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NON-IDENTITY OF CYSTINE LYASE WITH β-CYSTATHIONASE IN TURNIP ROOTS

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Key Word Index—*Brassica rapa*; Cruciferae; turnip root; cystine lyase; β-cystathionase; cystine degradation.

Abstract—An active preparation of cystine lyase (EC 4.4.1.–) was prepared from turnip roots and its substrate specificity examined. Only L-cysteine, cysteine-S-SO_3, and the sulphoxides of L-djenkolic acid, *S*-methyl- and *S*-ethyl-L-cysteine were substrates. L-Cystathione, L-djenkolic acid, *S*-methyl- and *S*-ethyl-cysteines were not cleaved by this enzyme. The K_m for L-cystine was 1.3 mM and L-cystathionine acted as an effective competitive inhibitor with a K_i of 0.7 mM. After dialysis against 10 mM potassium phosphate buffer pH 7.5, added pyridoxal phosphate was absolutely necessary for activity. In addition a marked stimulation was observed in the presence of ammonium sulphate. The products of the reaction were cysteine persulphide, pyruvate and presumably ammonia. The persulphide was easily demonstrated by cleavage with CN^- to yield SCN^- under conditions in which elemental sulphur was unreactive.

INTRODUCTION

A number of years ago a cystine lyase was reported in several *Brassica* species including cabbage, cauliflower, turnip and rutabaga [1]. A partially purified enzyme from rutabaga (*B. napobrassica*) roots was characterized in some detail [1]. This relatively impure preparation cleaved L-cystine by the following reaction:

$$\text{L-cystine} + H_2O \rightarrow \text{L-cysteine-S-SH} + \text{pyruvate} + NH_3.$$

The only other substrates utilized of those tested were *S*-methyl-L-cysteine sulphoxide and L-cysteine-S-SO_3.

Recently an enzyme from turnip roots capable of a similar degradation of L-cystine has been purified 277-fold and its properties reported [2]. In contrast to the rutabaga enzyme the turnip preparation was reported to degrade a large number of substrates even if less effectively than cystine [2]. Among the substrates cleaved at a significant rate were cystathionine and other thioethers such as *S*-methyl- and *S*-ethyl-cysteine, and L-djenkolic acid. Since turnip and rutabaga are closely related species, it seemed unusual to find such a marked variation in the properties of a similar enzyme.

Because of its reported ability to cleave cystathionine, it was suggested that the metabolic role of the turnip root cystine lyase was to function as a β-cystathionase in the biosynthesis of methionine [2].

Due to the significance of this conclusion we have re-examined the specificity of the enzyme from turnip. In our hands there is no marked distinction in the specificity of this enzyme from that previously reported for rutabaga [1]. Only L-cystine, *S*-alkyl-L-cysteine sulphoxides, and L-cysteine-S-SO_3 were substrates. L-cystathionine and other thioether derivatives of L-cysteine were inactive as substrates. Both enzymes showed a marked stimulation of activity in the presence of ammonium sulphate.

RESULTS

Enzyme activity and recovery and stimulation by NH_4^+

Using the preparative method [2] described in the Experimental, a very active cystine lyase was obtained from turnip roots. Anderson and Thompson [2] had dialysed their step 2 (ammonium sulphate fraction) enzyme prior to assay. Our previous experience with rutabaga [1] had shown that dialysis yielded very low activities in comparison to an undialysed enzyme preparation. We dialysed aliquots of the original extract and the ammonium sulphate-precipitated fraction against 150 volumes of the phosphate–PLP (pyridoxal-5′-phosphate) suspension buffer for 3 hr. After each hour new phosphate–PLP buffer was used as the dialysis medium. Since the major salt removed during dialysis of the step 2 fraction was entrained ammonium sulphate, the activity of the dialysed fractions was determined in the presence and absence of 50 mM ammonium sulphate. The results are summarized in Table 1. With both step 1 and step 2 fractions, dialysis yielded active preparations which were stimulated *ca* 50% by added ammonium sulphate. For convenience step 2c enzyme was used in the remainder of the experiments unless otherwise stated, because of the significant amount of ammonium sulphate entrained in the precipitate. Essentially 100% of the total activity was

Table 1. Summary of the recovery and purification from turnip roots

Step	Fraction	Total protein (mg)	Total activity nkat	Recovery (%)	Specific activity (nkat/mg protein)	Purification
1a	Potassium phosphate extract*	2068	1716	100	0.83	1.0
1b	Potassium phosphate extract + $(NH_4)_2SO_4$	—	2688	157	1.3	1.6
2	$(NH_4)_2SO_4$ precipitate	354	—	—	—	—
2a	Dialysed	—	1727	101	4.88	5.9
2b	Dialysed + NH_4	—	2502	146	7.07	8.5
2c	Undialysed	—	1901	111	5.37	6.5

*Extract obtained from 600 g peeled sliced turnip roots. Dialysed aliquot against 165 volumes of 10 mM potassium phosphate pH 7.5 containing 50 μM PLP for 3 hr. Changed dialysis medium every hour.

recovered in step 2. The rutabaga enzyme showed a similar striking stimulation of activity on addition of ammonium sulphate to dialysed fractions. K^+ or Mg^{2+} could not replace NH_4^+.

pH optimum

The pH optimum of the turnip lyase was determined using Bicine buffer. For each point at least one other buffer was tested instead of Bicine. The enzyme had an optimum pH in Bicine between 8.7 and 9.0. At each point Bicine gave more active preparations than the other buffers used. Table 2 gives the comparison between Bicine and several other buffers at the optimum pH.

Specificity of turnip lyase

A number of sulphur-substituted cysteines were tested as potential substrates and compared in activity to L-cystine. The results are summarized in Table 3. Only L-cystine, L-cysteine–S–SO_3, L-djenkolic acid sulphoxide, and *S*-methyl- and ethyl-L-cysteine sulphoxides were cleaved by this enzyme preparation. Other than cystine itself only substrates in which the sulphur was oxidized to a sulphoxide or linked to an oxidized sulphur atom could act as substrates. For example djenkolic acid was inert but its sulphoxide was an excellent substrate. Similarly the thioethers *S*-methyl- and *S*-ethyl-L-cysteine were inert but their sulphoxides were cleaved at a significant rate. For comparison the previous results with rutabaga [1] are also listed. The two enzymes appear identical in their choice of substrates. L-Cystathionine not only was inactive as a substrate, but it acts as a competitive inhibitor of the cleavage of L-cystine (Fig. 1). A K_i of 0.7 mM is derived from the data presented. L-Djenkolic acid also inhibited the cleavage of L-cystine in apparently the same manner. The K_i of 0.7 mM is of the same order of magnitude as the K_m for L-cystine lyase of 0.94 mM [2] and in our present studies 1.3 mM for the turnip enzyme and 1.0 mM for the rutabaga enzyme [1].

Table 2. Comparative lyase activity in various buffers at pH 8.7

Buffer	Relative activity
Bicine	100
Tricine	94
Glycylglycine	86
Tris–acetate	80
CHES	72

The reaction mixture is the same as described in the Experimental except for the buffer used.

Table 3. Relative activity of lyase with various substrates

Substrate	Relative activity Turnip	Rutabaga*
L-Cystine	100	100
L-Djenkolic acid sulphoxide	116	—
L-Cysteine–S–SO_3^{2-} Na_2^+	129	156
S-Methyl-L-cysteine sulphoxide	25	58
S-Ethyl-L-cysteine sulphoxide	32	—
L-Cystathionine	0	0
L-Djenkolic acid	0	0
S-Ethyl-L-cysteine	0	—
D-Cystine	0	—
S-Methyl-L-cysteine	0	0

*Reproduced from [1] for comparative purposes.

PLP requirement

Step 2c enzyme was very stable to storage at −15°. During the course of these studies it was thawed and refrozen frequently. After 3 months of this treatment, an aliquot of the enzyme solution was dialysed for 3 hr against 125 volumes of 10 mM potassium phosphate buffer, pH 7.5 and another aliquot against the same volume of this buffer containing 50 μM PLP. A stimulation by ammonium sulphate and an absolute requirement for exogenous PLP were easily shown (Table 4). Using the standard assay reaction mixture which contains 50 μM PLP, the activities of enzyme from both dialysis conditions were equal. In the absence of PLP, the enzyme dialysed against buffer alone demonstrated almost a complete loss of activity. The addition of 50 mM ammonium sulphate to the reaction mixture returned the activity of the dialysed enzyme preparations in the presence of PLP to the activity of the undialysed enzyme at this time. Comparison of step 2c enzyme activity at the beginning (Table 1) and after 3 months storage and use (Table 4) showed 90% retention of the activity under these conditions of storage.

Thiocysteine as a reaction product

The reaction with L-cystine is considered to have the following stoichiometry:

$$\text{L-cystine} + H_2O \rightarrow \text{L-thiocysteine} + \text{pyruvate} + NH_3.$$

L-thiocysteine (also known as cysteine persulphide) can be assayed by cleavage of the persulphide bond by CN^- to give rise to SCN^- which can be determined colorimetrically. In five different determinations involving differing times of incubation,

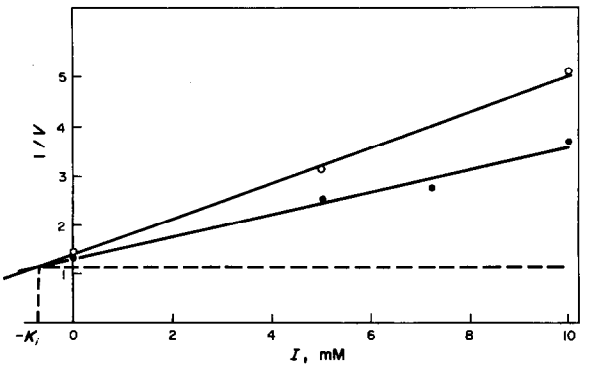

Fig. 1. A Dixon plot of the inhibition of L-cystine cleavage by L-cystathionine. O—O, 5 mM L-cystine; ●—●, 7.5 mM L-cystine; — — —, $1/V_{max}$ for the uninhibited enzyme. K_i from this plot is 0.7 mM.

Table 4. Cofactor requirements for lyase reaction

Assay reaction mixture	Specific activity (nkat/mg protein)		
	Step 2c enzyme treatment		No treatment
	Dialysis buffer only	Dialysis buffer + PLP	
Complete*	4.61	4.2	5.7
$-(NH_4)_2SO_4$, − PLP	0.16	—	—
− PLP	0.46	4.95	—
$-(NH_4)_2SO_4$	2.55	2.77	4.83†

*Complete reaction mixture is that described in the Experimental + 50 mM $(NH_4)_2SO_4$.
†No added $(NH_4)_2SO_4$ but some salt is present from fractionation step.

levels of cystine, and varying amounts of enzyme the amount of persulphide as SCN^- was compared to the amount of pyruvate produced. The mean ratio of thiocysteine–pyruvate was $1.01 \pm 0.06 : 1.00$. Since the assay procedure was that described by Flavin [3] only a compound of the structure R–S–SH will form SCN^- under these conditions; S^0 reacts too slowly to yield positive values under the conditions of assay.

DISCUSSION

Turnip root cystine lyase appears to be almost identical in its action and properties to the C–S lyase described from rutabaga roots [1]. It cleaves relatively few substrates being active with L-cystine and L-cysteine–S–SO_3 and the sulphoxides of L-djenkolic acid; *S*-methyl- and *S*-ethyl-L-cysteine of the substrates tested (Table 3). Its stoichiometry with L-cystine is that of a β-elimination reaction of an α-amino acid viz:

$$\mathrm{R\text{–}S\text{–}CH_2CH(NH_2)\text{–}COOH + H_2O \rightarrow RSH + MeC(=O)\text{–}COOH + NH_3.}$$

The RSH in this particular case is thiocysteine or cysteine persulphide.

An interesting finding is the activation of the enzyme by NH_4^+. Our prevous study with rutabaga [1] had reported that dialysis or gel filtration had decreased the activity of the enzyme almost completely. Anderson and Thompson [2] had dialysed their preparations extensively before assay and had found activity. Since the most obvious loss on dialysis with buffers containing PLP of the step 2 enzyme is the ammonium sulphate entrapped in the protein pellet, addition of ammonium sulphate to the dialysed enzyme was tested and found to increase the activity of both the steps 1 and 2 enzymes. A rutabaga preparation was prepared and the stimulatory effect of added ammonium sulphate on the dialysed enzyme was the same. The ammonium sulphate present in the step 2c enzyme might also have a role in the preservation of activity over a long period in frozen storage. An aliquot of step 2a enzyme stored at −15° for 3 months was almost completely inactive on testing under any assay condition, whereas as pointed out in the results above, step 2c enzyme retained at least 90% of its activity under the same conditions.

This stimulation by NH_4^+ was not replaceable by K^+ or Mg^{2+} and at the moment there is not sufficient information to suggest its mode of action. This will have to wait for further work with homogeneous enzyme.

Our results differ to a large degree with those obtained by Anderson and Thompson [2]. Using their extraction procedure we obtain a step 2 enzyme having approximately six-fold their specific activity. Conceivably this might be explained partially by the removal of the ammonium sulphate in their preparation and thus lowering the activity. Compounding this decrease in activity is the use of Tris–acetate as the assay buffer and the lower activity with it compared to Bicine. However no facile explanation comes to mind for the wide specificity of their enzyme preparation and the final products obtained viz. elemental sulphur.

One possible explanation of the difference in behaviour would be if there are two enzymes present, one of wide specificity and low activity, and the other of limited specificity and high activity. The dialysis and lyophilization step used in their purification procedure might inactivate the latter enzyme. Under these circumstances their customary incubation time (30 min) would allow the low activity enzyme to be demonstrated. The present results, however, should have given some indication of this second activity since our preparation would have contained both enzymes.

Far from being a β-cystathionase the present results demonstrate the ineffectiveness of cystathionine as a substrate and its effectiveness as a competitive inhibitor of the cleavage of L-cystine. The question as to the role of this enzyme in the metabolism of the plant is not answered, except to state that it does not have a place in the biosynthetic pathway to methionine. On the other hand the cysteine persulphide product or those formed on cleavage of the alkyl cysteine sulphoxides would certainly be similar to those produced by *Allium* sp. or other Cruciferae on disruption of their tissue. These have been conjectured to act physiologically as fungistats [4]. At the present time only the enzyme described by Giovanelli and Mudd [5] has been shown to cleave cystathionine preferentially and it has a very limited specificity.

EXPERIMENTAL

Enzyme preparation. Turnip and rutabaga (*Brassica rapa* and *B. napobrassica* respectively) roots were purchased in local markets. Turnip roots were peeled and washed and then enzyme preparations were prepared by the method of Anderson and Thompson [2] through their step 2 $(NH_4)_2SO_4$ fraction. A slight modification of their method was to blend for only 40–60 sec rather than 2 min. The protein pellet obtained at step 2 was dissolved in 10 mM KPi buffer, pH 7.5, which was 50 μM to pyridoxal phosphate. This solution was stored at −15° and thawed for use as the enzyme source for the expts described above. It was very stable to repeated freezing and thawing for at least 2 months.

Rutabaga enzyme was prepared as previously described [1] but using the phosphate–PLP soln as the suspension medium as described above.

Protein was determined by the procedure of Lowry *et al.* [6].

Enzyme assay. The L-cystine, L-cystathionine, and L-djenkolic acid were prepared by soln in 0.05 M NaOH. All other substrates were dissolved in H_2O. The reaction mixture consisted of the following components (final vol. 1 ml): Bicine buffer pH 8.8, 0.1 M; substrate, 10 mM; pyridoxal phosphate, 50 μM; enzyme. Prior to the addition of enzyme to start the reaction, the pH of each reaction mixture was adjusted to lie between 8.6 and 8.9 by the addition of small vols of 5 N HCl or NaOH. With the turnip enzyme the usual reaction time was 10 min at room temp. and for the rutabaga enzyme 15 min. To stop the reaction 2 ml 10% TCA (trichloroacetic acid) was added. The ppt was brought down by centrifugation and an aliquot of the supernatant was assayed for pyruvate by the total keto acid method of Friedemann and Haugen [7]. The assay for thiocysteine was similar to that described previously [1] based on the procedure of Flavin [3].

Chemicals. L-Cystine was a product of Nutritional Biochemicals; D-cystine, L-djenkolic acid, *S*-ethyl- and *S*-methyl-L-cysteine were purchased from Sigma; L-cystathionine, Bicine, and glycylglycine were obtained from Calbiochem; L-djenkolic acid sulphoxide was a gift from Dr. L. Fowden; *S*-methyl- and *S*-ethyl-L-cysteine sulphoxides were synthesized as previously described [1]. Cysteine–S–SO_3 was prepared by the method of Segel and Johnson [8].

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