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Diamine oxidase of *Lathyrus sativus* seedlings. Purification and properties

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Abstract. Diamine oxidase (EC 1.4.3.6) was purified from 5-day-old etiolated seedlings of *Lathyrus sativus* by $MnCl_2$ treatment, $(NH_4)_2SO_4$ and acetone fractionations, DEAE-Sephadex chromatography followed by gel filtration on Sephadex G-200. A single step purification of the enzyme was achieved by using an immunoaffinity column, wherein rabbit antibodies to the homogeneous diamine oxidase were coupled to CNBr-activated Sepharose. The enzyme thus obtained was homogeneous by electrophoretic, immunological and ultracentrifugal criteria. It had an M_r of 148,000 (6·46S) and was a dimer with similar sub-units (M_r 75,000). Amino acid analysis showed the absence of cysteine residues although it contained five disulphide bonds. The enzyme had copper (2·7 g atom/mol enzyme) but was not a glycoprotein. No absorption maximum in the visible region was detectable. Ethylenediamine 1,3-diaminopropane and histamine were potent competitive inhibitors for the substrate putrescine. The addition of monospecific antibodies to the enzyme increased the K_m for benzyl amine without any change in the V_{max}. Diamine oxidase from pea seedling, partially purified, exhibited complete cross-reactivity with the antibodies to the *L. sativus* enzyme.

Keywords. Diamine oxidase; *Lathyrus sativus*; purification; properties; immunoaffinity procedure; sub-units; copper content.

Introduction

The major catabolic route of amines in various biological systems involves the participation of amine oxidases (Kapeller-Adler, 1970). In plants, monoamine oxidases are of relatively rare occurrence, while di- and polyamine oxidases are more widely distributed in dicots and monocots respectively (Smith, 1971), The physiological significance of plant amine oxidases has been the subject of much speculation. It seems reasonable to consider that they regulate the intracellular amine levels, which in turn are intimately associated with macromolecular metabolism and growth. Amine oxidases in plants have also been implicated in the biogenesis of various alkaloids and of the plant hormone indoleacetic acid (Smith, 1975).

Abbreviations used: sodium dodecyl sulfate, Na · dod · SO_4.

During the earlier investigations on the purification and properties of arginine decarboxylase (EC 4.1.1.19) from etiolated *L. sativus* seedlings (Ramakrishna and Adiga, 1975), it was intriguing to find that, with L-[U-^{14}C-]-arginine as the substrate, $^{14}CO_2$ production could be readily detected in the crude extracts but not the formation of ^{14}C-agmatine, the other product in stoichiometric amounts. Furthermore, these crude extracts also exhibited artifactual lysine decarboxylase (EC 4.1.1.18) (Ramakrishna and Adiga, 1976) and putrescine-dependent S-adenosyl-L-methionine decarboxylase (EC 4.1.1.21) (Suresh and Adiga, 1977) activities which were however catalase-sensitive. These anomalies were traced to the presence of a powerful semicarbazide-sensitive diamine oxidase in the crude extracts which oxidatively deaminated several aliphatic and arylamines but not histamine (Suresh *et al.,* 1976). In this paper, we describe the purification, physicochemical and immunological properties of *L. sativus* diamine oxidase and compare it with similar enzymes from other sources. An immunoaffinity method has been devised to eliminate this enzyme specifically from the crude extracts in order that the biosynthetic enzymes of polyamine metabolism could be studied.

Materials and methods

Materials

L. sativus seeds were obtained from the Plant Breeding Section of the Indian Agricultural Research Institute, New Delhi. *Pisum sativum* seeeds (pea) were obtained from the Karnataka Seeds Corporation, Bangalore. Horse-radish peroxidase (type II, R_Z 1–1·5 and type VI, R_Z 3), *o*-diansidine 3,3′-diamino, benzidine, 5,5′-dithiobis-(2-nitrobenzoic acid), $NaBH_4$ and N-bromosuccinimide were procured from Sigma Chemical Company, St. Louis, MO, USA. The sources of other biochemicals have been referred to earlier (Ramakrishna and Adiga, 1973-1975, 1976; Suresh and Adiga, 1977; Suresh *et al.,* 1976). [1–4, ^{14}C]-Putrescine dihydrochloride (specific activity 54 Ci/mol) was purchased from the Radio-chemical Centre, Amersham, UK. Analytical grade chemicals of the highest purity were used.

The conditions for the growth of seedlings have been described earlier (Rama-krishna and Adiga, 1973).

Enzyme assays

Diamine oxidase activity was routinely assayed by monitoring either the liberation of NH_3, H_2O_2 or the aldehyde product (Suresh *et al.,* 1976). One unit (*U*) of activity is defined as the amount of enzyme required for either the liberation of 1 μmol of NH_3 , benzaldehyde or guaicol oxidised/min. Specific activity is *U*/mg protein.

General methods

Protein was estimated by the procedure of Lowry *et al.* (1951) using bovine serum albumin as the standard. Polyacrylamide gel electrophoresis was performed in 7·5 % gels at pH 8·3 (Davis, 1964). After electrophoresis, the enzyme was stained

for the presence of carbohydrate residues by the method of Zacharius *et al.* (1969). Sodium dodecylsulfate (Na dod. SO_4) gel electrophoresis was performed in 7·5% gels for 8 h at 8 mA/tube (Weber and Osborne, 1969). The M_r of the enzyme was determined by gel filtration using Sephadex G-200 (40-100μ) (Andrews, 1964) or thin layer (10–40 μm, superfine) chromatography (Radola, 1968). The amino acid analysis of the protein hydrolysed with 6N HCl at 110°C for 22 h was carried out (Spackman *et al.,* 1958) using an automatic amino acid analyser. The cysteine content was determined by using 5,5′-dithiobis (2-nitrobenzoic acid) (Habeeb, 1972). The cysteine content was estimated as cysteic acid using the automatic amino acid analyser after performic acid oxidation of the protein followed by acid hydrolysis (Hirs, 1956). Tryptophan content was determined by spectrophotometric titration using N-bromosuccinimide (Spande and Witkop, 1967). Sedimentation analysis (Schachman, 1959) of the protein (15 mg/ml) was carried out in a single sector 12 mm. cell of a Beckman (Model E) analytical ultracentrifuge equipped with the Schlieren optics.

Enzyme staining on Polyacrylamide gels

Following electrophoresis at 0–4°C, the enzyme was stained for activity by incubating the gels for 2–4 h with 2·5 ml of a solution containing 25 mM Tris-HCl pH 8·4, 100 μg of horse radish peroxidase (R_z 1–1·5), 0·02% *o*-diansidine and 10 mM substrate (Hampton *et al.,* 1972).

Immunological techniques

Procedures for the preparation of antisera to the *L. sativus* diamine oxidase in rabbits, Ouchterlony immuno-double diffusion and immuno-electrophoresis were essentially those described earlier (Ramakrishna and Adiga, 1975).

Preparation of affinity matrices

A series of aminoalkyl-Sepharoses of different spacer lengths [$(CH_2)_2$ to $(CH_2)_6$] were prepared by coupling the appropriate amine to CNBr-activated Sepharose (March *et al.,* 1974).

The rabbit antibodies monospecific to diamine oxidase was partially purified and coupled to CNBr-activated Sepharose. The antibodies from 10 ml of antiserum were dissolved in 10 ml of 0·2 M $NaHCO_3$ pH 8 and mixed with 10 ml of CNBr-activated Sepharose. The mixture was gently stirred for 18 h at 0–4° C. Subsequently, 500 mg of glycine was added to block any reactive group present. More than 90% of the added immunoglobulins were found coupled to the insoluble matrix by this procedure.

Binding of [1,4, ^{14}C] *-putrescine*

Purified diamine oxidase and [^{14}C]-putrescine were kept at 30^o C for 1 min and 5 mg solid $NaBH_4$ added (Kumagai *et al.,* 1969). After 30 min it was acidified with an equal volume of 10% trichloroacetic acid, 400 μg carrier BSA added, cooled overnight and the precipitate washed five times with 5% trichloro acetic acid. The

final pellet was dissolved 1 M KOH and the radioactivity measured in a Beckman LS-100 liquid scintillation spectrometer using methyl cellosolve : toluene (1:1) containing 0·5 % 2,5-diphenyl oxazole.

Purification of pea-seedling diamine oxidase

For direct comparisons with the *L. sativus* enzyme, the pea seedling enzyme was partially purified upto step 4 (Hill, 1970) from 8-day-old etiolated seedlings.

Estimation of copper

The copper content of the pure protein (14 mg) was estimated spectrophotometrically using biscyclohexanone-oxalyldihydrazone reagent (Peterson and Bollier, 1955). Deionised water, double-distilled, was employed throughout for the preparation of the buffers and the reagents.

Results

Purification of the diamine oxidase from L. sativus seedlings

The initial steps in the purification of the enzyme from 5-day-old etiolated seedlings have been reported in detail elsewhere (Suresh *et al.,* 1976). Briefly, the steps involved were $MnCl_2$ precipitation of the homogenate, $(NH_4)_2SO_4$ fractionation (25–60%), acetone fractionation (30–55%), adsorption and elution from alumina Cγ-gel followed by a negative adsorption on DEAE-Sephadex. The enzyme at this stage of purification gave two protein bands which were obtained on polyacrylamide gel electrophoresis. In subsequent experiments, it was found that the acetone fraction could be directly loaded on to the DEAE-Sephadex column without much difference in recoveries or purity. The pooled DEAE-Sephadex fraction was concentrated over aquacide and loaded on to a Sephadex G-200 column (2·5 × 40 cm) equilibrated with 50mM Tris-HCl pH 8·4 containing IM KCl and eluted with the same buffer. The protein peak obtained after the void volume (figure 1, peak 1) contained the diamine oxidase activity. It was collected and concentrated over aquacide. The final preparation was purified 200-fold, with a yield of 33% and a specific activity of 10. About 50 mg of the homogeneous enzyme was obtained from 2 kg (fresh wt) of seedlings.

Criteria of purity

Polyacrylamide disc gel electrophoresis of the purified diamine oxidase (50–100 μg) at pH 8·3 on 7·5% gels showed a single band (figure 2). The mobility of the band was not altered when the enzyme was pre-incubated with either putrescine, cadaverine, 1,6-diaminohexane, spermidine, histamine, hydroxylamine or 4-bromo-3-hydroxybenzoyl-oxyamine dihydrogen phosphate. Scanning for the enzyme following gel electrophoresis also revealed that enzyme activity was coincident with the protein stain (figure 3); no other enzyme band could be observed. When the antiserum raised against the Cγ-gel fraction was reacted with the purified enzyme by the techniques of Ouchterlony double diffusion

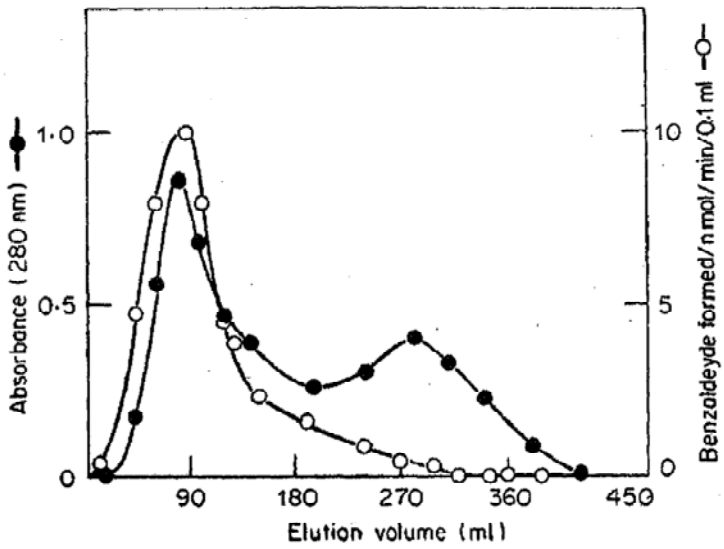

Figure 1. Resolution of *Lathyrus sativus* diamine oxidase on Sephadex G–200 column. The unadsorbed and concentrated DEAE-Sephadex fraction (2·5 ml) was loaded on to a Sephadex-G-200 column (2·5 × 40 cm) equilibrated with 50 mM Tris-HCl pH 8·4 containing 1 M KCl and eluted with the same buffer.

(data not given) or immunoelectrophoresis, a single precipitin line was obtained in both the cases while Cγ-gel fraction of the enzyme gave two or more precipitin lines.

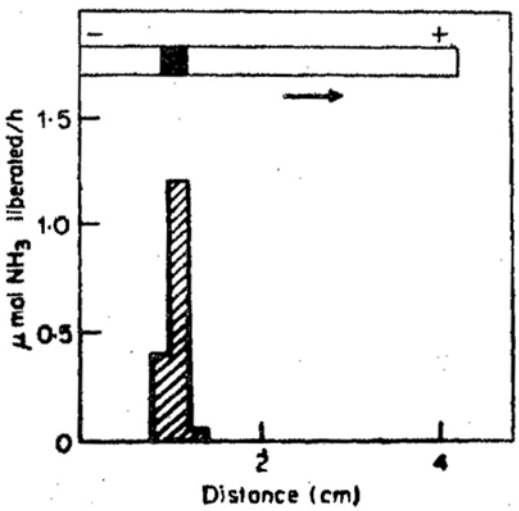

Figure 3. Distribution of enzyme activity and protein stain following electrophoresis on polyacrylamide gel. Protein (250 μg) was loaded on duplicate gels and electrophoresed in 0·2 M Tris-glycine buffer, pH 8·3. While one gel was stained for the proteins, the other gel was sliced (4 mm thick) and individual slices extracted with 0·2 M Tris buffer, pH 8·3 and supernatant assayed for enzyme activity.

The purified preparation in 50 mM sodium phosphate buffer pH 7·5 at 5 mg/ml sedimented during ultracentrifugation as a single symmetrical peak (6·4S, figure 4). A similar pattern was observed at 7·5 and 15 mg/ml protein concentrations. Treatment of the protein in 0·1 % Na·dod·SO_4 and 0·1 % 2-mercaptoethanol at 80° C resulted in the appearance of a single symmetrical peak with an *S* value of 1·27 (data not given).

Activity staining

Polyacrylamide disc gel electrophoresis at 0–4° C of the enzyme and subsequent staining for the activity (see Methods) showed the appearance of a brown band corresponding to the protein band (located by staining with Coomassie brilliant blue). Electrophoresis of the concentrated crude extracts obtained from embryo axes and cotyledons and subsequent staining revealed the appearance of only one brown band when either putrescine, benzylamine, spermidine, homoagmatine, N-carbamylcadaverine or lysine was used as the substrate. This excluded the presence of multiple forms of diamine oxidases in this system.

Stability of the enzyme

The pure enzyme in solution was stable for long periods of time (< 12 months) at – 20° C. However, lyophilisation led to considerable losses in activity.

Affinity chromatography

The purified enzyme when loaded (1 mg each) was not retained on the following aminoalkyl-Sepharose columns: aminoethyl-, aminopropyl-, aminobutyl-, amino-pentyl-, aminohexyl-, spermidine- and spermine-Sepharoses.

In view of the above, an immunoaffinity method was developed. The purified enzyme obtained through the conventional purification procedure was injected into rabbits and a patent antiserum obtained. This antiserum was monospecific to the plant antigen as revealed by immuno double-diffusion and immuno electrophoresis. The titer of the antibodies was high since 40 μg of diamine oxidase could be completely precipitated with 10 μ1 of the antiserum. The partially purified antibodies prepared from such an antiserum were coupled to CNBr-activated Sepharose (see Methods). The crude extract of *L. sativus* seedlings was passed through this immunoaffinity column at room temperature. The effluent was devoid of any detectable diamine oxidase activity and this fraction was employed for the study of the other biosynthetic enzymes (Suresh and Adiga, 1977). The column was washed and the enzyme eluted from the column (figure 5) with 0·1 M acetic acid, immediately titrated with 1 M NaOH to pH 7·5, dialysed and concentrated. This procedure afforded a single-step purification of diamine oxidase. The specific activity was approximately 7 *U*/mg as against 10 *U*/mg protein by the conventional procedure. That this decreased activity may be due to partial inactivation presumably as the result of exposure to 0·1 M acetic acid, is supported by the findings that the protein thus isolated was homogeneous by the criteria of Na·dod· SO_4-polyacrylamide gel electrophoresis and immunological techniques; in both cases, single protein species could be detected by these techniques. The efficiency of the column was decreased after each cycle of purification and storage of the affinity matrix,

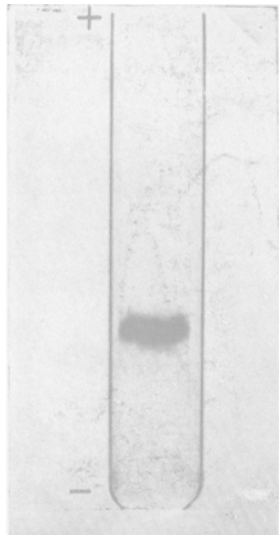

Figure 2. Polyacrylamide gel electrophoresis of purified *L. sativus* diamine oxidase.

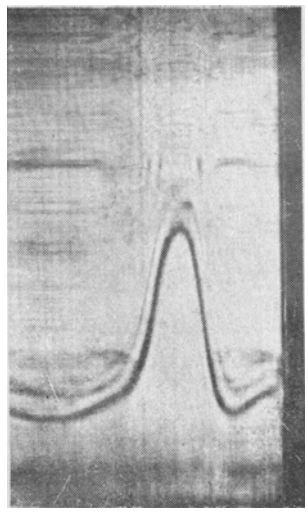

Figure 4. Analytical ultracentrifugal pattern of *Lathyrus sativus* diamine oxidase. The purified protein (15 mg/ml) in 50 mM sodium phosphate buffer (pH 7·5) was centrifuged at 59,780 rpm. The photograph was taken at 56 min after reaching maximum speed. For details see Materials and Methods.

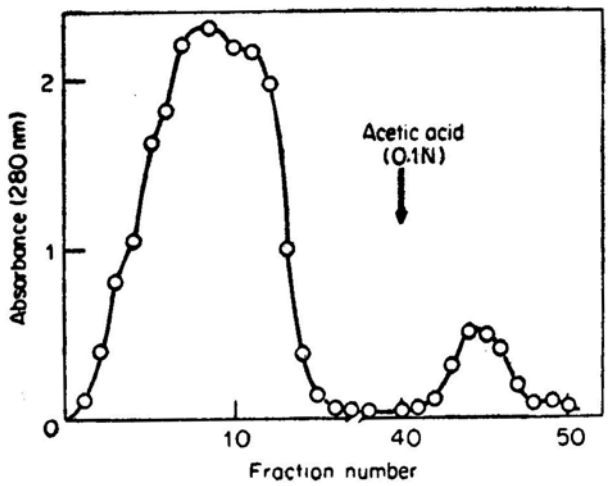

Figure 5. Immunoaffinity chromatography of *L. sativus* diamine oxidase. Partially purified rabbit antibodies to homogeneous diamine oxidase was coupled to CNBr-activated sepharose. The column (1·2 × 6 cm) was equilibrated with 50 mM sodium phosphate buffer pH 7·5 containing 0·9% NaCl and the crude homogenate of the seedlings passed at a rate of 20 ml/h at 25° C. The column was washed and the pure diamine oxidase was eluted with 0·1 M acetic acid and the eluate titrated to pH 7·5 with 1 M NaOH. The column was then equilibrated with phosphate buffered saline.

Immunological cross-reactivity

The antibodies to the homogeneous *L. sativus* diamine oxidase on immuno double-diffusion analysis showed total cross-reactivity with the pea-seedling enzyme and the two precipitin lines obtained were confluent.

Molecular weight and sub-unit composition

The M_r of the enzyme determined with reference to standard marker proteins using Sephadex G-200 column or thin layer were 148,000 and 160,000 respectively.

Polyacrylamide gel electrophoresis in the presence of Na· dod· SO_4 showed a single band corresponding to an M_r of 75,000. This result suggests that the enzyme is a dimer with similar sub-units.

Amino acid composition

The amino acid composition of the highly purified diamine oxidase is given in table 1, along with the values reported for the pea-seedling enzyme (Nylen and Szybek, 1974) for comparison. The relative numbers of residues in the enzymes from the two sources are comparable only with regard to a few amino acids such as lysine, threonine, valine, isoleucine, leucine and tyrosine. Cysteine residues were absent in both the enzymes. The absence of free sulphydryl groups in *L. sativus* diamine oxidase was confirmed by the reaction with 5,5′-dithiobis (2-nitrobenzoic acid). The pea enzyme was reported to have in addition, ornithine and a low methionine content while both the amino acids were absent in the

Table 1. Amino acid composition of *L sativus* diamine oxidase.

Amino acid	No. of residues[1]		No. of residues/ mol protein[2]
Lysine	7·83	(6·66)[6]	82·5
Histidine	1·00	(4·7)	10·5
NH_3	10·51	(..)	101·2
Arginine	1·79	(4·5)	18·9
Aspartic acid	9·95	(16·8)	104·9
Threonine	12·14	(9·2)	128·0
Serine	11·84	(26·5)	124·8
Glutamic acid	7·93	(24·3)	83·6
Proline	5·65	(..)	59·6
Glycine	5·88	(22·1)	62·0
Alanine	6·29	(11·2)	66·3
Cysteine[3]	0·0		0·0
Valine	9·22	(7·6)	97·2
Methionine	0·0	(1·0)	0·0
Isoleucine	8·5	(7·0)	89·6
Leucine	8·84	(8·2)	93·2
Tyrosine	4·15	(4·3)	43·7
Phenylalanine	9·02	(4·9)	95·1
Tryptophan[4]	..		5·5
Cysteic acid[5]	..		9·6
		Total	1178

Weight of protein used for the estimation of basic acidic and neutral amino acids = 1·675 mg each.

[1] Normalised to one histidine residues.

[2] Normalised to 150,000 M_r of the native protein.

[3] Estimated also by 5, 5'dithiobis-(2-nitrobenzoic acid) method (Habeeb, 1972).

[4] Estimated by N-bromosuccinimide titration (Spande and Witkop, 1967).

[5] Estimated by performic acid oxidation and acid hydrolysis of protein (Hirs, 1956).

[6] Amino acid composition of pea-seedling enzyme (Nylen and Szybek, 1974).

L. sativus diamine oxidase. The earlier claim (Nylen and Szybek, 1974) that cysteine residues were absent in the pea enzyme (by 6 N HCl hydrolysis alone) is inconclusive since performic acid oxidation was not undertaken unlike in the present study with the *L. sativus* enzyme which showed the presence of nearly 10 residues of cysteic acid. This is indicative of the presence of five disulphide bonds. Electrophoresis and staining for carbohydrates (Zacharias *et al.,* 1969) showed that the *L. sativus* enzyme was not a glycoprotein.

Copper content

The copper content of the enzyme was 0·11 % corresponding to about 2·7 g atom/ mol of the enzyme,

Absorption spectrum

The enzyme exhibited a typical protein absorption spectrum in the ultraviolet region with $\epsilon^{1\%}_{280nm}$ of 9·6. However, there was no absorption maxima in the visible region at l0 mg/ml (data not given).

Kinetics of substrate saturation

The broad substrate specificity of the enzyme was reported earlier (Suresh *et al.,* 1976). The K_m values for the various substrates obtained using the coupled assay procedure by Lineweaver-Burke plots are given in table 2. There was no correlation between the relative rates of oxidation of the various substrates (Suresh *et al.,* 1976) and the corresponding K_m values. Although putrescine was the best substrate, the K_m value was three times higher than that obtained with agmatine which was oxidised at l/5th the rate of the diamine.

The diamine oxidase-antibody complex obtained after precipitation of the enzyme with the monospecific immunoglobulins was found to be as active as the native (uncomplexed) enzyme. However, a study of the substrate saturation kinetics revealed that despite the unchanged V_{max}, the K_m for benzylamine was slightly yet significantly increased on addition of the specific antibody (figure 6). The antigen-antibody precipitate of the pea-enzyme obtained by using the antibody to *L. sativus* diamine oxidase was also enzymatically active.

Inhibition by substrate analogues

Employing a number of substrate analogues, the inhibition of putrescine oxidation in the coupled assay method was analysed. The inhibitors used were without effect on the oxidation of guaicol by horse radish peroxidase. Table 3 lists the K_i values for a few compounds that competitively inhibited the oxidation of putrescine. Ethylenediamine, 1,3-diaminopropane and histamine were potent competitive inhibitors. L-ornithine (10 mM) was slightly inhibitory (5–10%). No inhibition was obtained with γ-aminobutyric acid, *ß*-alanine, histidine, L-arginine, *L*-homoarginine and diaminobutyric acid even at 10 mM.

Table 2. K_m values for the various amine substrates of *L. sativus* diamine oxidase.

Substrate	K_m (mM)
Putrescine	0·09
Cadaverine	0·13
Spermidine	0·10
Spermine	0·17
Benzylamine	0·10
Agmatine	0·03
Homoagmatine	0·20
Tyramine	0·11

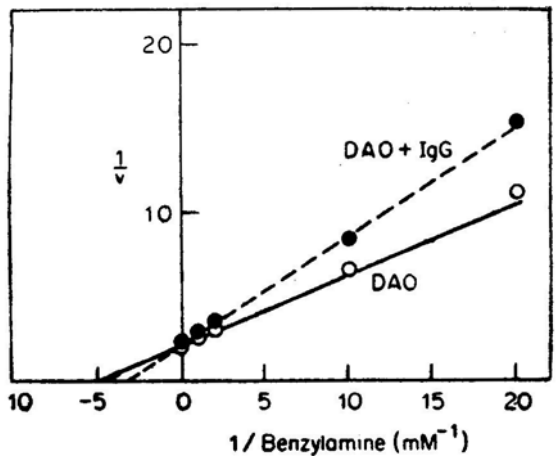

Figure 6. Effect of antibodies on the substrate saturation kinetics of *L. sativus* diamine oxidase. Aliquots (0·1 ml) of the enzyme were incubated with or without the monospecific antibodies to homogeneous *L. sativus* diamine oxidase for 30 min followed by the addition of benzylamine to start the reaction. Benzaldehyde formed was estimated. In a large scale reaction mixture incubating antigen and antibody in the same ratio, it was ascertained that all the enzyme was in the complexed state. Addition of non-immune antibodies had no effect.

Binding of ^{14}C-putrescine

The presumed Schiff base formed between the amine substrate and the carbonyl function in the active site was reduced with $NaBH_4$ (see Methods). The amount of ^{14}C-putrescine bound specifically was corrected for the non-specific interaction. Approximately 0·6 mol putrescine was bound/mol enzyme (table 4).

Table 3. K_i values for various competitive inhibitors of *L. sativus* diamine oxidase.

Inhibitor	Replot of slope *vs* inhibitor concentration	K_i (mM)
Ethylene diamine	Linear	0·26
1, 3-Diaminopropane	Linear	0·36
Histamine	Linear	0·32
Imidazole	Linear	8·2
α-Aminocaproic acid	Hyperbolic	1·6[1]

The product H_2O_2 was estimated by using horse radish peroxidase and *o*-diansidine. The inhibitors had no apparent effect on the peroxidase reaction.

[1] Determined from Dixon plots.

Table 4. Binding of ^{14}C-putrescine to *L. sativus* diamine oxidase.

Order of addition	Bound radio-activity (cpm)	nmol putrescine bound/mol enzyme[1]
Diamine oxidase (150 μg), $NaBH_4$, putrescine	1594	..
Diamine oxidase (150 μg), putrescine, $NaBH_4$	7510	0·6
Diamine oxidase (300 μg), putrescine, $NaBH_4$	12810	0·56

[1] M_r of diamine oxidase taken as 150,000. For experimental details see Materials and Methods.

Discussion

The demonstration of a highly active diamine oxidase in *L. sativus* seedlings is in line with the observations with other leguminous plants on the presence of soluble enzymes capable of oxidising diamines and related substances. The *L. sativus* enzyme shared a few common properties with the enzyme isolated from other sources such as sensitivity to carbonyl reagents, broad substrate specificity, copper content and the dimeric nature. Of particular interest are the striking differences despite complete immunological cross-reactivity between the pea and *L. sativus* enzymes (table 5). Presumably, this is related to the larger size of the pea enzyme.

In this paper, a simple and milder procedure has been adopted to obtain diamine oxidase in a homogeneous form as shown by various criteria. The chloroform-ethanol extraction step employed to purify the pea enzyme (Hill, 1970) was recently shown to be deleterious to an isoenzyme present in the embryo axis which was distinct from the cotyledonary enzyme in terms of kinetic properties and stability on polyacrylamide gels (Srivastava and Prakash, 1977). In *Cucumis sativus* seedlings, three amine oxidases resolved by ion-exchange columns have been described, one resembling monoamine oxidase and the other two, diamine oxidase (Percival and Purves, 1974). However, attempts to detect any isoenzymes of diamine oxidase in *L. sativus* seedlings were unsuccessful. It is pertinent that direct comparisons made with the pea enzyme would relate only to the cotyledonary protein, since in the present study the chloroform-ethanol procedure was employed to partially purify pea-diamine oxidase.

In terms of M_r, extinction and sedimentation coefficients, the pea enzyme exhibited significantly higher values than the *L. sativus* enzyme (table 5). The molecular properties of other highly purified diamine oxidases from *Aspergillus niger,* hog-kidney, bovine plasma and porcine plasma were more akin to the pea enzyme (Yasunobu *et al.,* 1976). Unlike several other diamine oxidases, the *L. sativus* enzyme was not a glycoprotein, The absence of ornithine, methionine and the

Table 5. Comparison of the properties of highly purified diamine oxidase from *L. sativus* and *P. sativum.*

Property	Source	
	P. sativum	*L. sativus*
1. Distribution	Absent in dry seed Appears on imbibition	Absent in dry seed Appears on imbibition
2. Subcellular distribution	Cytosol	Cytosol
3. M_r—Native Subunit	185,000 96,000	148,000 75,000
4. Sedimentation coefficient	7·7	6·46
5. Absorption maxima (nm)	280, 480, 505	280
6. $\epsilon^{1\%}_{280\,nm}$	14	9·56
7. Copper content	0·08–0·09 % (2·5 g atom/mol)	0·11 % (2·7 g atom/mol)
8. Decarboxylation during oxidative deamination	of β-oxoacids by cyclised products	of amino acids by H_2O_2 generated
9. Molecular forms	Two	One
10. Amino acid composition	Low methionine No disulphides Ornithine present	No methionine 5 disulphides No ornithine
11. pH optimum	Substrate-dependent	8·4 (Substrate-independent)
12. Substrate specificity	Histamine and ornithine also oxidised	Refractory to histamine and ornithine
K_m (μM) Putrescine Spermidine Spermine	 40 5 90	 90 100 166

presence of five disulphide bonds are the contrasting features of the *L. sativus* enzyme *vis-a-vis* pea diamine oxidase (Nylen and Szybek, 1974).

It is noteworthy that despite the presence of copper, the *L. sativus* enzyme had no absorption maxima in the visible region. The reason for this is not clear at present. The other diamine oxidases as exemplified by the pea enzyme had a pink colour which disappeared on reduction with sodium dithionite or turned yellow

in an anaerobic reaction on addition of substrate (Yasunobu *et al.,* 1976; Hill and Mann, 1964). Preliminary evidence from electron spin resonance studies suggested that the copper in the *L. sativus* enzyme was in the cupric state (Suresh, unpublished observations). It is pertinent to mention that despite being a copper enzyme, chelators more specific for copper were not potent inhibitors (Suresh *et al.* 1976).

The inability of the *L. sativus* enzyme to bind to amino-alkyl-sepharose columns contrasts with the observations on the human pregnancy plasma histaminase purified on aminopentyl-sepharose column (Baylin and Margolis, 1975) and the *A. niger* amine oxidase on amino-hexyl-sepharose column (Toraya *et al.,* 1976). However, the availability of the monospecific antibodies raised against the pure *L. sativus* enzyme enabled the development of a specific immunoaffinity column to purify the enzyme in a single step. Further, this method greatly facilitated the emoval of the interfering diamine oxidase and enabled the study of the biosyntheticr *S*-adenosyl-*L* -methionine decarboxylase (Suresh and Adiga, 1977).

The addition of antibodies had an effect on the slope of the curve of the Lineweaver-Burke plot for benzyl amine for *L. sativus* diamine oxidase, without any apparent change in the V_{max}, (figure 6), suggesting that the antigen-antibody complex was enzymatically active. This was further confirmed by measuring the activity in the precipitate. It appears that the antigenic sites are away from the active site. Alternatively, even if the catalytic site of the enzyme was involved, the antigen-antibody interactions seem to offer little hindrance to the oxidation of the highly water-soluble, small M_r amine substrate. The inability to obtain stoichiometric binding (^{14}C)-putrescine *(ca* 0·6 mol/mol enzyme) could be either due to the non-ideal conditions of the experiment and/or the presence of inactive enzyme in the final preparation. It is pertinent to mention that the pig-plasma enzyme bound 3 mol ^{14}C-histamine/mol enzyme (Buffoni, 1968) while the pig-kidney histaminase showed a very low binding capacity of 0·115 mol ^{14}C-histamine/mol enzyme (Kumagai *et al.,* 1969).

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