the α,β-elimination activity of pea enzyme II. (●) L-Djenkolate; (▲) L-cystathionine; (△) L-selenocystathionine; (■) L-cystine; (□) DL-selenocystine. Other details as for Fig. 2.

the other substrates. Enzyme I catalysed the production of pyruvate from 0.9 9 mM cystathionine and 5 mM selenocystathionine at constant rates for at least 30 min. Enzyme I did not support the production of pyruvate from serine, homoserine, methionine or selenomethionine. With djenkolate as substrate, enzyme I activity was inhibited by β-cyanoalanine (57% inhibition at 2.6 mM).

The substrate specificity of spinach enzyme II was similar to that of pea enzyme II except that the spinach enzyme exhibited highest activity towards cystine rather than selenocystine. It also supported the production of pyruvate from selenocystathionine but the rates, especially at low concentrations, were less than for cystine (Table 3). The rates with 9 mM djenkolate and 5 mM cystathionine were < 20% of the rate with 3 mM cystine.

α,β-*Cleavage activity of* Astragalus *and* Neptunia *species*

Extracts of acetone powders of leaves of *Astragalus sinicus*, *A. racemosus*, *A. bisulcatus*, *Neptunia gracilis*, *N. dimorphantha* and *N. amplexicaulis* were examined for α,β-cleavage activity. Extracts from *A. sinicus* were by far the most active, being comparable with those from spinach. With djenkolate as substrate the activity resolved into two activities during gel filtration on Sephacryl S200. Like peas, the higher molecular weight form (enzyme I) accounted for *ca* 80% of the total activity. Fractions containing this activity exhibited highest activity towards djenkolate and cystathionine (Table 3) and the K_m values for these substrates were similar to those for enzyme I from peas and spinach (Table 2). Enzyme I from *A. sinicus* also catalysed α,β-cleavage at slower rates with selenocystathionine, cystine and selenocystine. With djenkolate as substrate, enzyme I was competitively inhibited by β-cyanoalanine (K_i < 0.1 mM). Enzyme I activity with djenkolate as substrate was also inhibited by *N*-ethylmaleimide (100% inhibition at 2 mM) but not significantly by 5 mM DL-propargylglycine. Enzyme II from *A. sinicus* was relatively much more active towards cystine, selenocystine and selenocystathionine than djenkolate and cystathionine (Table 3). With selenocystine as substrate, the activity of enzyme II was not significantly decreased by *N*-ethylmaleimide (2 mM) or β-cyanoalanine (2.6 mM).

Extracts of the selenium accumulator species *A. bisulcatus* and *A. racemosus* exhibited very low levels of enzyme I activity. Djenkolate was the most active substrate but cystathionine and selenocystathionine were also cleaved at very slow rates (Table 3). The rates with these

Table 3. α,β-Cleavage activity of enzymes I and II from spinach, *Astragalus* spp. and *Neptunia* spp. towards various substrates*

Source	α,β-Cleavage activity (nmol/min/ml of extract)				
	L-Djenkolate (9 mM)	L-Cystathionine (5 mM)	L-Seleno-cystathionine (3 mM)	L-Cystine (3 mM)	DL-Selenocystine (2 mM)
Spinach enzyme I	4.3	3.7	2.5	1.6	2.6
Spinach enzyme II	0.3	0.2	1.4	1.7	1.0
A. sinicus I	3.5	2.8	1.8	0.9	1.6
A. sinicus II	0.3	0.2	1.2	1.2	2.2
A. racemosus I	1.1	0.7	0.5	0.7	1.1
A. racemosus II	0.3	0.2	0.6	0.5	2.3
A. bisulcatus I	0.8	0.4	0.3	0.7	0.7
N. amplexicaulis I	0.3	0.1	0.7	1.1	1.3
N. amplexicaulis II	0.2	0	0.6	1.1	2.1
N. dimorphantha†	0.5	0.3	1.1	5.1	3.0

*α,β-Cleavage activity was determined at the substrate concentrations shown in the Table. With the exception of L-cystine these concentrations supported the activity of pea enzyme I at approximately maximum rates (see Fig. 2).

†The extraction procedures did not resolve more than one enzyme from this species. The elution characteristics were similar to those of enzyme II from other species.

substrates were too slow to obtain kinetic data. Enzyme I from these species was also active towards cystine and selenocystine. A fraction with gel filtration characteristics on Sephacryl S200 similar to pea enzyme II was obtained from *A. racemosus*. This activity, which was barely detectable with djenkolate as substrate, exhibited high activity with selenocystine but relatively little activity towards other substrates (Table 3). An analogous activity in *A. bisulcatus* was not detected using djenkolate as substrate.

None of the three species of *Neptunia* yielded very satisfactory preparations of α,β-cleavage activity as determined by the production of pyruvate from djenkolate and cystathionine. Activity with these substrates was barely detectable in extracts of *N. gracilis* following gel filtration on Sephacryl S200. Extracts of *N. dimorphantha* also exhibited very low activity with djenkolate and cystathionine but were very active towards cystine, selenocystine and selenocystathionine. Extracts of *N. amplexicaulis*, after passage through Sephacryl showed similar properties except that activity with cystathionine was barely detectable (Table 3).

DISCUSSION

The results reported in this paper are relevant to the methionine biosynthetic pathway in plants and the synthesis of selenium isologues of the pathway in various selenium accumulator and non-accumulator plants. The data on the kinetics (Table 2), substrate specificity (Figs 2 and 4) and inhibition by β-cyanoalanine (Fig. 3) of enzyme I from peas, spinach and *A. sinicus* are in approximate agreement with those described previously for β-cystathionase from plants [3], including a highly purified enzyme from spinach [4]. Fractions from pea, spinach and *A. sinicus* containing enzyme I activity also support the production of pyruvate from selenocystathionine. Further, since the K_m values for cystathionine and selenocystathionine are similar (Table 2) this suggests that the production of pyruvate from selenocystathionine in these species can be attributed to β-cystathionase activity. By inference, this affords a mechanism for the synthesis of selenohomocysteine from selenocystathionine in peas, spinach and *A. sinicus* and presumably other species which do not accumulate selenium. Plants contain the enzymes glutathione reductase and cysteine synthase which catalyse the incorporation of SeO_3^{2-} into selenocysteine [12–14]. Thus, provided some mechanism exists for the synthesis of selenocystathionine from selenocysteine [Dawson, J. C. and Anderson, J. W., unpublished], it follows that plants which do not accumulate selenium contain mechanisms for the incorporation of inorganic selenium into selenohomocysteine and, potentially, selenomethionine. The latter compound is an important incorporation product in many non-accumulator plants when supplied with inorganic selenium [15, 16].

The capacity of β-cystathionase to support a reaction with selenocystathionine extends the list of enzymes of the sulphate assimilatory pathway which catalyse reactions with selenium isologues of intermediates of the pathway. ATP sulphurylase and cysteine synthase provide other examples of this principle [12, 17–19] although the former enzyme is unlikely to be important physiologically since selenate is not a stable form of selenium in the physical environment [9]. The similar kinetic values of enzyme I for cystathionine and selenocystathionine (Table 2, Fig. 2) imply that, in equimolar mixtures, the two substrates would be metabolized in approximately equal amounts. *In vivo*, under conditions conducive to the presumed assimilation of selenium into selenocystathionine, this would have important consequences unless mechanisms for the intracellular separation of the isologues existed.

The very low activity of enzymes I and II in fractions from *A. racemosus*, *A. bisulcatus*, *N. amplexicaulis* and *N. dimorphantha* only permit the formulation of tentative conclusions concerning the properties of enzyme(s) sup-

porting α,β-elimination in these species. The first three of these species are reportedly selenium accumulators [20] and the growth of *N. dimorphantha* exhibits considerable tolerance to inorganic selenium [Anderson, J. W., unpublished]. Fractions with elution characteristics similar to those of enzyme I from peas supported the production of pyruvate from djenkolate, cystathionine and selenocystathionine albeit at very low rates (Table 3). These data suggest that selenium accumulator plants, like the non-accumulators, possess the capacity to metabolize selenocystathionine to selenohomocysteine. Further experiments are required to establish whether the very slow rates of cystathionine-cleavage activity in these species are attributable to very inefficient extraction and/or purification procedures or whether they are an inherent property of selenium accumulator plants. The latter possibility brings into question whether the transsulphurylation pathway is an important mechanism for the synthesis of methionine as reported for other species [1–3] and contributes to the accumulation of selenocystathionine in some selenium accumulators, e.g. *N. amplexicaulis* [21]. The relatively higher activity with cystine and selenocystine is not understood.

The data reported in this paper, while relevant to the debate on the substrate specificity of β-cystathionase and cystine lyase [6–8], do not permit resolution of this issue. As already noted, the characteristics of enzyme I are similar to those of the β-cystathionase described in ref. [4]; it has relatively low affinity towards cystine. Enzyme II on the other hand exhibited highest activity and affinity with cystine and its selenium isologue; it had very low activity with djenkolate and little if any activity with cystathionine. It is more typical of the cystine lyases reported in ref. [7]. A further study is required to establish whether a single enzyme catalyses the reaction with cystine and selenocystine or whether a specific selenocystine lyase is also involved.

EXPERIMENTAL

Plant material and preparation of extracts. Pea seedlings (*Pisum sativum* cv Massey Gem) were raised in growth cabinets and plants of *Astragalus sinicus, A. racemosus, A. bisulcatus, Neptunia amplexicaulis, N. gracilis* and *N. dimorphantha* were raised from seed as per refs [12] and [22]. Me_2CO powders were prepared from freshly harvested leaf material of each of these species and from field grown spinach (*Spinacia oleracea*) and stored at 2°. When required, Me_2CO powder (10 g) was extracted in a slurry of Al_2O_3 (10 g) in 200 ml of medium 1 (0.1 M KPi buffer pH 7.3, 0.1 mM Na_2EDTA and 5 mM DTT) in a blender for 1.5 min. Insoluble material was removed by centrifuging (10000 *g* for 10 min) and solid $(NH_4)_2SO_4$ was added (0.144 g/ml). After removing insoluble material by centrifuging (20000 *g* for 10 min) more $(NH_4)_2SO_4$ (0.098 g/ml) was supplied. Precipitated material [25–40% $(NH_4)_2SO_4$] was recovered by centrifugation and redissolved in medium 2 (0.1 M triethanolamine–HCl buffer pH 8, 1 mM Na_2EDTA, 0.1 mM NaCl, 0.1 mM pyridoxal phosphate and 5 mM DTT) and subjected to gel filtration on a Sephacryl S200 column (bed vol. *ca* 150 ml) in medium 3 (as for medium 2 but without DTT). Fractions (*ca* 3 ml) eluting at or near the void volume which showed activity with djenkolate as substrate (enzyme I, see Fig. 1) were pooled and applied to a DEAE-cellulose column (bed vol. 30 ml) equilibrated in medium 3. The column was flushed with medium 3 before applying a linear gradient of 0–0.4 M NaCl in medium 3. Fractions exhibiting α,β-cleavage activity with djenkolate as substrate were pooled and dialysed to remove NaCl before use. The minor band of activity eluting from Sephacryl S200 columns (enzyme II) was usually used without further treatment. Unlike enzyme I, this activity did not bind to DEAE-cellulose.

Determination of α,β-cleavage activity. Pyruvate produced by α,β-cleavage of appropriate substrates was determined by the fluorometric estimation of NAD [23] produced by the oxidation of NADH in the presence of lactate dehydrogenase (LDH). Reaction mixtures (200 μl) contained substrate (9 mM djenkolate unless specified otherwise), 0.5 mM NADH, 3 units LDH, 66 mM triethanolamine–HCl buffer pH 8, 0.66 mM Na_2EDTA, 0.07 mM pyridoxal phosphate and enzyme. Incubations were conducted at 30° and terminated after 30 min by addition of 50 μl 1 M HCl. After 30 sec, 0.75 ml of 8.1 M NaOH was added and the mixtures incubated at 100° for 5 min. H_2O (4 ml) was added and fluorescence determined at $\lambda_{Ex} = 365$ nm and $\lambda_{Em} = 460$ nm. Enzyme activity, corrected for zero time controls and 30 min incubations lacking substrate, is expressed as nmol/min/ml of enzyme extract. Specific activity is expressed as nmol/mg protein/min.

Substrates. L-Selenocystathionine was synthesized from benzyl chloride, L-serine and Se essentially as described by Zdansky [24, 25]. However, several modifications were introduced to minimize losses due to the instability of some products and the occurrence of uncontrolled side reactions. Not more than 0.15 mol of benzyl chloride was reacted with Se at a time. The benzyl selenol formed in this reaction (carried out under N_2 in the presence of $NaBH_4$) was not purified owing to its instability in air but was extracted along with the diselenide in $CHCl_3$. The $CHCl_3$ extract was immediately reacted with 0.15 mol acrolein to form β-*Se*-benzyl selenopropanol. Subsequent batches of this product were pooled at this stage. It was also essential to conduct the debenzylation of L-γ-*Se*-benzylselenohomocysteine and its subsequent coupling to L-β-chloroalanine HCl under N_2. The R_f values for intermediates and products upon TLC in solvents I-VII were as follows: L-β-chloroalanine HCl, I 0.13, II 0.21; meso-5-(β-*Se*-benzylselenoethyl)-hydantoin, II 0.74; meso-γ-*Se*-benzylselenohomocysteine, II 0.51; meso-*N*-acetyl-γ-*Se*-benzylselenohomocysteine, II 0.72; L-*N*-acetyl-γ-*Se*-benzylselenohomocysteine anilide, II 0.89; L-γ-benzylselenohomocysteine, II 0.51; L(+)-selenocystathionine, IV 0.65, V 0.15, VI 0.33, VII 0.69. (Found: C, 30.8; H, 5.6; N, 10.4%. Calc. for $C_7H_{14}O_4N_2Se$: C, 31.2; H, 5.2; N, 10.4%.)

L-Djenkolate, DL-selenocystine, L-cystine, L-cystathionine and DL-propargylglycine were obtained from Sigma and adjusted to pH 7 immediately prior to use.

Chromatography. TLC in solvents I–V was conducted on silica gel plates. Cellulose plates were used for solvents VI and VII. The solvents were as follows: I, *iso*-PrOH–*n*-BuOH–H_2O (1:3:1); II, *n*-BuOH–HOAc–H_2O (4:1:1); III, EtOH; IV, $CHCl_3$–MeOH–H_2O (2:2:1); V, PhOH–H_2O (3:1); VI, PhOH–0.025% NaCN in H_2O (3:1); VII, *iso*-PrOH–satd NH_3–H_2O (12:1:12). Benzyl, amino and acidic groups were visualized by UV quenching, ninhydrin and bromophenol blue respectively.

Other methods. Protein was determined as in ref. [17].

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