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DIAMINE OXIDASE FROM *LENS ESCULENTA* SEEDLINGS: PURIFICATION AND PROPERTIES

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Key Word Index—*Lens esculenta*; Leguminosae; lentil seedlings; copper protein; diamine oxidase.

Abstract—A diamine oxidase (DAO) (EC 1.4.3.6) has been purified to homogeneity from lentil seedlings. The purified protein has a MW of 154 000 and is composed of two apparently identical subunits. It contains two Cu^{2+} atoms and one carbonyl-like group per mol. The purified enzyme is pink-red in concentrated solution and shows a broad, well-defined, absorption band in the visible region centered at 498 nm. The ESR spectrum is typical of Cu^{2+} in a tetragonal symmetry. The enzyme oxidizes only aliphatic diamines and spermidine with formation of the corresponding aldehydes, hydrogen peroxide and ammonia. Putrescine and cadaverine are oxidized most rapidly and the oxidation rate decreases when longer diamines are tested.

INTRODUCTION

Diamine oxidases (DAO), which are copper containing enzymes [1], are found in all forms of life. They catalyse the following general reaction:

$$R\text{–}CH_2NH_2 + O_2 + H_2O \rightarrow R\text{–}CHO + NH_3 + H_2O_2.$$

In the plant kingdom DAO has been purified and well characterized from pea [2, 3], *Vicia faba* [4] and *Euphorbia* [5]. This paper describes a DAO isolated from lentil seedlings (L-DAO), whose properties can be helpful in a comparative study to the understanding of the yet unknown role of plant DAO.

RESULTS

Criteria of purity

L-DAO obtained by the reported purification procedure (Table 1) was tested for homogeneity by polyacrylamide gel electrophoresis (PAGE) and by ultracentrifugation. Only one protein band with enzymatic activity was present on PAGE and analytical gel electrofocusing. The isoelectric pH L-DAO is 7.4. Only one band was observed in SDS-PAGE, in the presence and absence of mercaptoethanol. Ultracentrifuge experiments showed a single symmetrical peak sedimenting with $S_{20,w} = 7.28$.

Reaction stoichiometry, substrate specificity and inhibitors

Stoichiometry was determined as follows: (a) oxygen uptake was determined polarographically in the presence of catalase; (b) aldehyde production was followed with the 2-aminobenzaldehyde method [6] using putrescine as substrate; (c) ammonia production was determined by the Nessler method [7]; and (d) hydrogen peroxide was determined in the absence of catalase with the peroxidase–benzidine method.

As reported in Table 2, among the compounds tested, only aliphatic diamines and spermidine were oxidized. The enzymic activity is high for putrescine and cadaverine

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Table 1. Purification of DAO from lentil seedlings

Step	Total vol. (ml)	Protein (mg/ml)	Sp. act. (nkat/mg)	Total act. (μk at)	Purification (fold)	Yield %
(1) Crude homogenate	2150	38.90	1.3	111.2	1.0	100
(2) Controlled heat denaturation	2100	14.70	3.5	108.0	2.6	97
(3) Ammonium sulfate fractionation	315	4.60	65.5	95.0	49.2	85
(4) DEAE-cellulose column	370	0.73	265.5	71.7	200.0	65
(5) AH-Sepharose 4B column	164	0.25	1180.0	48.3	880.0	43

Table 2. Substrate specificity of DAO from lentil seedlings

Substrate	Relative reaction rate
p-Dimethyl-aminomethylbenzylamine	20
1,4-Diaminobutane	100
1,5-Diaminopentane	91
1,6-Diaminohexane	31.5
1,7-Diaminoheptane	25
1,10-Diaminodecane	11.5
Spermidine	43

No activity was found with histamine, diaminoethane, 1,3-diaminopropane, spermine, lysine, arginine, butylamine, benzylamine, tyramine, epinephrine and ascorbic acid.

and sharply decreased with increasing chain length of diamines.

It is worth noting that L-DAO, like the *Euphorbia* enzyme [5], does not oxidize histamine. The velocity of the oxidative deamination was investigated as a function of cadaverine and putrescine concentration. A decrease of the reaction rate at high substrate concentration ($> 2 \times 10^{-2}$ M) was observed. K_m and V_{max} values were estimated from the linear portion of the double reciprocal plots. The K_m for putrescine and cadaverine were 2.3×10^{-4} M, and 4×10^{-4}, respectively. The K_m values are very similar to those reported for other diamine oxidases acting on the same substrates, the V_{max} values are similar to those reported for the pea enzyme [2] but three times higher than those of the *Euphorbia* enzyme [5].

Potential inhibitors tested on L-DAO are listed in Table 3. Copper ligands and carbonyl group-directed reagents, were found to inhibit the enzyme, as already found for other amine oxidases. Sulfhydryl group reagents had no effect on diamine oxidase activity.

Effect of pH, buffer and temperature

The effect of pH on L-DAO activity was tested in the pH range 5.4–8.3 with putrescine dihydrochloride as substrate. 0.1 M buffers used were: sodium acetate (pH 5.4–5.8), potassium phosphate (pH 6.1–7.8) and Tris–HCl (pH 7.4–8.3). The pH curves are broad, with an optimum at 7.1 in 0.1 M potassium phosphate buffer. An inhibition of the enzymatic activity was noticed in Tris–HCl buffer. The effect of temperature on the rate of reaction was studied under standard assay conditions over the range 20–70°. A straight line was obtained in the Arrhenius plot with an activation energy of 11 Kcal/mol.

Table 3. Effect of various reagents on enzyme activity

Reagent	K_i (M)	Nature of inhibition
Carbonyl reagents		
Isoniazid	2×10^{-3}	Irreversible
Phenylhydrazine	1.5×10^{-9}	Irreversible
Semicarbazide	5.5×10^{-6}	Irreversible
Copper ligands		
Sodium cyanide	3×10^{-3}	Non-competitive
Diethyldithio-carbamate	4.5×10^{-5}	Non-competitive
Sodium azide	5×10^{-2}	Non-competitive
Miscellaneous		
Arcaine sulfate	10^{-2}	Competitive
Histamine	1.7×10^{-2}	Competitive
β-Bromoethylamine	1.5×10^{-6}	Irreversible

No inhibition was found with the sulfhydryl reagents: iodoacetic acid, iodoacetamide, *N*-ethylmaleimide, *p*-chloromercuribenzoic acid and 4,4′-dithiodipyridine

Copper and sugar content

The purified enzyme contains 0.082% of copper. On this basis a minimum MW of 77 500 may be calculated. The presence of 14% (w/w) neutral sugar was also determined.

Amino acid composition

The amino acid composition of L-DAO reported in Table 4 is similar to those reported for other plant amine oxidases [3, 5].

Table 4. Amino acid composition of DAO from lentil seedlings

Amino acid		% by wt of residues
Aspartic acid	60	10.3
Threonine	49	7.5
Serine	50	6.8
Glutamic acid	55	10.4
Proline	36	5.3
Glycine	35	3.4
Alanine	27	3.1
Valine	38	5.7
Methionine	6	1.1
Isoleucine	48	8.1
Leucine	38	6.4
Tyrosine	23	5.4
Phenylalanine	30	6.4
Histidine	20	4.0
Lysine	36	6.8
Arginine	21	4.7
Half-cystine	6	0.9
Tryptophan	13	3.4
Total number	591	—

Samples containing 0.5–0.6 mg of the purified enzyme were hydrolysed for 24, 48 and 72 hr in 1 ml 6 M hydrochloric acid: (a) obtained by extrapolation to zero hydrolysis time; (b) determined as cysteic acid, and (c) determined by the method of ref. [18].

Molecular weight determination

The MW of DAO was determined by SDS-PAGE and sedimentation velocity. SDS-PAGE showed a single band with a MW of 78 000. Sedimentation velocity experiments with homogeneous enzyme preparation give a M_r = 187 000. L-DAO seems, therefore, to be a dimer made of identical subunits with a MW in the range 80 000–90 000.

Spectroscopic properties

The absorption spectrum of L-DAO in the visible and near UV is very similar to that of the pea DAO and shows two well-defined bands centered at 498 nm ($E^{M}_{1\,cm} = 3.3 \times 10^3$) and 278 nm ($E^{M}_{1\,cm} = 1.78 \times 10^5$). Upon addition of substrate, under aerobic or anaerobic conditions, the pink-red enzyme solution becomes colorless and the absorption band at 498 nm is replaced by low intensity bands with maxima at 460 and 430 nm. Oxygenation restores the pink-red color to its original intensity. Similar spectral changes were described for the pea DAO [8]. Also, addition of phenylhydrazine to the enzyme solution gives absorption spectra modifications very similar to those described for all other DAO, studied [9], the adsorption band at 498 nm disappearing and being substituted by a single band with a maximum at 440 nm.

The enzyme shows a typical ESR spectrum of copper(II) in a tetragonal field with spectroscopic parameters very similar to those already reported for other DAOs [10].

EXPERIMENTAL

Chemicals. Obtained as pure commercial products. Standard proteins and DEAE-cellulose were from Serva; AH-Sepharose 4B from Pharmacia.

Plant. Lentil (*Lens esculenta*) seeds (1 kg) were soaked for 24 hr in H_2O and germinated for 8 days in moist sawdust, contained in plastic trays, in the dark at room temp.

Purification. 8-Day-old lentil seedlings (*ca* 900 g) were homogenized in a Waring blendor with 2 l. deionized H_2O for 4 min. The homogenate was brought to 48° and kept at this temp. for 10 min under continuous stirring. After rapid cooling in ice–water, the suspension was centrifuged at 9000 rpm for 30 min and the ppt discarded. To the supernatant, solid $(NH_4)_2SO_4$ was added to 70% satn with constant stirring at 4° over a period of 10 min. The mixture was stirred for an additional 30 min and centrifuged at 9000 rpm for 30 min. The ppt was dissolved in *ca* 300 ml deionized H_2O, dialysed against 15 l. deionized H_2O and the insoluble material removed by centrifugation at 19 000 rpm for 30 min. The supernatant (*ca* 300 ml) was made 15 mM in KPi buffer, pH 7, and loaded onto a DEAE-cellulose column (2.5 × 15 cm) equilibrated and washed (flow rate 1.5 l./hr; 10 ml fractions) with the same buffer until the *A* at 280 nm of the effluent became < 0.1. The eluate, containing the enzymatic activity, was diluted with an equal vol. of H_2O and applied to an AH-Sepharose 4B column (2 × 10 cm) equilibrated with 7.5 mM KPi buffer, pH 7. After washing with 10 mM KPi buffer, pH 7, the bound DAO was eluted with 50 mM KPi buffer, pH 7 (flow rate 150 ml/hr; 10 ml fractions). The active fractions were pooled and concd by ultradialysis.

A summary of the purification procedure is presented in Table 1. The overall purification achieved was 880-fold with a yield of *ca* 43%. The purified enzyme in concd solns was pink-red, like other DAOs. The enzyme is indefinitely stable when stored in H_2O or diluted KPi buffer at −20°, but loses 50% of its activity after 15 days at 4°. The purified L-DAO does not withstand lyophilization.

Enzyme assay. DAO activity was tested polarographically by a Gilson oxygraph equipped with a Clark electrode. The reaction was carried out at 38° using air as the gaseous phase. The standard reaction mixture (1 ml) contained 50 μg catalase and the required amount of enzyme in 100 mM KPi buffer pH 7. The reaction was started by addition of a small vol. of amine substrate (as hydrochloride) after at least 10 min preincubation.

Protein bands with DAO activity were detected by PAGE and isoelectric focusing by staining the gels, after the run, in 5 ml 0.1 M KPi buffer, pH 7, containing 100 μg peroxidase, 1 mg benzidine and 17 mM cadaverine.

Analytical PAGE. Electrophoresis under non-denaturing conditions was performed as described in ref. [11]. The pI of L-DAO was measured by the polyacrylamide-gel-electrofocusing technique in the pH ranges 3–10 and 5–8, as described in ref. [12]. Continuous SDS-PAGE was carried out according to ref. [13].

Determination of molecular weight. Sedimentation velocity expts were carried out at 13.5° and 56 000 rpm using a Spinco Model E ultracentrifuge. The sedimentation coefficients are expressed in Svedberg units, corrected to H_2O at 20°. Diffusion coefficients were calculated from the areas of the Schlieren diagrams and corrected for radial dilution and for the movement of the boundary in the centrifugal field, according to ref. [14]. A partial specific vol $\bar{V} = 0.732$ was used, calculated from the amino acid composition [15].

Spectroscopic measurements. Absorption spectra of the enzyme were taken with a Cary Model 219 spectrophotometer using 1 cm light-path cells. ESR spectra were taken at *ca* 9 GHz and at 77 K with a Varian E9 spectrometer.

Amino acid composition. Determined according to ref. [16] with an amino acid analyser LKB Model 4101. The standard, single column method was used. Half cystine was determined as cysteic acid after oxidation with performic acid [17]. Ser and Thr contents were extrapolated to zero hydrolysis time. Trp was estimated spectrophotometrically as described in ref. [18].

Metal determinations. Cu was determinated by atomic absorption using an IL 951 atomic absorption spectrophotometer equipped with a graphite furnace type F.A.S.T.A.C. The spectral line chosen was 3247 Å.

Other analytical methods. Protein was determined by the method of ref. [19], using BSA as standard. Protein concn in column eluates and in soln of purified enzyme was determined by *A* at 280 nm using an $E^{1\%}_{1\,cm} = 13$. Neutral sugars were estimated by the PhOH–H_2SO_4 method [20] using D-glucose, D-galactose and D-mannose as reference standards.

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