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DIAMINE OXIDASE OF *LATHYRUS SATIVUS* SEEDLINGS

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Key Word Index—*Lathyrus sativus*; Leguminosae; chick-pea; diamine oxidase; distribution; purification; putrescine; cadaverine; spermidine; spermine.

Abstract—A diamine oxidase has been purified (*ca* 40-fold) from 5-day old etiolated seedlings of *L. sativus* by $MnCl_2$ treatment, $(NH_4)_2SO_4$ and Me_2CO fractionations, positive adsorption on alumina Cγ-gel followed by column chromatography on DEAE-Sephadex. This cytosol enzyme oxidatively deaminates a number of aliphatic and aryl alkylamines but not histamine. NSD-1055, semicarbazide and other carbonyl reagents, α,α'bipyridyl, 1,10-phenanthroline and 8-hydroxyquinoline inhibit the enzyme. Pargyline, SKF trans-385, atabrine were without effect on the enzyme.

INTRODUCTION

Diamine oxidases (diamine oxidoreductase (deaminating) EC 1.4. 3.6) oxidatively deaminate a number of aliphatic- and arylalkyl-amines to the corresponding aldehydes, NH_3 and H_2O_2. Diamine oxidases have been demonstrated in the Leguminosae [1–3] and in *Cucumis sativus* seedlings [4]. The extensively studied pea seedling diamine oxidase is a Cu-protein, like the diamine oxidase of animal origin [5–7].

The present paper describes the partial purification of this enzyme and compares the properties with those of the pea-seedling and other plant enzymes.

RESULTS AND DISCUSSION

Distribution of diamine oxidase in L. sativus *seedlings with growth*

In the whole seedlings as well as in the isolated parts of the embryo, the enzyme was barely detectable up to day 1, and activity increased rapidly thereafter (Fig. 1a) as in the case of diamine oxidase of other legumes [8]. The total activity in the whole seedlings increased progressively up to day 5 and declined thereafter. The pattern that emerged with cotyledons was similar, while in the embryo-axis, the enzyme attaining highest level around 5th day remained unchanged up to day 12. At day 5, the contribution of cotyledons to the total enzyme complement of the seedlings was about 60% (Fig. 1b). However, the specific activity was higher in the embryo axis than in the cotyledons during the entire growth period. At day 5, the difference between the two tissues was *ca* 9-fold. The pattern of distribution of diamine oxidase during seedling growth parallels the changes in the levels of guanido-, di- and polymines [9] and arginine decarboxylase [10] in the same plant tissues.

Subcellular distribution and purification

Most of the activity (*ca* 80%) was in the cytosol fraction which also had the highest sp. act. As the total activity in the 100 000 *g* pellet was low (*ca* 8%)—the sp. act. being comparable to the cytosol fractions (80–82 nmol NH_3 liberated/min/mg protein)—post-mitochondrial supernatants with *ca* 86% of the total activity were utilized for the purification of the enzyme (see Experimental).

On resolution of the enzyme on a DEAE-Sephadex column, fractions with high diamine oxidase activity were pooled, concentrated over aquacide and dialyzed against 5 mM Na Pi buffer. This procedure afforded a 40-fold purification of the enzyme with a recovery of *ca* 50% (Table 1).

Effect of pH and temperature

The optimum pH for activity towards putrescine, cadaverine, lysine, agmatine, *N*-carbamylputrescine, and benzylamine was *ca* 8.4 in 100 mM Tris–HCl buffer unlike the pea-seedling diamine oxidase and polyamine oxidase of barley which have pH optima depending upon the substrate in question [11,12]. With putrescine, cadaverine, lysine and benzylamine, a broad pH optimum was obtained, in contrast with the sharp pH optimum obtained with agmatine and *N*-carbamylputrescine as substrates. At any given pH, the enzyme activity in

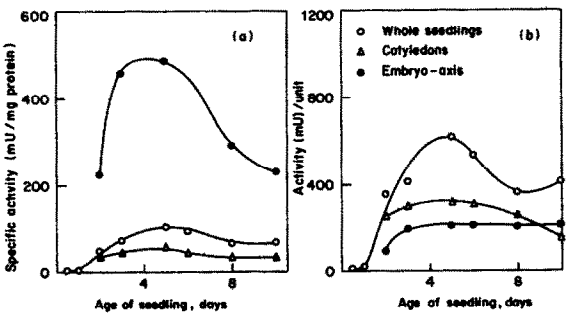

Fig. 1. Distribution of diamine oxidase in *Lathyrus sativus* seedlings during germination and development. (a) Specific activity. (b) Total activity. A unit represents either a whole seedling, a pair of cotyledons or an embryoaxis. The data represent an average of triplicate values.

Table 1. Purification of diamine oxidase of *Lathyrus sativus* seedlings

Fraction	Protein (mg)	Total activity (units)	Specific activity (units/mg protein)
1. Crude extract	3950	188	0·04
2. $MnCl_2$ supernatant	2980	180	0.06
3. 25–55% Ammonium sulphate fraction	1400	159	0.11
4. 30–55% Acetone fraction	566	155	0.27
5. Alumina Cγ-gel eluate pool	171	122	0.71
6. DEAE-Sephadex pool	49	92	1.89

Tris–HCl buffer was always higher than in Na Pi buffer. Under standard assay conditions (see Experimental) the oxidation of putrescine and benzylamine was optimal at 30°. At 60° activity was 45% of that at 30°.

Substrate specificity

A number of substrates with primary amino group and of general formula RCH_2NH_2 were attacked (Table 2). Putrescine was the best substrate, followed by cadaverine and the guanidoamine homoagmatine in that order. However, 1,3-diaminopropane was not attacked while 1,6-hexamethylenediamine was oxidized only slowly. The polyamines viz. *sym.* homospermidine, non-*sym.* homospermidine, spermidine and spermine also served as substrates in decreasing order. Among the aliphatic monoamines, *n*-propylamine and *n*-butylamine, but not methyl- and ethylamine, were attacked and very high concentrations were needed for saturation (> 40 mM). Norepinephrine, epinephrine, serotonin, *N*-α-acetyl-L-lysine, *N*-ε-*t*-BOC-L-lysine, as well as lysyl-lysine did not serve as substrates for the enzyme, as tested with both assay procedures. While the *L. sativus* enzyme attacks only lysine among the amino acids, the *Pisum*-enzyme oxidizes ornithine, in addition [13]. The enzyme from *Lupinus luteus* does not oxidize ornithine, L-lysine, tryptamine and benzylamine [2]. More significantly, histamine was completely refractory to the *Lathyrus* enzyme even at 20 mM in the sensitive coupled assay procedure. Using the same procedure, histamine was oxidized by the pea enzyme at 5% of the rate of oxidation observed for putrescine in agreement with the earlier observation [11]. The pea-seedling enzyme oxidized homoagmatine at a rate 40% that observed when putrescine was the substrate. Since all other diamine oxidases attack the short chain diamines and histamine, the *Lathyrus* enzyme is the first instance of a constitutive diamine oxidase which fails to show histaminolytic activity. By contrast hog-kidney histaminase does not attack the short chain diamines [14].

Table 2. Substrate specificity of diamine oxidase from *Lathyrus sativus* seedlings

Substrate	Relative efficiency of oxidation (% of Putrescine)
Putrescine	100
Cadaverine	81
1,6-Diaminohexane	21
Homoagmatine	62
Agamatine	19
Spermidine	31
Spermine	18
sym. Homospermidine	42
non-*sym.* Homospermidine	31
N-Carbamylcadaverine	26
N-Carbamylputrescine	18
Benzylamine	5
Tyramine	31
Tryptamine	8
L-Lysine	24
D-Lysine	19
n-Butylamine	8
n-Propylamine	4

Values computed are by the coupled assay procedure with 10 mM substrate concentration. Although an essentially similar pattern of substrate oxidation was obtained by NH_3 estimation method, the % values would not represent the true picture since some of the reaction products distil with NH_3 to interfere during nesslerization [8].

Effect of metal ions

Although the enzyme did not show an absolute requirement for any added metal ion, Fe^{2+}, Fe^{3+} and Mn^{2+} at concentrations $10^{-5} - 10^{-3}$ M stimulated the enzyme activity to a significant extent. Cu^{2+} did not influence activity; at higher concentrations (10^{-2}M) it was inhibitory (*ca* 80%). With the exception of Fe^{3+} several other metal ions tested were also deleterious at higher concentrations. Further, even at 10^{-4}M, Zn^{2+}, Hg^{2+}, Cd^{2+} and Ag^{+} were inhibitory (*ca* 10–40%), while Mg^{2+}, Ca^{2+} and Co^{2+} were ineffective.

Effect of metal chelators

A pronounced inhibition of activity was obtained with 8-hydroxyquinoline (*ca* 80%) at 10^{-4}M. Among the other metal chelators α,α′-dipyridyl, salicylaldoxime and 1,10-phenanthroline inhibited to a comparable degree at 10^{-4}–10^{-3}M. However, the more specific copper chelators viz. diethyldithiocarbamate (DIECA), at 10^{-3}M and bathocuproine sulphonate at 10^{-2}M, did not show appreciable inhibition despite long periods of preincubation with the enzyme. Only at 10^{-2}M did DIECA suppress the enzyme activity by 80%. EDTA and NaCN inhibited only marginally at high concentrations. Direct comparisons in experiments with the pea enzyme showed that 5×10^{-4}M DIECA (30 min preincubation) could completely inhibit the enzyme as reported by Hill and Mann [15].

Effect of carbonyl and other reagents

Around 50–100% inhibition of *L. sativus* diamine oxidase could be achieved with phenylhydrazine, semicarbazide, hydroxylamine and hydrazine at a concentration of *ca* 10^{-5}M; however, isoniazid and iproniazid exhibited only marginal inhibition at concentrations 10^{-4}M. NSD-1055 (4-bromo-3-hydroxybenzoyloxyaminedihydrogen phosphate) a known specific inhibitor of pyridoxal phosphate-requiring enzymes [16] greatly decreased activity at 10^{-5} in both the assay systems. But this inhibition could not be either reversed or prevented by exogenous pyridoxal phosphate even at a concentration of >1 mM.

Addition of pyridoxal-5-phosphate, NAD^+, FAD or FMN to the dialysed enzyme preparation did not enhance activity significantly. Further, the specific flavin inhibitor atabrine, the pyridoxal phosphate analogs like pyridoxal and pyridoxine and the monoamine oxidase inhibitor pargylin failed to interfere with the enzyme catalysis even at higher concentrations (10^{-2}M). SKF *trans*-385 (trans-2-phenylcyclopropylamine) yet another potent monoamine oxidase inhibitor [17] was only marginally inhibitory. Similarly neither thiol compounds nor thiol inhibitors had any influence on the enzyme, consistent with the observations that diamine oxidases in general are not sulfhydryl enzymes.

EXPERIMENTAL

Materials. Sources of *L. sativus* seeds, amines and other reagents have been described earlier [18]. *Pisum sativum* seeds were purchased from the Karnataka Seeds Corporation, Bangalore. Pea-seedling diamine oxidase was isolated from 8-day old seedlings as described by Hill [5].

Purification of the Lathyrus *diamine oxidase.* Seeds were germinated in the dark at 25–28° and the etiolated 5-day-old seedlings were ground in a chilled pestle and mortar with one vol of 50 mM Na_2HPO_4. The homogenate was filtered and centrifuged (17 000 *g*) and the supernatant represents the crude extract. Unless otherwise mentioned, all operations were done at 2–5° maintaining the pH around 7 with dilute NH_4OH, and centrifugations at 25 000 *g* for 30 min. Nucleoproteins were precipitated by addition of $MnCl_2$ to the crude extract (7.5 mM final concentration) and centrifuged. From the above supernatant protein precipitating between 25–55% $(NH_4)_2SO_4$ saturation was collected and redissolved in 5 mM NaPi buffer (pH 7.5). To this $(NH_4)_2SO_4$ fraction, precooled (−20°) Me_2CO was added and the protein precipitating between 30–55% in Me_2CO was collected by centrifugation and redissolved in 5 mM NaPi buffer and dialysed. A batchwise positive adsorption on alumina Cγ-gel was performed (protein–gel ratio, 1:6) and the enzyme eluted with 50 mM NaPi buffer (pH 7.5). The pooled eluates were concentrated and dialysed against 50 mM NaPi buffer. The Cγ-fraction was loaded onto a DEAE-Sephadex (A-50, medium) column (20 × 1.5 cm, 35 ml bed vol) pre-equilibrated with 50 mM NaPi buffer (pH 7.5), and the enzyme eluted with the same buffer, while the decarboxylating activities for L-arginine, L-lysine and L-homoarginine were retrained on the column [10].

Assays were performed in 10 ml capacity vials provided with a tight-fitting rubber cork having a centrally placed glass rod (1 cm) towards the inside. The reaction mixture consisted of 10 μmol of substrate, 100 μmol of Tris–HCl buffer (pH 8.4) and enzyme in a final vol. of 1 ml and was incubated at 30° for 30 min. The reaction was terminated by the addition of saturated K_2CO_3 (0.4 ml) and the NH_3 liberated during the post incubation period of 6–8 hr was trapped in a droplet of 4 M H_2SO_4 at the tip of the glass rod above the reaction mixture. The droplet was washed into a test tube with 5 ml H_2O and the NH_3 estimated by nesslerization [19]. A comparison of direct NH_3 standards with that obtained with the above vial procedure gave superimposable curves with excellent reproducibility. These values were also comparable when the reaction was performed either in a Warburg flask or in a Conway Microdiffusion apparatus.

Due to the interference by various factors in the NH_3 estimation method [8] the results were compared and confirmed by the spectrophotometric method using benzylamine sulphate as substrate [20,21]. The liberation of H_2O_2 during the oxidation of all the substrates, was shown employing the coupled assay procedure of ref. [12] in 0.1 M Tris–HCl buffer.

Enzyme unit and specific activity. One unit is defined as the amount of enzyme required for the liberation of 1 μmol of NH_3 liberated, benzaldehyde formed or guaiacol oxidized/min (in the three different assay systems used). Sp. act. is in units/mg protein.

Protein estimation. Protein was estimated in the extracts after suitable dilutions by the method of ref. [22].

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