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DEMONSTRATION AND CHARACTERIZATION OF CYSTEINE SULFOXIDE LYASE IN THE CRUCIFERAE

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Abstract—An enzyme which degrades cysteine sulfoxides has been found in members of the genus *Brassica* of the family Cruciferae. The name 3-alkylsulphinylalanine alkyl sulphenate lyase (deaminating) (cysteine sulfoxide lyase) is proposed for this enzyme.* The enzyme has been purified about eleven-fold from broccoli buds. The broccoli lyase is similar in its mode of action to alliinase which has been described in *Allium* species. The pH optimum of the broccoli enzyme is between 8·4 and 8·6 in borate buffer. The products of the reaction are pyruvate, ammonia, and alkyl alkane thiosulfinates. The *Km* is $2{\cdot}7 \times 10^{-3}$ *M* with L-methylsulphinylalanine as the substrate. Studies with acetone powders and aged preparations, and inhibition studies indicate that pyridoxal-5'-phosphate is a coenzyme. s-methyl-L-cysteine was not a substrate, nor did it inhibit the reaction when present in equimolar concentration with the sulfoxide. Equimolar amounts of L-cysteic acid and L-cysteine sulfinic acid resulted in inhibitions of 30–40 per cent in the utilization of L-methylsulphinylalanine.

INTRODUCTION

L-3-ALKYLSULPHINYLALANINES* (alkyl cysteine sulfoxides) have been found as major constituents of the free amino acid pool of many members of the Liliaceae and the Cruciferae.[1,2] L-3-Methylsulphinylalanine is the most widely distributed, being found in members of both families, and indeed is the only 3-alkylsulphinylalanine derivative so far demonstrated in the Cruciferae. The propyl and allyl analogues have been observed in various species of the Liliaceae and the ethyl derivative has been found in a single species of this family.[1] Stoll and Seebeck[3] first demonstrated the enzymatic degradation of these compounds by showing that a preparation from garlic (*Allium sativum*) could convert alliin (L-3-allylsulphinylalanine) to pyruvic acid, ammonia, and allicin (s-allylthio-2-propene-1-sulfinate). Enzyme preparations from onion (*Allium cepa*) have also been obtained which carry out the same reaction and will utilize the other L-3-alkylsulphinylalananines as substrates.[4,5] Since L-3-methylsulphinylalanine has been found to be widespread in the Cruciferae, attempts to demonstrate a similar enzyme in members of this family have been made. These efforts have been unsuccessful, and the enzyme thus reported to be absent in the Cruciferae.[1,6]

This paper is a report of the occurrence of such an enzyme in members of the genus *Brassica* in the Cruciferae. The name 3-alkysulphinylalanine alkyl sulphenate lyase (deaminating) (cysteine sulfoxide lyase) is proposed to conform with the nomenclature suggested by the Commission on Enzymes of the International Union of Biochemistry,[7] and since alliin is not present in these plants, the trivial name alliinase would not be apropos. The enzyme from broccoli buds has been purified to some extent and some of its characteristics and properties described.

* s-alkyl-L-cysteine sulfoxides are better named L-3-alkylsulphinylalanines. This nomenclature is used throughout this paper (but see Report of the Commission on Enzymes of the I.U.B[7]., enzyme 4 : 4 : 1 : 4).

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[6] S. TSUNO, *Bitamin* **14**, 671 (1958); *Chem. Abst.* **55**, 12569c (1961).
[7] *Report of the commission on enzymes of the International Union of Biochemistry*, Pergamon Press, New York (1961).

RESULTS

Demonstration of cysteine sulfoxide lyase activity in acetone powder extracts

Preliminary tests of turnip and cauliflower acetone powder extracts showed that lyase activity could be exhibited at an alkaline pH. Table 1 shows that the enzyme activity was easily demonstrated in members of the genus *Brassica* at pH 10. In each case where there was a significant lyase activity, there was a large stimulation on the addition of pyridoxal-P.

TABLE 1. LYASE ACTIVITY IN PLANT ACETONE POWDERS

Source	Specific activity (μmole pyruvate/mg protein/hr)		
	Complete reaction mixture pH 10	Minus pyridoxal-P pH 10	Complete reaction mixture pH 7
Brassica oleracea var. botrytis (cauliflower)	4·0	2·4	
B. oleracea var. capitata (cabbage)	2·0	0·8	
B. oleracea var. pompejana (broccoli)	2·1	1·0	0·4
B. oleracea var. gemmifera (brussel sprouts)	1·7	1·3	0·2
B. rapa (turnip)	2·0	1·0	
B. alba (mustard)	0·9	0·7	
Raphanus sativa (radish)	0·2	0·2	0·2
Allium sativum (garlic)	0·3		1·9
Ipheon uniformum	0·1	0·1	0·2

This stimulation varied from 30–150 per cent. Acetone powder extracts of kidney bean, carrot, barley, corn, squash, lettuce, and chard had no activity. Radish, a cruciferous plant, contains L-3-methylsulphinylalanine,[2] but an insignificant amount of lyase activity was observed. Similarly, in the case of *Ipheon uniformum*, a member of the Liliaceae, which is known to contain L-3-ethylsulphinylalanine.[1] At pH 10 garlic preparations had very little activity, but at pH 7 these preparations were quite active. In comparison, the activity of the cruciferous plants, broccoli and brussel sprouts, was quite high at pH 10 and almost non-existent at pH 7.

Partial purification of enzyme from broccoli

Broccoli buds were used as a source from which to purify the enzyme from a cruciferous source. Early experiments showed that the enzyme was localized in the supernatant solution obtained after centrifugation of broccoli homogenates at $35{,}000 \times g$. An example of a typical purification follows. All operations were carried out in the cold. A known weight of broccoli buds was blended in 0·05 *M* sodium borate solution ($Na_2B_4O_7$) for 1 min at line voltage. One ml of solution was used for each gram of broccoli buds. After straining the homogenate through cheesecloth 15 ml of a 1% protamine sulfate solution in 0·2 *M* acetic acid was added for each 100 ml of filtrate. The mixture was centrifuged at $35{,}000 \times g$ for 30 min. The yellowish green supernatant solution was decanted and 25 g solid $(NH_4)_2SO_4$ per 100 ml of solution added. The precipitate obtained was discarded and 14·5 g $(NH_4)_2SO_4$ per 100 ml of the original solution added. The precipitate was collected by centrifugation and dissolved in 0·005 *M* sodium borate. Glacial acetic acid was added dropwise to pH 4, and the precipitate formed was removed immediately and discarded. The clear solution was brought to pH 8 at once with 2·5 *N* NaOH. A saturated solution of $(NH_4)_2SO_4$ at pH 7·4 was added slowly until 35 per cent saturation was reached. After 10 min with stirring, the precipitate present was removed by centrifugation and discarded. Saturated $(NH_4)_2SO_4$ at pH 7·4 was added until the solution was 55 per cent saturated. The precipitate was collected by centrifugation and dissolved in 0·005 *M* sodium borate. This enzyme solution

(AS) was used as the source of enzyme in some experiments. In most cases, however, the solution was dialyzed against several changes of 0·005 *M* sodium borate for 6 hr. The dialyzed solution (DAS) was divided into small aliquots and stored at −10°. The aliquots

TABLE 2. PURIFICATION OF BROCCOLI LYASE

	Total activity	Specific activity	Purification	% Yield
Protamine supernatant	2436	0·5	1·0	100
$(NH_4)_2SO_4$ Fraction 1	2858	1·9	3·8	117
Acid-treated solution	2629	2·2	4·2	104
35–55% $(NH_4)_2SO_4$ Fraction (AS)	1439	4·1	8·1	59
Dialyzed $(NH_4)_2SO_5$ Fraction (DAS)	1362	5·3	10·6	56

The reaction mixture consisted of 100 μmole of sodium borate at pH 8·4, 10 μmole L-3-methylsulphinylalanine, 0·1 μmole pyridoxal-P, enzyme fraction, and distilled water to a final volume of 2 ml Incubation time was 30 min. The reaction was terminated by the addition of 3 ml of 10% trichloroacetic acid. The assay was carried out for pyruvate as described under "Experimental".

were thawed as needed. DAS has retained up to 80 per cent of its activity after 72 days at −10°. Some preparations, however, were less stable and after 3 weeks had lost 50 per cent of the original activity. Table 2 is an outline of the procedure and the purification at each

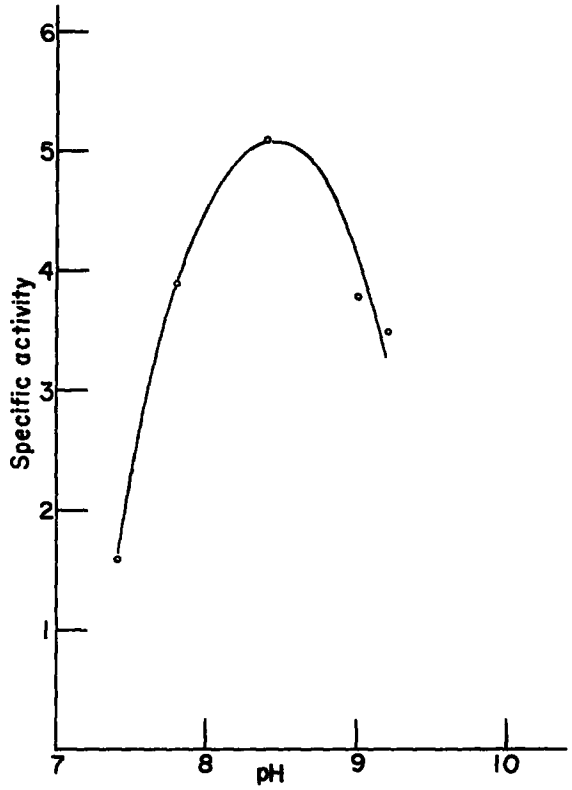


FIG. 1. EFFECT OF pH ON BROCCOLI LYASE ACTIVITY

The reaction mixture was the same as that given in Table 2 except 200 μmole of sodium borate to give the final pH was added. 1·8 mg AS was used as the enzyme source.

step. The reaction was linear with respect to the amount of enzyme added in the experiments which will be described. The enzyme reaction was anaerobic since its activity in a helium atmosphere was the same as that under aerobic conditions.

Lyase activity and pH

The activity of the enzyme as a function of pH is shown in Fig. 1. A fairly sharp optimum at 8·4 was obtained in borate buffer. When pyrophosphate buffer was used, a similar pH optimum was reached but the level of activity was less than at the same pH using borate. With glycine as the buffer, the optimum pH was shifted to about 9·2. Borate was the most effective buffer in terms of the activity demonstrated by the enzyme.

Substrate concentration and lyase activity

The *Km* of the reaction with L-3-methylsulphinylalanine as the substrate was determined by the graphical method depicted in Fig. 2. The *Km* was $2{\cdot}7\times10^{-3}$ *M*.

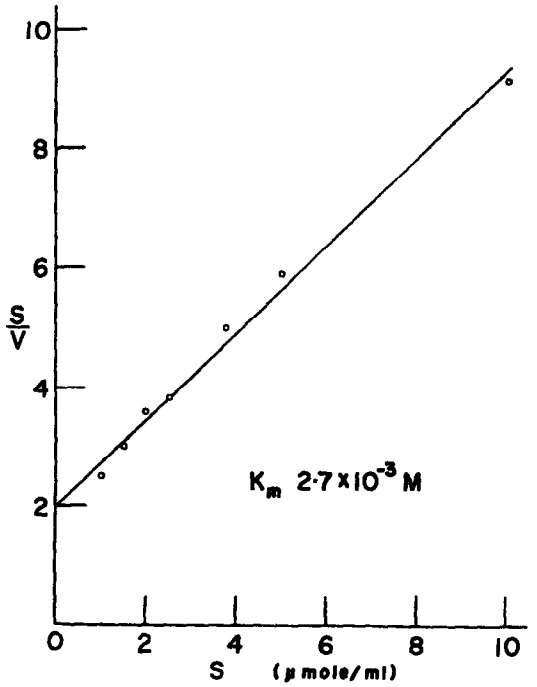


FIG. 2. EFFECT OF SUBSTRATE CONCENTRATION ON PYRUVATE FORMATION
Reaction mixture same as that in Table 2. Time of incubation was 10 min. 1·2 mg DAS was used as the enzyme.

Pyridoxal-P requirement for lyase activity

The acetone powder extracts of broccoli and the other crucifers showed a marked stimulation of activity on the addition of small amounts of pyridoxal-P (Table 1). The purified broccoli enzyme (DAS), however, did not show any large stimulation with the addition of pyridoxal-P. Indeed in several freshly prepared DAS preparations there was even a slight inhibition of activity on the addition of the cofactor. The non-dialyzed AS solutions when kept several days at ordinary refrigerator temperatures between 8° and 10°,

exhibited considerable stimulation on addition of the pyridoxal-P. Table 3 shows the large stimulation obtained after 22 days in this temperature region. Fifty per cent of the original activity was recovered upon addition of the coenzyme. Without the added cofactor the preparation was almost totally inactive. The dialyzed enzyme did not show this aging effect. It appears that the $(NH_4)_2SO_4$ remaining before dialysis can resolve the enzyme and bound

TABLE 3. STIMULATION OF AGED BROCCOLI AS PREPARATIONS BY PYRIDOXAL-P

Days	Pyruvate (μmole) −pyridoxal-P	+pyridoxal-P
0	2·5	3·3
6	1·1	2·9
22	0·2	1·7

Reaction mixture was the same as that in Table 2, except that pyridoxal-P was not added where indicated. 1·8 mg of protein was present in each case.

coenzyme. Inhibition experiments also indicated that pyridoxal-P was the natural cofactor. Studies with KCN and hydroxylamine revealed that both of these were extremely effective inhibitors of lyase activity. KCN at a final concentration of 5×10^{-4} *M* gave almost complete inhibition. Hydroxylamine was even more effective since 5×10^{-5} *M* inhibited over 90 per cent. Isonicotinic acid hydrazide did not have as great an effect giving only 50 per cent inhibition at a concentration of 5×10^{-3} *M*.

Products of the reaction

The 2,4 dinitrophenylhydrazine assay is non-specific under these assay conditions as to the carbonyl compound measured. The product was shown to be an α-keto acid by the fact that an assay using D-lactate : NAD oxidoreductase and $NADH_2$ gave equivalent results to the dinitrophenylhydrazine procedure. Rigorous proof that pyruvic acid was a product of the reaction came by isolating the 2,4 dinitrophenylhydrazone of pyruvic acid from a large scale reaction mixture. This mixture consisted of 5 mmole of sodium borate at pH 8·4, 1 mmole L-3-methysulphinylalanine, 2·5 μmole pyridoxal-P, and 78 mg DAS with distilled water to a final volume of 100 ml. The mixture was incubated with constant stirring at room temperature and aliquots removed at intervals for dinitrophenylhydrazine assay. After 3 hr the reaction had reached completion. Fifty ml of 10% trichloroacetic acid was added, the precipitate removed by centrifugation, and 200 ml of a saturated solution of 2,4-dinitrophenylhydrazine in 2 *N* HCl added. This was left at refrigerator temperature for several days, the precipitate collected by filtration and dried over P_2O_5 *in vacuuo*. The dried precipitate was recrystallized from ethanol–water. One hundred and five mg of product were obtained. Found: C, 40·3; H, 3·3; N, 20·5. Calc. for $C_9H_8O_6N_4$: C, 40·3; H, 3·0; N, 20·9%. Comparison of the infra-red spectra of the isolated product and an authentic sample of pyruvate 2,4-dinitrophenylhydrazone showed complete identity. Using L-3-propylsulphinylalanine as substrate, the equivalence of pyruvate formation and ammonia production is shown in Table 4. The colorimetric test for alkyl thioalkane sulfinates described by Schwimmer *et al.*[4] was carried out on aliquots of the same reaction mixture after addition of the trichloroacetic acid. The propyl derivative was used since the thiosulfinate produced is more stable than that produced by L-3-methylsulphinylalanine. A positive test for the formation of S-propylthiopropane-1-sulphinate was obtained.

Specificity of the enzyme

Several L-3-alkylsulphinylalanines were tested as substrates for the broccoli lyase. Serine and threonine were also tested and showed no activity. L-2-amino-4-methylsulphinylbutyric acid (L-methionine sulfoxide) was not a substrate, and in equimolar amounts with L-3-methylsulphinylalanine was only very slightly inhibitory. The ability of S-methyl cysteine to act as a substrate or inhibitor was examined. More highly oxidized cysteine derivatives such as L-cysteic acid and L-cysteine sulfinic acid were also investigated as to substrate or inhibitory properties. Table 5 shows the results of these experiments. It is obvious that only alkylsulphinyl derivatives will act as substrates. An equimolar amount of S-methyl cysteine does not inhibit the utilization of the sulphinyl compound to any great extent. However, more highly oxidized sulfur derivatives of cysteine appear to have a significant effect on the decomposition of the alkylsulphinyl derivative.

TABLE 4. BALANCE STUDIES ON ENZYME REACTION PRODUCTS

Time (min)	Pyruvate (μmole)	Ammonia (μmole)
30	1·6	1·8
60	2·4	2·3

Reaction mixture was the same as described in Table 2. 0·8 mg protein was used as the enzyme. A 2 ml aliquot of the solution after trichloroacetic acid addition was taken for the ammonia determination.

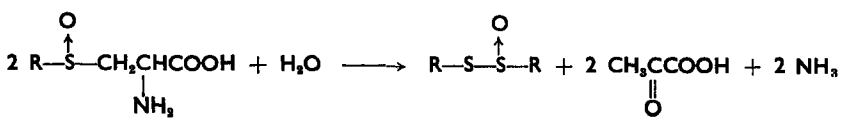
TABLE 5. SUBSTRATE SPECIFICITY OF BROCCOLI LYASE

Substrate	Relative activity
L-3-methyl-sulphinylalanine	100
L-3-ethyl-sulphinylalanine	140
L-3-propyl-sulphinylalanine	128
L-3-butyl-sulphinylalanine	105
L-3-allyl-sulphinylalanine	115
L-3-benzyl-sulphinylalanine	138
S-methyl-L-cysteine	0
L-cysteic acid	0
L-cysteine sulfinic acid	0
L-3-methylsulphinylalanine+S-methyl-L-cysteine	89
L-3-methylsulphinylalanine+L-cysteic acid	67
L-3-methylsulphinylalanine+L-cysteine sulfinic acid	61

Reaction mixture was the same as that described in Table 2 except 10 μmole of the listed substrates were used in every case.

DISCUSSION

The overall enzymatic breakdown of L-3-alkylsulphinylalanines (substituted cysteine sulfoxides) by broccoli lyase can be depicted as follows:



The products are the same as those given by the enzyme alliinase with the same substrates. The C–S lyase of *Albizzia lophanta* will act on these substrates to give pyruvate and ammonia,

but the sulfur-containing moiety produced has not been described.[8] The latter enzyme differs from broccoli lyase and alliinase in being able to utilize the thioether as readily as the sulfoxide. Alliinase from garlic has a broad pH optimum from pH 5 to pH 8.[3] The enzyme from *Allium bakeri* and *A. odorum* has an optimum activity between pH 5·6 and 6·4.[9] Since in this pH range the cruciferous enzyme is inactive, this could explain the previous reports of its absence in this family.[1,6]

From the evidence that pyridoxal-P is the coenzyme and pyruvate and NH_3 are products the reaction mechanism would appear to be an α, β elimination which has been discussed by Snell[10] with reference to the serine dehydrase and cysteine desulfhydrase reactions.

From an evolutionary viewpoint it is interesting that two such widely separated families, viz. Liliaceae and Cruciferae, have the same unique class of free amino acids as constituents and have developed a similar mechanism for their metabolism. The failure to demonstrate a similar enzyme in radish and *I. uniformum* could be a reflection on the methodology rather than an indication of another route of metabolism or the inert nature of these compounds in these plants.

EXPERIMENTAL

Chemicals

The L-3-methyl-ethyl, and butyl-sulphinylalanine derivatives were purchased from Calbiochem. The benzyl derivative was a product of Nutritional Biochemical Co. S-propyl-L-cysteine and the corresponding sulfoxide were a gift from Dr. J. F. Carson. S-allyl-L-cysteine was prepared by the method of Theodoropoulos.[11] The alkylsulphinylalanines, sulfoxides corresponding to the above cysteine derivatives, were prepared from the latter by oxidation with acid hydrogen peroxide and recrystallization from ethanol–water solutions. Pyridoxal-5′-phosphate was a product of Sigma Chemical Company.

Analytical procedures

Protein was determined by a spectrophotometric assay.[12] Pyruvate was assayed by the total keto acid method of Friedemann and Haugen.[13] Ammonia production was measured by microdiffusion followed by nesslerization. The determination of alkylthio alkane sulfinates was made qualitatively by the adaptation of Schwimmer *et al.*[4] of the method of Carson and Wong.[14]

Preparation of acetone powders

The plant materials were purchased customarily at local markets. Garlic bulbs were a gift from Dr. Louis Mann. Except for garlic the procedure was as follows: All operations were carried out in the cold. The washed plant material, usually 250 to 300 g, was cut into small pieces and blended in a Waring blendor for 1–2 min in a solution 0·3 *M* to sucrose and 0·2 *M* to sodium phosphate pH 7·0. Enough solution was used to allow smooth blending of the plant material at 70–90 V. The blended mixture was strained through two layers of cheesecloth and centrifuged at $1000 \times g$ for 5 min. The sediment was discarded and solid $(NH_4)_2SO_4$ added to the homogenate to 75 per cent saturation. The precipitate was

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[13] T. E. FRIEDEMANN and G. E. HAUGEN, *J. Biol. Chem.* **147**, 415 (1943).
[14] J. F. CARSON and F. F. WONG, *Nature* **183**, 1673 (1959).

collected by centrifugation and resuspended in cold, distilled water. This suspension was clarified by centrifugation and 4 vol of cold (−20°) acetone added slowly with stirring. The precipitate was collected by filtration and dried in vacuo over P_2O_5. Garlic acetone powder was prepared in the same manner except that after centrifuging the homogenate at $1000 \times g$ the supernatant solution was filtered by use of a filter aid and vacuum filtration. The filtrate was then treated with $(NH_4)_2SO_4$ as above. After resuspension in distilled water and centrifugation, the protein solution obtained was dialyzed against 0·02 *M* K_2HPO_4 for 7 hr with several changes of dialyzing medium. The dialyzed solution was then treated with acetone as described before.

Incubation procedure for acetone powder activity

Enough powder was used to make a 10–20 per cent suspension in 0·02 *M* phosphate buffer pH 7·0 and extracted for several minutes in the cold. The suspension was centrifuged and the supernatant solution tested for enzyme activity. The activity was assayed at pH 10 in every case and also at pH 7 in some instances. The complete reaction mixture consisted of 100 μmole sodium borate pH 10 or phosphate pH 7, 5 μmole L-3-methylsulphinylalanine (S-methyl-L-cysteine sulfoxide), 0·2 μmole pyridoxal-P, enzyme solution, and distilled water to a final volume of 2 ml. The reaction mixture was incubated for 30 min at room temperature with occasional shaking, and was terminated by the addition of 3 ml 10% trichloracetic acid. After centrifugation, aliquots of the supernatant solution were assayed for pyruvic acid.

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