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AN ALKYL-CYSTEINE SULFOXIDE LYASE IN *TULBAGHIA VIOLACEA* AND ITS RELATION TO OTHER ALLIINASE-LIKE ENZYMES*

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Abstract—A carbon-sulfur lyase has been found in *Tulbaghia violacea* Harv. which is capable of acting on L-cysteine sulfoxides in a manner similar to the alliinase reaction described in the genus *Allium*. The enzyme has a substrate specificity similar to that of alliinase, and its activity is enhanced by pyridoxal phosphate. Cupric ion also stimulates its activity, but the enzyme does not appear to require copper as a co-factor. Amino acid sulfoxides found in the tissue give rise to pyruvate when treated with an enzymic preparation from *T. violacea*, indicating that the mechanism responsible for odor production in *Tulbaghia* is the same as that in *Allium*. Data are presented to support the classification of alliinase-like enzymes into two groups on the basis of optimal pH.

INTRODUCTION

Tulbaghia, a genus of plants of South African origin which is closely related morphologically to *Allium*,[1,2] contains species described as having garlic-like odors.[3–5] *Tulbaghia violacea* Harv., a strong-smelling member of the genus, produces odorous compounds only when cells are damaged. In this respect it is similar to *Allium* species.

The chemical nature of the odor-producing system in *Allium sativum* L. (garlic) has been known for some time.[6] Alliin (*S*-allyl-L-cysteine sulfoxide) is cleaved enzymatically, and allicin (allyl ester of allylthiosulfinic acid), pyruvic acid, and ammonia are the ultimate reaction products.

Allicin is the principal odorous volatile of garlic. The odorous characteristics of *T. violacea* indicate that a similar reaction may occur in this species. In garlic the enzyme mediating the reaction is alliinase (alliin lyase),[6] an alliin allylsulfenate-lyase (EC 4.4.1.4).

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Enzymes of this type have been demonstrated in *A. sativum*[6–8] and *A. cepa*,[9,10] and their presence in many other species of *Allium* has been indicated by detection of one or more of the reaction products.[11–16] Presumably, similar enzymes and appropriate substrates are present in all alliums, because production of odor is common to all members of the genus.

Other enzymes capable of catalyzing a similar reaction have been found in *Brassica* species,[17,18] *Albizzia lophantha*,[19] *Pseudomonas cruciviae*,[20] and *Bacillus subtilis*,[21,22] although in the latter the enzyme is probably adaptive.

This paper reports the occurrence in *T. violacea* of alkyl-cysteine sulfoxide lyase, its characteristics, comparison with other allium *C-S* lyases, and possible taxonomic significance.

RESULTS

Localization and Purification of Enzyme

About 79 per cent of the original activity was recovered in the supernatant fluid from centrifugation at 100,000 × *g*. Hence, the enzyme activity was associated with the soluble cytoplasmic proteins. The specific activity was 0·13 unit per mg protein. Ninety-eight per cent of the soluble enzyme was recovered in the 0·55 to 0·75 saturation ammonium sulfate fraction. This fraction had a specific activity of 0·80, which approximates a six-fold purification.

Identification of Products and Stoichiometry of Reaction

The 2,4-dinitrophenylhydrazone (2,4-DNPHone) of the carbonyl product formed during cleavage of (±)-*S*-ethyl-L-cysteine sulfoxide gave an i.r. spectrum almost identical with that of synthetic pyruvic acid 2,4-DNPHone, showing that the reaction product is pyruvic acid.

The i.r. spectrum of ethyl ethanethiosulfinate (Fig. 1) prepared by the method of Small *et al.*[23] is characterized by strong absorption at 9·14 μ, a characteristic of the S–O linkage in this compound. All absorption bands of this spectrum are represented in the spectrum of the product extracted by benzene (Fig. 1), but additional bands are also present. These are accounted for by the strong absorption bands of the lower spectrum in Fig. 1, which is the compound synthesized by the method of Stoll and Seebeck.[24] The latter compound is probably ethyl ethanethiosulfonate, since the strong absorptions at 7·47 and 8·79 μ were reported by Barnard[25] to be characteristic of the S–O linkages in this compound. Apparently

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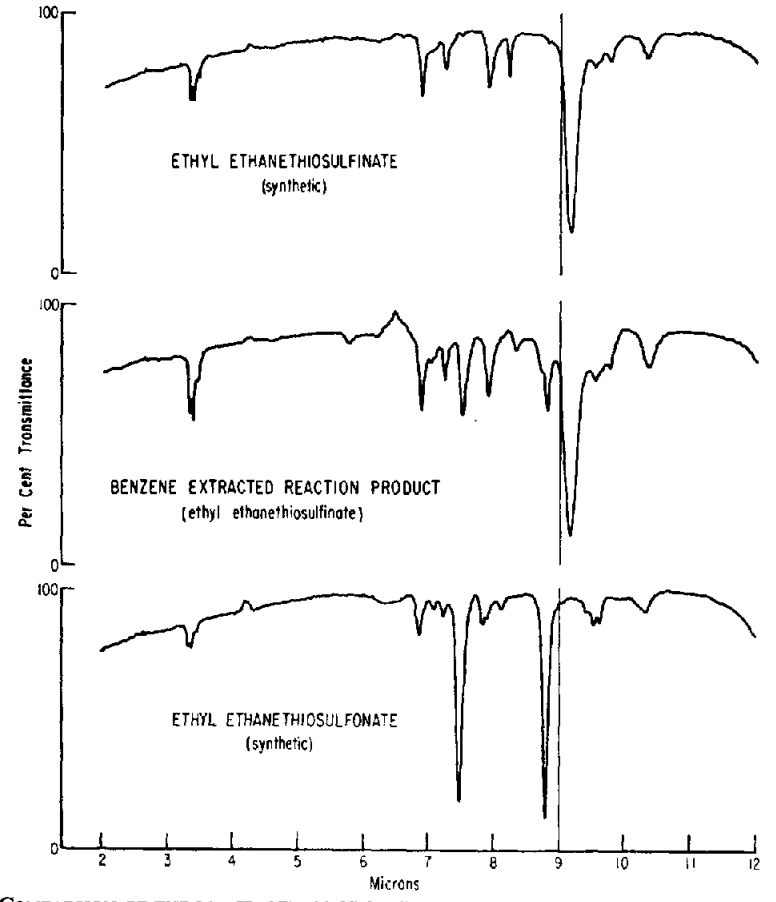

FIG. 1. COMPARISON OF THE I.R. SPECTRUM OF SYNTHETIC ETHYL ETHANETHIOSULFINATE, SYNTHETIC ETHYL ETHANETHIOSULFONATE, AND THE PRODUCT OF *Tulbaghia* ENZYME ACTION ON (±)-*S*-ETHYL-L-CYSTEINE SULFOXIDE.

TABLE 1. STOICHIOMETRY OF THE ACTION OF *Tulbaghia* ENZYME ON (±)-*S*-ETHYL-L-CYSTEINE SULFOXIDE

Compound	Change (μmoles)
(±)-*S*-ethyl-L-cysteine sulfoxide	−1·60
Pyruvic acid	+1·50
Ammonia	+1·56
Ethyl ethanethiosulfinate	+0·89

the principal reaction product obtained by benzene extraction is ethyl ethanethiosulfinate. A small amount of the thiosulfonate probably formed by disproportionation of thiosulfinate, as described by Barnard,[26] also is present.

Determination of the stoichiometry of the reaction (Table 1) revealed that cleavage of 1 μmole of substrate produced about 1 μmole of ammonia, 1 μmole of pyruvic acid, and 0·5 μmole of ethyl ethanethiosulfinate.

[26] D. BARNARD, *J. Chem. Soc.* **4**, 4675 (1957).

pH *Optimum*

The pH optimum of the enzyme was determined in several buffer types (Fig. 2). Optimum pH was 6·6, 6·2, and 6·7 in phosphate, citrate, and pyrophosphate buffers, respectively. Ionic strength, as examined in phosphate buffer, had no effect on pH optimum; however, it did affect the activity at this pH.

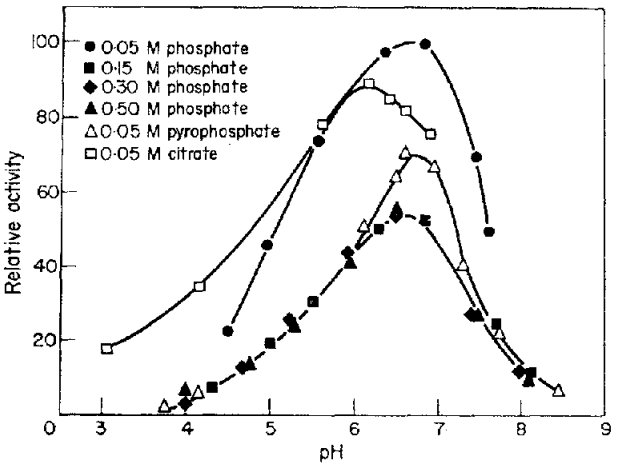

FIG. 2. EFFECT OF BUFFER STRENGTH AND TYPE ON THE pH OPTIMUM OF THE CLEAVAGE OF (±)-*S*-ETHYL-L-CYSTEINE SULFOXIDE. REACTION TIME WAS 15 MIN.

Substrate Specificity

Of the sulfur-containing amino acids tested, only *S*-substituted-L-cysteine sulfoxides were substrates for the enzyme (Table 2). Although not given in the table, other sulfur-containing compounds (L-cysteine hydrochloride, L-cysteine sulfinic acid, L-cysteic acid, DL-homocysteic acid, and reduced glutathione) gave negative results. However, in these instances the pH of the reaction mixture was about 5.

TABLE 2. LYASE ACTIVITY WITH VARIOUS SULFUR-CONTAINING AMINO ACIDS AS SUBSTRATES

Amino acid*	Relative activity†
(±)-*S*-Methyl-L-cysteine sulfoxide	21
(±)-*S*-Ethyl-L-cysteine sulfoxide	100
(±)-*S*-Propyl-L-cysteine sulfoxide	27
(±)-*S*-Allyl-L-cysteine sulfoxide	138
S-Ethyl-L-cysteine	—
L-Djenkolic acid	—
S-Methyl-L-cysteine	—
DL-Methionine	—
DL-Methionine-(±)-sulfoxide	—
DL-Methionine sulfone	—
L-Cystine	—
Lanthionine	—

* Concentration of all amino acids in the reaction mixtures was 40 μmoles per 3·0 ml reaction mixture. pH of reactions was 6·2.

† Relative activity was measured by the method used for pyruvate determination. Reaction time was 20 min. Activity of (±)-*S*-ethyl-L-cysteine sulfoxide was set at 100.

Effect of Pyridoxal Phosphate

The activity of freshly prepared enzyme was enhanced about 20 per cent by pyridoxal phosphate (PalP), but this value varied with different enzyme preparations. If PalP is required for enzyme activity, it is tightly bound. PalP concentrations of about 5×10^{-5} M appear to be at a saturation level as there were no responses at higher concentrations. Dialysis of the enzyme preparation for 17 hr against 0·1 M phosphate buffer, pH 6·2, followed by the addition of PalP to the reaction mixture ($4{\cdot}2 \times 10^{-5}$ M final concentration) caused no significant increase in enzyme activity compared to undialyzed enzyme. However, the enzyme appeared to resolve during long storage at 0°. After 19 days, the stimulation from PalP addition increased almost twelve-fold. Resolution apparently was accompanied by denaturation, because in this time the activity had decreased to about 2 per cent of the original level.

Irradiation of vitamin B_6 enzymes with u.v. light has been shown to reduce their activity,[7,27] due to destruction of pyridoxine.[28] Freshly prepared enzyme was irradiated $1\frac{3}{4}$ hr by the method of Meister *et al.*, except that the distance between the surface of the enzyme solution and the lamp was 15 cm. After treatment, the enzyme was incubated at 30° for 15 min in the presence of various pyridoxine compounds at a concentration of $2{\cdot}0 \times 10^{-4}$ M, and then assayed. The final concentration of pyridoxine compounds in the reaction mixtures was $6{\cdot}7 \times 10^{-5}$ M. Untreated enzyme was incubated and assayed in the same way. Without u.v. treatment, PalP enhanced activity 24 per cent, while the effects of other pyridoxine derivatives were less than 12 per cent. U.v. treatment reduced enzyme activity 30 per cent, but this was completely restored by the addition of PalP. Other pyridoxine compounds tested were ineffective.

Effects of Metal Ions and Metal Complexing Agents

Enzyme activity was enhanced by the addition of cupric ion to the reaction mixture (Table 3), but other metals caused no significant stimulation. $CuSO_4$ and $CuCl_2$ produced

TABLE 3. EFFECTS OF METAL IONS ON LYASE ACTIVITY

Salt	Relative activity	
	No preincubation*	Preincubation†
Control	100	100
$MgCl_2$	99	99
$MnCl_2$	92	101
$CuSO_4$	165	—
$CuCl_2$	156	179
$ZnCl_2$	—	79
$FeSO_4$	—	0
$Fe_2(SO_4)_3$	—	117
$CoCl_2$	—	116

* All metal ions were present in the reaction mixtures at a concentration of $0{\cdot}94 \times 10^{-3}$ M.

† Enzyme was preincubated at 30° for 15 min in the presence of $3{\cdot}0 \times 10^{-3}$ M metal ion. Reactions were run for 15 min at 30°.

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comparable effects, so that any participation of the anion can be eliminated. The Cu^{2+} was partly precipitated in phosphate buffer. However, re-examination of the effect of Cu^{2+} in citrate buffer produced comparable results.

With Cu^{2+} present, irrespective of the buffer used, color development was abnormal. On addition of sodium hydroxide, blanks became colorless and reaction samples turned pink instead of the characteristic reddish-brown. To determine whether this interfered with pyruvate determination, the following experiment was done. A reaction and its control (heat-killed enzyme) were prepared. Cu^{2+} was added to one set of samples but not to another set. Pyruvate was determined as usual. There was no difference between treatments in the amount of pyruvate assayed. Hence, the procedure gives valid results in the presence of Cu^{2+} provided the correct blank is used.

The increase in product formation with Cu^{2+} was enzymatic, because no pyruvate was formed when Cu^{2+} was added to a reaction mixture containing heat-killed enzyme.

The greatest stimulation was produced by preincubation of the enzyme with $3{\cdot}0 \times 10^{-3}$ M Cu^{2+}. At concentrations less than $3{\cdot}0 \times 10^{-5}$ M, no increase in activity occurred, and above $3{\cdot}0 \times 10^{-3}$ M, Cu^{2+} became inhibitory and enzyme activity decreased.

It is possible that Cu^{2+} removed an inhibitor of the enzyme and caused an apparent stimulation. Such an inhibition could have occurred during homogenization of the plant tissue. To test this possibility, enzyme solution was made $0{\cdot}94 \times 10^{-3}$ M in Cu^{2+} and then dialyzed at 0° for 92 hr to remove the Cu^{2+}. The activity of the enzyme was measured with and without the addition of Cu^{2+} to the reaction mixture. For comparison, similar assays were made of enzyme which was untreated and also of enzyme which had been dialyzed for 92 hr (Table 4). Pretreatment with Cu^{2+} did not significantly affect the ability of the enzyme

TABLE 4. EFFECT OF ENZYME PRETREATMENT WITH Cu^{2+}, FOLLOWED BY DIALYSIS, ON THE RESPONSE OF THE ENZYME TO Cu^{2+} ADDED TO THE REACTION MIXTURE

Pretreatment	% stimulation by Cu^{2+}*
None (not dialyzed)	47
Dialyzed at 0° for 92 hr	66
Cu^{2+}-treated,† then dialyzed at 0° for 92 hr	45

* Enzyme solution was made $3{\cdot}0 \times 10^{-3}$ M in Cu^{2+} 15 min before commencement of assay. Assay period was 10 min.

† Enzyme solution was made $0{\cdot}94 \times 10^{-3}$ M in Cu^{2+} prior to dialysis.

to be stimulated by Cu^{2+}. Hence, the Cu^{2+} effect, which can be removed by dialysis, may be regarded as a true stimulation of enzyme activity. The decrease in activity during dialysis was probably due to resolution and denaturation of the enzyme with time, as previously described.

Reactions were run in the presence of EDTA (disodium salt) in concentrations increasing from $2{\cdot}3 \times 10^{-5}$ M to $4{\cdot}6 \times 10^{-2}$ M. There was a slight reduction in activity at the higher concentrations of EDTA. Incubation of enzyme at 0° for 24 hr in the presence of $2{\cdot}4 \times 10^{-2}$ M EDTA in 0·10 M phosphate, pH 6·2, had no effect on activity. Enzyme was also incubated with $5{\cdot}0 \times 10^{-2}$ M sodium diethyldithiocarbamate (NaDIECA), pH 10·0, and 10^{-2} M NaDIECA in 0·05 M phosphate, pH 6·5, for 17 hr. After NaDIECA was removed by dialysis,

measurements of activity indicated that these treatments also had no effect. However, the enzyme could be inhibited if NaDIECA, Na_2S, or potassium ethylxanthate was incorporated into the reaction mixture.

Dependence of the Cupric Ion Effect on Active Enzyme

It was of interest to know whether Cu^{2+} stimulated only holoenzyme or whether it could exert its effect only in conjunction with apoenzyme. To determine this, an enzyme solution was irradiated with u.v. light for 0, 2½, and 4½ hr to partially destroy PalP. The response of irradiated and nonirradiated enzyme to PalP, Cu^{2+}, and both together was then measured. Increasing time of irradiation caused increased loss of enzyme activity, but addition of PalP ($7{\cdot}1 \times 10^{-5}$ M final concentration) restored activity completely to all treatments. This represented an increased stimulation by PalP with increased time of irradiation. The effect of Cu^{2+} on activity of all treatments was virtually the same in the presence or absence of PalP. Hence, it seems that Cu^{2+} stimulates only active enzyme and cannot act in conjunction with apoenzyme only.

Effect of Inhibitors

Pyridoxal phosphate enzymes are inhibited by carbonyl reagents. When hydroxylamine was present in the reaction mixture at $8{\cdot}4 \times 10^{-6}$ M, activity was reduced 90 per cent. Cyanide at a concentration of $8{\cdot}4 \times 10^{-4}$ M reduced activity 42 per cent. However, when the enzyme was preincubated for 15 min with $2{\cdot}5 \times 10^{-3}$ M cyanide before reaction, activity was reduced 97 per cent.

Inhibition of enzyme activity by cyanide could be removed by dialysis at 0° for 134 hr. Prior to dialysis, enzyme was held at 0° for 2 hr in $2{\cdot}0 \times 10^{-2}$ M cyanide in water. This treatment has been shown to essentially eliminate enzyme activity. Dialysis caused the recovery of 89 per cent of the activity of control enzyme, which was dialyzed only.

DISCUSSION

The cysteine sulfoxide lyase in *Tulbaghia* mediates the same type of reaction as does the alliinase of garlic. Also, it is similar to the enzymes of *Brassica*[17,18] and *Allium* species[6–8,10] in its apparent requirement for PalP and its substrate specificity. It is also soluble like other enzymes of this type. Probably it is significant from an evolutionary standpoint that similar enzymes occur in the genera *Allium* and *Tulbaghia*, which, on morphological and anatomical characteristics, are close relatives in the family Amaryllidaceae.[1,2]

Although it is not known whether the cysteine sulfoxide lyase of *T. violacea* is responsible for the characteristic odor of this species, some evidence has been obtained to support this possibility. According to preliminary data, this species contains three amino acids which are sulfoxides.[29] These, when acted on by an enzyme preparation from *Tulbaghia*, produce pyruvic acid, as occurs in the alliinase reaction. The sulfoxides have not yet been identified. Attempts to identify the volatiles from crushed *T. violacea* tissue were largely unsuccessful; however, it was clear from gas chromatographic analysis that there were two main volatile compounds. Elemental analysis indicated that both compounds contained C, H, and S. Judging by their odor and retention times in gas chromatography, these volatiles differ from those commonly found in *Allium*.

[29] J. V. JACOBSEN, Ph.D. Dissertation, Univ. California, Davis (1965).

Just as Saghir[30] has used analyses of sulfur-containing volatiles of *Allium* to evaluate the existing taxonomy of this genus, a survey of the occurrence of cysteine sulfoxide lyases could be of value in taxonomy of the family Amaryllidaceae. Reports of enzyme occurrence and production of volatiles lead to the belief that the enzyme is ubiquitous in *Allium*. Although not all species of *Tulbaghia* produce garlic-like odors,[4,5] it is possible that the enzyme is present in all species but appropriate substrate is not present. Garlic odors are also produced by crushed tissues of some species of *Nectaroscordum*, *Leucocoryne*, and *Tristagma* (syn. *Ipheion*).[2,31] Most probably these genera contain cysteine sulfoxide lyases also. If so, this would support the occurrence of the enzyme as a taxonomic parameter. In this vein, the present report supports the reclassification of *Tulbaghia* from the tribe Agapantheae to Allieae (with *Allium*), as proposed by Traub.[2]

Although the C–S lyases of *Allium*, *Tulbaghia*, and *Brassica* mediate similar reactions and appear to be similar in nature, the pH optima for these enzymes differ. Consideration of the literature indicates that these lyases can be separated into two groups (Table 5)—those with

TABLE 5. PROPOSED CLASSIFICATION OF CYSTEINE SULFOXIDE LYASES BASED ON THEIR pH OPTIMA FOR ACTIVITY

Group		Optimum pH	Buffer*	Reference
1 (Opt. near pH 6)	*Allium sativum* (garlic)	6·4	Phosphate	Goryachenkova[7]
	Allium sativum (garlic)	5·8	0·05 M citrate–0·033 M phosphate–0·05 M borate	Stoll and Seebeck[35]
	Allium sativum (garlic)	5·6–6·4	Acetate–phosphate–Bellonard	Tsuno[33]
	Tulbaghia violacea	6·6	Phosphate	—
	Allium chinense (Rakkyo)	5·6–6·4	Acetate–phosphate–Bellonard	Tsuno[33]
	Bacillus subtilis	About 7	Phosphate	Murakami[22]
2 (Opt. near pH 8·5)	*Allium cepa* (onion)	8·5–8·8	Pyrophosphate	Schwimmer and Mazelis[32]
	Allium cepa (onion)	8·0–8·2	Tris	Schwimmer and Mazelis[32]
	Allium cepa (onion)	8·0–8·2	Acetate	Schwimmer and Mazelis[32]
	Allium cepa (onion)	7·4–7·6	Phosphate	Schwimmer and Mazelis[32]
	Allium cepa (onion)	8·4	0·07 M pyrophosphate	Schwimmer *et al.*[10]
	Allium cepa (onion)	7·4	0·125 M phosphate-tris	Kupiecki and Virtanen[9]
	Albizzia lophantha	8·5	Borate	Gmelin *et al.*[19]
	Pseudomonas cruciviae	8·8	0·08 M tris	Nomura *et al.*[20]
	Brassica oleracea	8·4	0·01 M borate	Mazelis[17]
	var. *pompejana* (broccoli)	9·2	Glycine	Mazelis[17]
		8·4	Pyrophosphate	Mazelis[17]
	Brassica napobrassica (rutabaga)	8·5–9·0	0·2 M tris-HCl	Mazelis *et al.*[18]

* Where buffer strengths are not given, data are lacking or cannot be deduced from the reference.

pH optima near 6·0 (group 1) and those with optima near 8·5 (group 2). Other enzymes with cysteine sulfoxide lyase activity, but having broader substrate specificity, have been included in the table.

[30] A. R. SAGHIR, L. K. MANN, M. OWNBEY and R. Y. BERG, *Am. J. Botany* **53**, 477 (1966).
[31] H. P. TRAUB and H. N. MOLDENKE, Plant Life, *Herbertia* **11**, 125 (1955).

It has been shown (Fig. 2) that the pH optimum of the *Tulbaghia* enzyme is unaffected by buffer strength but is affected by buffer type. The latter has also been demonstrated by Schwimmer and Mazelis[32] for onion alliinase. Hence, comparisons of pH optima within groups of the proposed classification must be made with this in mind. The similarity of the optima of the enzymes from *A. sativum* (pH 6·4) and *T. violacea* (pH 6·6) in phosphate buffer is noteworthy in group 1. In group 2, similar comparisons can be made between the enzymes of *A. cepa* (pH 8·4 and 8·5–8·8) and *Brassica oleracea* (pH 8·4) in pyrophosphate buffer, of *Albizzia lophantha* (pH 8·5) and *B. oleracea* (pH 8·4) in borate buffer, and of *Allium cepa* (pH 8·0–8·2) and *Pseudomonas cruciviae* (pH 8·8) in *tris* buffer.

Tsuno[33] found that the activities of the cysteine sulfoxide lyases from *A. sativum* and *A. chinense* were stimulated by Mg^{2+}, but it cannot be concluded that Mg^{2+} is required by these enzymes. However, it is interesting that the activities of the higher plant enzymes of group 1 (Table 5) are stimulated by metal ions, whereas the onion enzyme in group 2 is not stimulated by Mg^{2+}, Al^{3+}, Cu^{2+}, Co^{2+}, or Mn^{2+}.[9]

The effect of Cu^{2+} is difficult to explain. The increased activity is real and not an artifact resulting from enzyme preparation or assay procedure. The fact that Cu^{2+} can only enhance the reaction in the presence of active enzyme indicates that it acts either in conjunction with pyridoxal phosphate or on the protein part of the enzyme. A Cu^{2+}–PalP enzyme (plasma amine oxidase) has been demonstrated by Yasunobu and Yamada;[34] the *Tulbaghia* enzyme could be similar in its Cu^{2+} requirements.

Schwimmer and Mazelis[32] pointed out that the anion form of the amino acid was the active species of cysteine sulfoxide in the onion enzyme reaction. They based their conclusion on the alkaline pH optimum of the enzyme and on the requirement for a free amino group to combine with PalP. On the basis of pH optima of the enzymes in group 1, the active substrate species probably is the zwitterion. Even though reactions at both high and low pH appear to be α, β elimination reactions, presumably the difference in charge of the substrate acted on reflects a difference in the active sites of the two enzyme groups, and perhaps a difference in mechanisms. Presumably, also an unprotonated amino group is not necessary for combination with PalP. Conceivably, such a difference could be involved with stimulation by metal ions.

EXPERIMENTAL

Enzyme Preparation

Shoots of *Tulbaghia violacea* grown at Davis were used for enzyme preparation. The tissue was washed and chilled to 0°. All subsequent operations were done at 0° with materials prechilled to this temperature.

About 30 g of tissue were homogenized in a Waring blendor for 3 min. For each gram of material, 2 ml of extracting medium were added. The latter consisted of 0·2 M phosphate, pH 6·2, containing 0·3 M sucrose. The homogenate was ground to a fine slurry in a mortar with 1 to 2 g of acid-washed sand. The preparation was centrifuged at 300 × *g* for 10 min to remove coarse particles, and the supernatant fluid recentrifuged at 31,000 × *g* for 30 min.

To localize the enzyme in the cell, the supernatant fluid from this centrifugation was recentrifuged at 100,000 × *g* for 1 hr. To precipitate the soluble protein from the solution, crystalline ammonium sulfate was added until the solution was 0·55 saturated. Stirring was continued for 30 min. The precipitate was discarded after centrifugation. The supernatant fluid was made 0·75 saturated with ammonium sulfate, and the precipitate was dissolved in deionized water. Unless otherwise stated, this solution was used for all enzyme reactions.

[32] S. SCHWIMMER and M. MAZELIS, *Arch. Biochem. Biophys.* **100**, 66 (1963).
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Procedure for Enzyme Assay

Immediately before use, the enzyme solution was diluted (usually 1:10) with distilled water to an appropriate level of activity, and the diluted solution mixed with an equal volume of 0·1 M phosphate buffer, pH 6·2. The substrate, usually (±)-S-ethyl-L-cysteine sulfoxide (20 μmoles/ml), was dissolved in 0·05 M phosphate buffer, pH 6·2. This substrate was chosen because preliminary mass spectroscopic analysis of sulfur-containing volatile compounds from *T. violacea* indicated the presence of ethyl radicals, and it was possible that these were derived from *S*-ethyl-L-cysteine sulfoxide by analogy with the system in *Allium*.

Enzyme and substrate solutions were equilibrated separately at 30° for 10 min before commencing reactions, which also were run at 30°. If there was a preincubation period of the enzyme with PalP, Cu^{2+}, or inhibitors, temperature equilibration took place during this period. Reactions were begun by adding 1·0 ml of enzyme solution to 2·0 ml of substrate solution. Under these conditions, with 0·1 unit of enzyme,[36] the reaction rate was essentially constant for the duration of the assay period (up to 20 min). Reactions were stopped by adding 2 ml of 10 per cent trichloracetic acid (TCA). It was not necessary to deproteinize the mixtures, because the amounts of protein present were small and did not interfere with subsequent determinations. For controls, the TCA was added to the substrate fraction before the enzyme. However, if PalP was present during reaction, the amount of nonenzymic reaction was significant, especially if metal ions were added at the same time. In such instances, controls were prepared by substituting heat-killed enzyme (100° for 4 min) for active enzyme.

Pyruvate production, determined by the 2,4-dinitrophenylhydrazone method of Schwimmer and Weston,[37] was used as a measure of enzymatic activity. Activity is expressed as mole per cent conversion of substrate to pyruvate.

Experiments were usually done twice in duplicate. The same preparation of enzyme was used for replicates because different preparations responded differently. Although this produced some apparent irregularities in the data, the qualitative aspects of response were consistent from preparation to preparation.

Quantitative Determinations

Amino acids were determined by the method of Kornberg and Patey,[38] using synthetic (±)-*S*-ethyl-L-cysteine sulfoxide as the standard. Ammonia was measured by the microdiffusion method of Brown *et al.*[39] The method of Lowry *et al.*[40] was used for protein determinations. Bovine serum albumin was used as the standard. The procedure of Schwimmer and Mazelis [32] was used for determination of ethyl ethanethiosulfinate, except that the thiosulfinate was extracted from the reaction mixture with chloroform instead of benzene. As indicated by i.r. spectra, benzene appeared to accelerate breakdown of thiosulfinate to thiosulfonate. The quantities of thiosulfinate determined in reaction mixtures were about half of the expected values calculated from pyruvate production. $CHCl_3$ could be evaporated much more quickly than benzene in recovering the thiosulfinate, and the amounts determined were much closer to the expected values.

Syntheses of Substrates and Products

Cysteine sulfoxides used for substrates were prepared from the corresponding *S*-alkyl or *S*-alkenyl-L-cysteines by oxidation with H_2O_2 in glacial acetic acid, according to the method of Stoll and Seebeck.[41] All sulfoxides used as substrates were diasteriomeric mixtures. For identification of pyruvate as a product, the 2,4-dinitrophenylhydrazones (2,4-DNPHones) of the carbonyl product of reaction and of commercial pyruvate were prepared by the methods of Schwimmer and Mazelis.[32] The hydrazones were pressed in KBr pellets for i.r. absorption studies. For ethyl ethanethiosulfinate identification, the compound was synthesized by two methods, one of Small *et al.*[23] and the other of Stoll and Seebeck.[25] The reaction product was isolated by extraction with benzene. These compounds were dissolved in spectro-grade CCl_4 for analysis. I.r. spectra of the compounds were measured on a Beckman IR-5 spectrophotometer.

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