Purification of the Alliin Lyase of Garlic, *Allium sativum* L.

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1. Alliin lyase (EC 4.4.1.4) was purified up to sevenfold from garlic-bulb homogenates. The enzyme was unstable to storage at -10° , particularly in dilute concentrations, but the addition of glycerol (final concentration 10%, v/v) stabilized the activity completely for at least 30 days. 2. The purified enzyme had an optimum pH for activity at 6.5. The addition of pyridoxal phosphate stimulated the reaction rate and the stimulation became more marked as the purification proceeded. 3. Hydroxylamine ($10\mu\text{M}$) and cysteine (0.5mM) inhibited the enzyme activity by more than 80%. Spectral studies indicated that cysteine reacted with pyridoxal phosphate bound to the protein. 4. The K_m values for *S*-methyl-, *S*-ethyl-, *S*-propyl-, *S*-butyl- and *S*-allyl-L-cysteine sulfoxides were determined. With *S*-allyl-L-cysteine sulfoxide the K_m was 6mM and the V_{max} was greater than those with the other substrates tested. 5. The thioether analogues of the substrates were competitive inhibitors for the lyase reaction. The K_i decreased with increasing chain length of the alkyl substituent. With *S*-ethyl-L-cysteine sulfoxide as substrate the K_i was 33, 8 and 5mM respectively for *S*-methyl-, *S*-ethyl- and *S*-propyl-L-cysteine. 6. The addition of EDTA or Mg^{2+} , Mn^{2+} , Co^{2+} or Fe^{2+} stimulated the reaction rate. Other bivalent cations either had no effect or gave a strong inhibition. In the presence of EDTA no further increase of activity was observed with added Mg^{2+} .

S-Alkyl-L-cysteine sulfoxides are constituents of the non-protein amino acid pool of a number of species of the Liliaceae and Cruciferae (Fowden, 1964; Virtanen, 1965). The first to be identified was alliin (*S*-allyl-L-cysteine sulfoxide) from garlic, *Allium sativum* L. (Stoll & Seebeck, 1951). The same workers also showed that an enzyme was present in the garlic tissue that degraded alliin to allicin, pyruvate and ammonia according to the reaction shown in Scheme 1. This enzyme, alliin alkylsulphenate lyase (EC 4.4.1.4), is known trivially as alliin lyase or alliinase.

Subsequently enzymes that can catalyse the same

reaction have been found in other *Allium* species (Tsuno, 1958; Schwimmer, Carson, Makower, Mazelis & Wong, 1960; Kupiecki & Virtanen, 1960), in *Tulbaghia violaceae* (Jacobsen, 1965) and in a number of *Brassica* species (Mazelis, 1963). The enzymes from onion, *Allium cepa* (Schwimmer & Mazelis, 1963; Kupiecki & Virtanen, 1960), *T. violaceae* and *B. oleracea* have been studied in some detail. However, the enzyme in garlic has not been well characterized, though this was the first source in which the activity was described. The observations by Stoll & Seebeck (1951) were made with a crude cell-free fraction. Later Goryachenkova

Scheme 1.

(1952) showed that the garlic enzyme was stimulated by the addition of pyridoxal phosphate. The properties of the enzyme from onion must be different from that from garlic, since they differ markedly in optimum pH for the reaction (8.5 for onion, 6.5 for garlic).

The present paper describes the properties of partially purified alliin lyase from garlic.

EXPERIMENTAL

Chemicals. *S*-Methyl-, *S*-ethyl- and *S*-butyl-L-cysteine were purchased from Calbiochem, Los Angeles, Calif., U.S.A. *S*-Allyl-L-cysteine was prepared by the method of Theodoropoulos (1959). *S*-Propyl-L-cysteine and its sulphoxide were a gift from Dr J. F. Carson. The sulphoxides were prepared from the thioethers by oxidation with acid H_2O_2 and recrystallization from ethanol-water solutions (Stoll & Seebeck, 1951). Pyridoxal 5'-phosphate was a product of Sigma Chemical Co., St Louis, Mo., U.S.A. DEAE-Sephadex and Sephadex G-75 and G-200 were obtained from Pharmacia, Uppsala, Sweden.

Analytical procedures. Protein was determined by the procedure of Lowry, Rosebrough, Farr & Randall (1951). Pyruvate was assayed by the total keto acid method of Friedemann & Haugen (1943).

Enzyme assay. The standard reaction mixture consisted of the following in the final concentrations given: sodium-potassium phosphate buffer, pH 6.5 (made by combining 0.2M- NaH_2PO_4 with 0.2M- K_2HPO_4 until the pH was 6.5), 0.1M; pyridoxal 5'-phosphate, 25 μM ; *S*-alkyl-L-cysteine sulphoxide, 10–60mM. The final volume was 2ml., and 10–100 μg . of enzyme protein was used. The reaction mixture was incubated at room temperature (23°) for the desired time, usually 5 or 10min., and then terminated by the addition of 3ml. of 10% (w/v) trichloroacetic acid. Samples of this final solution were removed for the assay of pyruvate.

One unit of enzyme activity was defined as that which produced 1 μmole of pyruvate/min. at 23°.

Plant material. Garlic bulbs were purchased from local markets as needed. The bulbs were sometimes stored in a refrigerator for up to several weeks.

RESULTS

Purification of enzyme. A typical preparation is summarized in Table 1. In this instance 170g. of

garlic bulb (with the loose dead outer leaves removed) was blended for 1min. in 230ml. of cold 0.02M-sodium-potassium phosphate buffer, pH 7.5, containing 10% (v/v) of glycerol. The homogenized material was strained through several layers of cheesecloth. All further operations on the strained homogenate were carried out in the cold.

The homogenate was centrifuged for 30min. at 25000g and the supernatant solution decanted. Then 15ml. of protamine sulphate solution (1%, w/v) was added/100ml. of supernatant fluid, and the precipitate that formed in 10min. was removed by centrifugation. Solid ammonium sulphate was added to the supernatant to give 35% saturation, and the precipitate was collected by centrifugation and suspended in the above phosphate-glycerol solution. The suspension was clarified by centrifugation.

Fig. 1. Chromatographic elution pattern of the 0–35%-saturated $(\text{NH}_4)_2\text{SO}_4$ fraction (fraction III) obtained with a Sephadex G-200 column. The details of the elution are given in the text. $\circ$, Protein (E_{280}); $\bullet$, specific activity (units/mg. of protein). The substrate was *S*-methyl-L-cysteine sulphoxide (10mM).

Table 1. *Purification of garlic alliin lyase*

The reaction mixture was the standard one described in the Experimental section. The substrate was 10mM-*S*-methyl-L-cysteine sulphoxide and incubation was at room temperature for 10min.; 1 unit of enzyme formed 1 μmole of pyruvate/min.

Fraction		Volume (ml.)	Total protein (mg.)	Total activity (units)	Specific activity (units/mg.)	Recovery (%)	Purification
I	Centrifuged homogenate	210	3066	19622	6.4	(100)	(1.0)
II	Protamine supernatant	220	2728	19642	7.2	100	1.1
III	0–35%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction	40	1600	13920	8.7	71	1.4
IV	Sephadex G-200 filtrate	150	233	8750	37.6	45	5.9

Further purification was achieved by passing 5ml. samples of the enzyme through a 2.5cm. $\times$ 40cm. Sephadex G-200 column that had been equilibrated with phosphate-glycerol solution. The protein was eluted with the same buffer. Fractions (approx. 3ml. each) were collected and those having specific activities at least twice that of the starting material were combined. A typical elution is shown in Fig. 1. The recovery shown in Table 1 is the sum of all the fractions that met this criterion. A 5.9-fold purification with 45% recovery was obtained. However, individual tubes from the gel filtration had specific activities of 47, equivalent to more than sevenfold purification. Further treatment by DEAE-Sephadex, CM-Sephadex, isoelectric precipitation, ammonium sulphate fractionation or calcium phosphate gel adsorption gave no further purification.

The 0-35%-saturated ammonium sulphate fraction could, however, be further purified by the use of DEAE-Sephadex (Fig. 2). The specific activities of the active fractions were similar to those from the Sephadex G-200 filtration. If the combined active fractions from the Sephadex G-200 column

were placed on a DEAE-Sephadex column, the activity was eluted as one major peak with a long trailing edge and without significant improvement in purification.

Stabilizing effect of glycerol. Dilute solutions of the 0-35%-saturated ammonium sulphate fraction in 0.02M-phosphate buffer, pH 7.5, lost their activity rapidly even on storage at -10° . A concentrated solution lost its activity more slowly, but even then after 57 days at -10° more than 50% of the original activity was lost.

The presence of glycerol (10%, v/v) appeared to stabilize the activity almost completely: after 30 days at -10° no loss in activity was observed. Enzyme preparations that were diluted with phosphate-glycerol solution could be stored, and thawed and refrozen many times, without loss of activity.

The stabilizing effect of glycerol was also indicated when the enzyme was eluted from a DEAE-Sephadex column with a 0.05-0.5M-phosphate buffer gradient at pH 7.0 or with a similar phosphate buffer gradient containing 10% (v/v) of glycerol. In the absence of glycerol the highest specific activity recovered was only one-half to one-third that of the original enzyme solution, whereas in the presence of glycerol it was more than twice that of the starting material.

Optimum pH. The pH at which the optimum activity occurred was 6.5-6.7 with tris-maleate or phosphate as buffer (Fig. 3). When EDTA was used as the buffer the rate was greater than with phosphate or tris-maleate buffer, but the optimum remained near pH 6.5.

Fig. 2. Elution pattern of fraction III from a DEAE-Sephadex column. A 5ml. sample of fraction III was put on a 2.5cm. $\times$ 40cm. column and eluted with a linear gradient from 10% glycerol-0.05M-phosphate buffer, pH 7.0, to 10% glycerol-0.5M-phosphate buffer, pH 7.0. Fractions (approx. 3ml.) were collected. $\circ$, Protein (E_{280}); $\bullet$, specific activity (units/mg. of protein). The substrate was *S*-methyl-L-cysteine sulfoxide (10mM) and incubation was for 10min.

Fig. 3. Effect of pH on the reaction rate. The reaction mixture composition was that described in the Experimental section except for the buffer indicated: $\circ$, tris-maleate buffer; $\bullet$, sodium-potassium phosphate buffer. The substrate was *S*-methyl-L-cysteine sulfoxide (10mM) and incubation was for 10min. at room temperature with 22 μ g. of fraction IV.

Effect of enzyme concentration and incubation time on rate of reaction. The reaction rate was proportional to the amount of enzyme added. The rate was approximately linear with time only during the first 10 min. The rapid onset of a non-linear rate appeared to be due to the inactivation of the enzyme, since on the addition of fresh enzyme the reaction once more resumed its initial rate. The inactivation appeared to be related to the absolute amount of pyruvate formed, with the result that the fraction of substrate utilized depended on its initial concentration.

Requirement for pyridoxal phosphate. It has been suggested that pyridoxal phosphate is probably a coenzyme for the alliin lyase reaction (Goryachenkova, 1952) on the basis of inhibition studies with hydroxylamine, the relief of this inhibition by added pyridoxal phosphate and the use of ultraviolet radiation to destroy the bound coenzyme. In our preparations added pyridoxal phosphate stimulated activity to an extent that became greater as the purification proceeded. Thus added pyridoxal phosphate (final concentration 25 μ M) stimulated the activity of fraction III enzyme (see Table 1) by only 25% and that of fraction IV enzyme by more than 70%. Further addition of pyridoxal phosphate had no increasing stimulatory effect.

The alliin lyase was sensitive to preincubation for 10 min. with added carbonyl reagents such as hydroxylamine or cyanide: 10 μ M-hydroxylamine inhibited the reaction by 84% and 1 mM-sodium cyanide inhibited it by 70%.

Another extremely effective inhibitor of the reaction was L-cysteine: inhibition of over 80% was obtained on preincubation of the enzyme with 0.5 mM-L-cysteine. L-Cysteine is known to react with pyridoxal phosphate to form a thiazolidine.

Fig. 4. Spectra of a solution of fraction IV enzyme (1.2 mg. of protein/ml. in 10% glycerol-0.02M-phosphate buffer, pH 7.5) in the presence (----) and absence (—) of 1 mM-L-cysteine.

Proteins that contain bound pyridoxal phosphate have an absorption maximum at about 415 m μ (Schirch & Mason, 1962). In the presence of L-cysteine the pyridoxal phosphate forms a thiazolidine with the cysteine, the 415 m μ peak is lost and a new maximum at about 340 m μ is produced. Fig. 4 shows the spectrum of the fraction IV protein and the change that occurs almost instantaneously on the addition of 1 mM-L-cysteine. There appears to be little doubt that fraction IV enzyme has endogenous pyridoxal phosphate attached to the protein.

Michaelis constants. Stoll & Seebeck (1951) showed that a large number of *S*-alkyl-L-cysteine sulfoxides could be cleaved by the lyase, and that the natural isomer, (+) - *S* - allyl - L - cysteine sulfoxide, was acted on most rapidly. The (–)-isomer was also a substrate, but was cleaved at a much lower rate. Synthesized alkyl-L-cysteine sulfoxides have a racemic sulphur atom [50% (+) and 50% (–); Stoll & Seebeck, 1951] so that the measured rate of cleavage with such substrates is a combination of the rates at which the (+)- and (–)-sulfoxides are utilized. We have determined the apparent K_m values for ($\pm$)-*S*-methyl-, *S*-ethyl-, *S*-propyl-, *S*-butyl- and *S*-allyl-L-cysteine sulfoxides from plots of the reciprocals of velocity and substrate concentration. The V_{max} with each substrate under identical conditions was also calculated. The results are summarized in Table 2. The values for V_{max} confirm the findings of Stoll & Seebeck (1951) on the rate of cleavage of the allyl derivatives compared with that of the other alkyl derivatives.

Inhibition by thioether analogues. The onion alliin lyase is known to be inhibited by *S*-alkyl-L-cysteines in a partially competitive fashion (Schwimmer, Ryan & Wong, 1964). Similar studies were made with the garlic enzyme. The inhibition of the cleavage of *S*-methyl-L-cysteine sulfoxide and of *S*-ethyl-L-cysteine sulfoxide by *S*-ethyl-L-cysteine was examined. The inverse plots of velocity against sulfoxide concentration in the presence and absence of the thioether showed that the inhibition was apparently competitive. However, the K_i calculated for *S*-ethyl-L-cysteine with *S*-methyl-L-

Table 2. Values of K_m and V_{max} for cleavage of various ($\pm$)-*S*-alkyl-L-cysteine sulfoxides

Alkyl group	K_m (mM)	V_{max} (units/mg. of protein)
Methyl	15	76
Ethyl	6	86
Propyl	3	30
Butyl	5	50
Allyl	6	123

cysteine sulfoxide as substrate was 18mM, and with *S*-ethyl-L-cysteine sulfoxide as substrate it was 8mM. The inhibition appears to be only partially competitive, as with the onion enzyme. The K_i values for *S*-methyl-L-cysteine and *S*-propyl-L-cysteine with *S*-ethyl-L-cysteine sulfoxide as substrate were determined by measuring the velocity of the reaction with a series of inhibitor concentrations at constant substrate concentration. Since the inhibitor is competitive and the K_m had previously been determined, a graphical determination could be made of K_i by the method of Dixon (Dixon & Webb, 1964) and is shown in Fig. 5. The K_i values for *S*-methyl-L-cysteine and *S*-propyl-L-cysteine were 33mM and 5mM respectively.

Effect of EDTA, Mg^{2+} and other cations. It was mentioned above that when EDTA was used as the buffer there was a stimulation of enzyme activity, but no change in the optimum pH. This was examined further by comparing the rate of the reaction with incubation mixtures containing 0.1 M-phosphate buffer, 0.1 M-EDTA or 5mM-EDTA in 0.1 M-phosphate buffer, final pH 6.5–6.7. With *S*-methyl-L-cysteine sulfoxide as substrate the relative rates were 100, 113 and 121 respectively. The effect of EDTA therefore seemed to be related, not to its buffering ability, but to some other attribute.

The effect of the addition of 5 μ moles of various bivalent cations (as the chloride or sulphate) to the reaction mixture was tested: Fe^{2+} , Mg^{2+} , Mn^{2+} , and Co^{2+} gave stimulations of 27–35%; Cd^{2+} and Ca^{2+} were without effect; Ni^{2+} inhibited by 33% and Cu^{2+} by 52%.

Fig. 5. Determination of the K_i values for *S*-methyl-L-cysteine (●) and *S*-propyl-L-cysteine (○) with *S*-ethyl-L-cysteine sulfoxide as substrate. The reaction mixture was as described in the text with 30mM-substrate and various amounts of the thioethers added as indicated. The reaction time was 5 min. at room temperature. The enzyme used was 10 μ g. of fraction IV protein, which was preincubated with the thioether in the reaction mixture at room temperature for 10 min. before the reaction was started by the addition of substrate. The reaction was then terminated after 5 min.

The effect of jointly adding EDTA and Mg^{2+} was investigated with *S*-ethyl-L-cysteine sulfoxide as substrate. The addition of EDTA (5mM), magnesium chloride (5mM) or EDTA (5mM) plus magnesium chloride (5mM) increased the rate by 34, 26 and 34% respectively compared with a control system.

In the absence of added pyridoxal phosphate freshly diluted enzyme preparations were much more strongly stimulated by EDTA or Mg^{2+} . The rate of reaction was almost doubled compared with the control reaction mixture without EDTA or Mg^{2+} . On storage, even at 0°, this effect was diminished.

DISCUSSION

A number of alliin lyases have now been reported from different higher-plant species and in some instances studied in some detail. The enzyme has so far been found restricted to *Allium* species and *Tulbaghia violaceae* in the Liliaceae and to *Brassica* species in the Cruciferae. A lyase has been purified from *Albizzia lophanta* (Schwimmer & Kjaer, 1960) that will cleave alkylcysteine sulfoxides, but this enzyme utilizes the thioether at a much better rate and appears to be much less specific than the alliin lyases mentioned above. Comparison of the properties of the alliin lyases from different sources shows a division into two distinct classes based on pH optima for activity (Jacobsen, 1965). The enzymes from *A. sativum*, *A. odorum*, *A. bakerii* and *T. violaceae* have pH optima from pH 5.6 to 6.5. The lyases from *A. cepa* and *Brassica* species show an optimum activity at pH 8.5. Assuming that the pK of the protonated amino group is 9.5 (which is reasonable for α -amino acids), then even at pH 8.5 90% of the substrate would be in the zwitterion form. Thus the difference in pH optima must be concerned with the charge at the binding site or the maintenance of the correct conformation of the protein rather than the ionic species of the substrate.

Kinetic comparisons between the garlic enzyme and that from onion show considerable similarity, despite the large difference in pH optimum. Table 3

Table 3. Comparison of K_m values for various ($\pm$)-*S*-alkyl-L-cysteine sulfoxides as substrates for garlic and onion alliin lyase

Values for the onion enzyme are from Schwimmer *et al.* (1964).

Alkyl group	K_m (mM)	
	Garlic enzyme	Onion enzyme
Methyl	15	16.6
Ethyl	6	5.7
Propyl	3	3.8
Butyl	5	4.7

compares the K_m values for various cysteine sulfoxide derivatives for the garlic enzyme with those obtained by Schwimmer *et al.* (1964) for the onion enzyme. The K_m values are essentially identical. The onion enzyme is also inhibited by the thioether analogues of the substrate, but the K_i values are considerably smaller than those for the garlic enzyme. Thus with *S*-ethyl-L-cysteine sulfoxide as substrate the K_i values for *S*-methyl-L-cysteine were 2.6 and 33 mM for the onion and the garlic enzyme respectively, and those for *S*-ethyl-L-cysteine were 1.9 and 8 mM for the onion and the garlic enzyme respectively.

Stimulation of activity with Mg^{2+} is in accord with the findings by Tsuno (1958) with other *Allium* species. Similarly the stimulation by EDTA is in agreement with the results found with other chelating agents and the onion enzyme (Schwimmer & Mazelis, 1963). Our present findings indicate that the above stimulation effects are related. It is possible that EDTA stimulates by removing trace heavy-metal ions in the enzyme preparations or reaction mixture, and that Mg^{2+} acts by displacing such ions from sensitive regions of the protein.

It is clear that the presence of glycerol during the isolation of the enzyme is beneficial. A number of enzymes, some of them cold-labile, have now been found to be stabilized or protected by the presence of glycerol (Jarabak, Seeds & Talalay, 1966). In preliminary work on purification of the onion alliin lyase there is a similar requirement for glycerol in the isolation medium for the retention of enzyme activity in dilute solutions (J. Brosin & M. Mazelis, unpublished work).

Since *A. sativum* and *A. cepa* are very close

taxonomically further purification and study of their markedly different alliin lyases might prove of value in understanding species adaptation at the molecular level.

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