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Cleavage of Alkyl Cysteine Sulphoxides by an Enzyme in Onion (*Allium cepa*)

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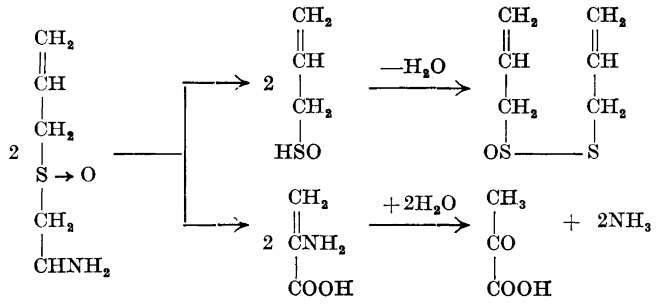
An enzyme which is specific for alkyl cysteine sulphoxides has been partially purified from onion. The enzyme appears to require pyridoxal phosphate as a cofactor, but shows no stimulation in activity by various metals. It shows a very low order of inhibition by various sulphydryl group reagents. Electrophoresis shows a single protein peak at two pH levels.

The first report of the enzymatic degradation of an alkyl cysteine sulphoxide was made by Stoll and Seebeck [1]. They have shown that an enzyme in garlic (*Allium sativum*) catalyzed the splitting of alliin (allyl cysteine sulphoxide) to pyruvic acid, ammonia, and allicin according to the following equation:



These authors postulated that the enzyme, alliinase, brought about the initial cleavage and that the primary products were split spontaneously to allicin, pyruvic acid, and ammonia.

Of the alkyl cysteine sulphoxides occurring naturally, methyl cysteine sulphoxide appears to have the widest distribution [2]. It occurs in several members of the *Liliaceae* and *Cruciferae* families as well as sporadically in the families *Compositae* (in *Lactuca sativa* L.), *Umbelliferae* (in *Cryptotaenia japonica* Hasskare) and *Leguminosae* (*Phaseolus vulgaris* L.) [3]. Propyl- and allyl

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cysteine sulphoxides have been reported only in *Liliaceae*, where they are less widely distributed than is the methyl homologue. Ethyl cysteine sulphoxide has thus far been reported only in *Ipheon uniformum*, a member of *Liliaceae*. The reported occurrence of specific alkyl cysteine sulphoxide splitting enzymes is restricted to the *Liliaceae* family.

The known occurrence in appreciable quantities of methyl- and propyl cysteine sulphoxides in onion [4], and the release of pyruvic acid in fresh onion extracts [5,6] has led us to choose this plant for studying the cleavage of alkyl cysteine sulphoxides. The following is a report on the partial purification of the enzyme and some of its properties.

EXPERIMENTAL

Preparation of enzyme. All steps were carried out at 0° unless otherwise indicated. 800 g of mature onions freed of skins, tops, and roots were homogenized in a "Waring type" blendor with 400 ml of 0.10 M potassium phosphate, pH 7.4, for 3 min in four equal portions. The thick suspension was stirred for 30 min and filtered through cheese cloth. The filtrate was centrifuged at 12 000 × *g* for 15 min. 40 mg per ml of charcoal was added to the bright yellow solution. After stirring for 15 min the charcoal was removed by centrifugation. This step removes a large amount of the coloured non-protein material as well as about one-half of the proteins. The resulting supernatant solution is almost colourless.

Calcium phosphate gel [7] (1 mg for each 3 ml of solution) was added and after stirring 15 min the suspension was centrifuged. There is only a small increase in specific activity following this step, but more of the non-protein material is removed and the solution becomes colourless.

The enzyme solution was brought to 0.8 saturation by the addition of solid ammonium sulphate during a 20 min period. After stirring 45 min Hyflo Super Cel (1 g per each 200 g of onion) was added and stirring was continued 15 min longer. The precipitate was removed by centrifugation and the Hyflo was washed twice with 0.05 M potassium phosphate pH 7.4 to give a final protein concentration of 30—35 mg per ml. The enzyme solution was brought to 0.40 saturation with ammonium sulphate, stirred 30 min and centrifuged. The precipitate was dissolved in the same phosphate buffer as above to give a solution containing 40—45 mg protein per ml. This was again treated with ammonium sulphate. The fraction obtained at 0.25 saturation was discarded, the solution was brought to 0.60 saturation and the precipitate was dissolved in 0.05 M potassium phosphate, pH 7.4. The resulting enzyme solution was clear and bright yellow. The enzyme is stable at all stages of purification to storage at −20° for several weeks. All fractions, except the extract, retain almost full activity when stored at 0° for 1 to 2 weeks. Protein concentration of the enzyme solutions was determined spectrophotometrically by light absorption at 280 and 260 mμ [8].

Enzyme assay: The enzyme was assayed by measuring the amount of pyruvic acid liberated according to the method of Friedemann and Haugen [9]. The standard assay system contained 125 μmoles of potassium phosphate buffer, pH 7.4, 2.5 μmoles $MgCl_2$, 25 μg of pyridoxal phosphate, 13 μmoles alliin (or other alkyl cysteine sulphoxide), and enzyme in a final volume of 1.0 ml. All components except the substrate were preincubated 10 min at 20°. Addition of substrate was followed by incubation at 20° for 20 min. A control tube without substrate was included for each sample of enzyme. No pyruvate was generated from the substrate in the absence of enzyme. 0.25 ml of 40 % TCA was added after incubation and 0.40 ml aliquots were used in the determination of pyruvic acid. A unit of enzyme is defined as that amount which yields 1 μmole of pyruvic acid under the assay conditions. The specific activity of the enzyme is expressed as the number of units per mg of protein.

RESULTS

Electrophoresis. The enzyme solution was subjected to electrophoresis at two stages of preparation and at pH 6.8 and 7.8. The apparatus was the Perkin-

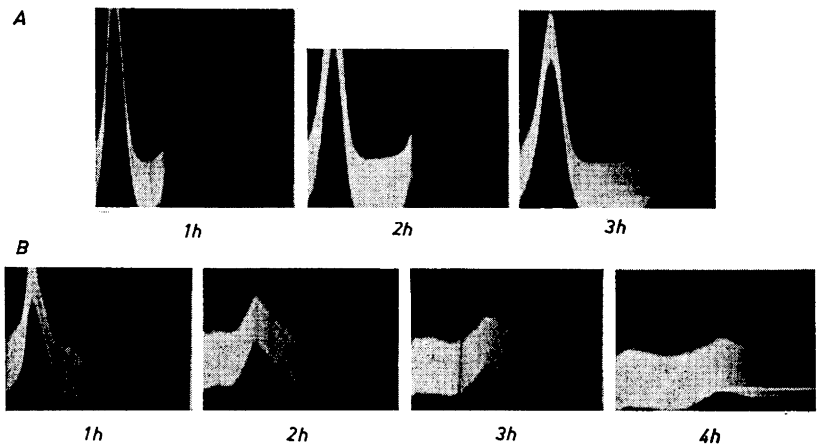

Fig. 1. Electrophoresis patterns. A shows the pattern obtained after calcium phosphate gel treatment (see text), B after 0.25—0.60 ammonium sulphate precipitation.

Elmer Model 38. Fig. 1A shows the pattern given after calcium phosphate gel treatment. To concentrate the protein by a factor of 10 the enzyme was subjected to lyophilization, dialyzed against distilled water and lyophilized again before dialysis against the buffer used for electrophoresis. The fraction obtained at 0.25—0.60 saturation with ammonium sulphate was also subjected to electrophoresis. Both preparations showed similar results at both pH's. The calcium phosphate gel supernate, however, contained a second peak which did not move at either pH. Fig. 1B shows that this peak was removed by precipitation with ammonium sulphate.

Enzyme activity as a function of pH. Fig. 2 shows that the enzyme is active over the range of pH 5.6 to 9.0, with maximal activity at pH 7.4.

Substrate specificity. Table 1 shows the various substrates that have been tested with the enzyme. As can be seen in the table only sulphoxides that are derived from cysteine are cleaved by the enzyme.

Inhibition by carbonyl group reagents. Table 2 gives the results obtained when the enzyme was tested with the three carbonyl group reagents: hydroxyl-

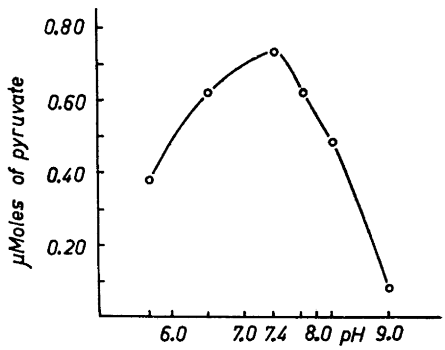

Fig. 2. Enzyme activity as a function of pH. The assay described in the text (with 250 µmoles of buffer) was used with 20 µg of the enzyme after treatment with charcoal. The substrate was synthetic methyl cysteine sulphoxide. Potassium phosphate buffers were used at pH 5.7, 6.5, 7.4, and 7.8. Tris buffer was used at pH 7.4, 8.2, and 9.0.

Table 1. Effect of carbonyl group reagents on enzymatic activity. The standard assay described in the text was used with some modifications. In experiments numbered 1 pyridoxal phosphate was omitted and the appropriate inhibitor was preincubated with all components except alliin for 20 min at 20°. In experiments numbered 2 the inhibitor was preincubated 10 min with the other components, the pyridoxal phosphate was added and preincubation was continued for 10 min. The order of preincubation of pyridoxal phosphate and inhibitor was reversed in experiments numbered 3. Pyridoxal phosphate concentration was 4×10^{-3} M.

No.	Substance added	Molarity of inhibitor	% inhib.	Molar conc. of inhibitor	% inhib.	Molar conc. of inhibitor	% inhib.
1	Hydroxylamine	10^{-6}	38	10^{-5}	75	10^{-4}	93
2	Hydroxylamine + pyridoxal—PO_4	10^{-6}	13	10^{-5}	58	10^{-4}	90
3	Pyridoxal—PO_4 hydroxylamine	10^{-6}	5	10^{-5}	38	10^{-4}	74
1	Hydrazine	10^{-6}	12	10^{-5}	25	10^{-4}	66
2	Hydrazine + pyridoxal—PO_4	10^{-6}	0	10^{-5}	17	10^{-4}	59
3	Hydrazine + pyridoxal—PO_4	10^{-6}	0	10^{-5}	2	10^{-4}	37
1	*Iso*nicotinic hydrazide	10^{-6}	0	10^{-5}	0	10^{-4}	0
2	*Iso*nicotinic hydrazide + pyr.—PO_4	10^{-6}	0	10^{-5}	0	10^{-4}	0
3	*Iso*nicotinic hydrazide + pyr.—PO_4	10^{-6}	0	10^{-5}	0	10^{-4}	0

amine, hydrazine, and *iso*nicotinic acid hydrazide, at three concentrations. At the concentrations used hydroxylamine was the most effective inhibitor. Hydrazine was somewhat less effective, while *iso*nicotinic acid hydrazide failed to show any inhibition at these concentrations.

Stimulation by pyridoxal phosphate. The stimulation due to pyridoxal phosphate is shown in Table 3. As can be seen in the table, very little increase in pyruvate generation is found when pyridoxal phosphate is added to the crude extract. The stimulation shown in other fractions is about 40 %. Dialysis reduces enzymatic activity to less than one-half (Expts. 4 and 5). The stimulation due to pyridoxal phosphate is, however, the same in each case.

The effect of sulphydryl group inhibitors on enzyme activity. The following inhibitors were tested at each of the molar concentrations, 10^{-6}, 10^{-5}, and 10^{-4}: *p*-chloromercuribenzoate, N-ethylmaleimide, iodosobenzoate; none inhibited at these concentrations. The latter two were also tested at a concentration of 10^{-2} M and were found to inhibit 30 % and 25 %, respectively. Iodoacetate and iodoacetamide failed to inhibit at 10^{-2} M but showed an inhibition of 25—30 % at 5×10^{-2} M. When corrected for an observed reaction with pyruvic acid, cyanide showed no inhibition even at 10^{-2} M while sulphide inhibited 30 % at this concentration.

Table 2. Specificity of enzyme. The standard assay described in the text was used except with benzyl cysteine sulphoxide. Because of the low solubility the substrate concentration was only half of the concentration used in the standard assay.

Substrate	μmoles of 2,4-dinitrophenylhydrazone
Alliin	2.49
Methyl cysteine sulphoxide	0.61
Ethyl » »	0.86
Propyl » »	0.53
i-Propyl » »	0.38
Benzyl » »	0.34
*Cyclo*alliin	0
Cysteine	0
Methyl cysteine	0
Ethyl »	0
Propyl »	0
Allyl »	0
S-Methylmethionine sulphonium bromide	0
S-Benzylmethionine » chloride	0
Thiazolidine carboxylic acid	0
Methionine sulphoxide	0
Methionine sulphone	0
Cysteine sulphinic acid	0

DISCUSSION

The experimental data presented here show the results of our attempts to purify the S-alkyl cysteine sulphoxide splitting enzyme. Renis [10] states that all attempts to purify the enzyme from onion were unsuccessful, although active crude extracts could be obtained in his laboratory. We have tried many of the usual procedures for enzyme purification, *e.g.* precipitation with ammonium sulphate, alcohol, or acetone. These procedures, in various combinations, together with gel absorption steps have given about 4—5 fold purification, but with serious losses in enzyme units. Dialysis was tried at various points in the purification and always resulted in a large loss of activity, only a part of

Table 3. The influence of pyridoxal phosphate on enzymatic activity. The standard assay was used except that pyridoxal phosphate was omitted from the experimental tube as well as the control tube (without substrate) where indicated.

Expt. No.	Enzyme fraction	μmoles pyruvate released		% Stimulation
		with pyridoxal—P	without pyridoxal—P	
1	Extract	1.09	0.99	10
2	Calcium PO_4 gel supernate	1.07	0.76	42
3	Extract	1.55	1.44	8
4	Dialyzed 0—0.40 $(NH_4)_2SO_4$ fraction	0.30	0.22	39
5	Undialyzed 0—0.40 $(NH_4)_2SO_4$ fraction	0.69	0.49	41

which could be restored with pyridoxal phosphate. Pretreatment of the extract with the relatively large amount of charcoal, as described, removes most of the interfering materials and makes some further purification possible. The series of steps described here results in preparations which have a specific activity about six times as great as the original extracts. However, the electrophoretic pattern of the most purified fraction and failure to increase the specific activity beyond 6 suggest that the purity of the enzyme may already be fairly high.

In all experiments reported here the Mg^{++} ion was used routinely, although no stimulation by this ion or Al^{+++}, Cu^{++}, Co^{++}, and Mn^{++} could be shown. Furthermore, a several fold excess of Versene failed to inhibit the reaction. The loss in units upon dialysis could be only partly regained by addition of pyridoxal phosphate. Addition of concentrated dialysate, boiled enzyme, or preparations from which protein was removed by alcohol did not further increase the activity.

The sharp pH optimum shown at pH 7.4 is in marked contrast to that reported by Stoll and Seebeck [1] for the alliin cleaving enzyme from garlic. Their enzyme was active between pH 4 and 9, but showed a broad optimum range from pH 5 to 8. Unlike the garlic enzyme, which could be precipitated at pH 4, the onion enzyme lost all its activity after this precipitation.

As shown in Table 2, the enzyme cleaves all the alkyl cysteine sulphoxides that were tested and alliin is the best substrate. This fact is somewhat surprising since alliin appears to be absent from onion. An alkyl cysteine sulphoxide splitting enzyme has recently been purified 200 fold from *Albizzia lophanta* by Schwimmer and Kjaer [11] but it splits the S-alkyl cysteines as well as the sulphoxides.

At the present time it is not possible to state conclusively whether one or more enzymes are involved in cleaving the sulphoxides to pyruvic acid, ammonia, and alkyl thiosulphinates. We have tried recombination of various fractions a number of times and have never found any indications that more than a single enzyme could be involved.

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