PERGAMON

Phytochemistry 52 (1999) 1–5

PHYTOCHEMISTRY

A thermostable diamine oxidase from *Vigna radiata* seedlings

A. Choudhary[a], I. Singh[b], R.P. Singh[a,*]

[a]*Department of Biosciences, M.D. University, Rohtak—124 001, Haryana, India*
[b]*Department of Chemistry, M.D. University, Rohtak—124 001, Haryana, India*

Received 15 April 1998; received in revised form 21 December 1998; accepted 24 February 1999

Abstract

Diamine oxidase (DAO) (EC. 1.4.3.6.) from the 1-day-old *Vigna radiata* cv. P105 seedlings grown in the dark was found to be thermotolerant, showing optimal activity at 60°, with an E_a of 2.75 kcal/mol estimated at temperatures from 25°–85° from the Arrhenius plot. The enzyme was purified up to 22- fold by ammonium sulphate fractionation, heat denaturation and hydroxylapatite column chromatography. The M_r was estimated by gel filtration to be 139 kDa and the SDS–PAGE yielded a single band at M_r 68 kDa, indicating the occurrence of two identical subunits in the mungbean DAO. The K_m value for putrescine, as the preferred substrate, was 0.15 mM and V_{max} was 0.065 μmol/min. The DAO was inhibited by sulphydryl reagents, copper binding agents, and other mono- and divalent ions.

Keywords: *Vigna radiata*; Leguminosae; Mungbean seedlings; Diamine oxidase; Protein purification

1. Introduction

Amine oxidases have been found in bacteria, fungi and various plants and animals. However, their physiological importance has not yet been completely understood (Zajoncova et al., 1997; McIntire & Hartmann, 1992). The Cu-amine oxidases (diamine oxidase) are widespread in the Leguminaceae. The enzyme catalyses the oxidative deamination of amines to their respective aldehydes, H_2O_2 and NH_3 (Smith, 1985). Diamine oxidase was first purified by Mann (1955) from pea seedlings. Thereafter, the enzyme has been purified to homogeneity and characterized by various workers from different plant sources (Sharma, 1997; Medda, Padiglia & Floris, 1995). Recently we reported DAO from light and dark grown cultivars of *Vigna radiata* (L.) Wilczek, which is active during seed germination and early seedling growth (Choudhary & Singh, 1997).

V. radiata (L.) Wilczek is a widely grown tropical legume rich in protein. This paper reports a simple and rapid method (Luhova, Slavik, Fredort, Halata & Pec, 1995) for the partial purification of the enzyme from *V. radiata* seedlings. The enzyme has been characterized and shown to be stable at high temperatures.

2. Result and discussion

We have reported earlier that diamine oxidase in *V. radiata* cv. P105, grown in the dark, is more active at 1 day and the activity reappears on day 7 (Choudhary & Singh, 1997). Thus, 1 day and 7 day etiolated mungbean seedlings were used separately for isolation and purification of the enzyme.

2.1. Purification of mungbean DAO

The purification was done following the method given by Luhova et al. (1995) with slight modification. One-day-old mungbean seedlings (53.5 g) were homogenized in a total volume of 107 ml of 25 mM potassium phosphate buffer (pH 7). The total protein contents were 12.305 mg, having a total DAO activity

* Corresponding author.
E-mail address: mdu@mdul.ernet.in (R.P. Singh)

0031-9422/99/$ - see front matter
PII: S0031-9422(99)00149-1

of 87.15 μkat and a specific activity of 7.07 μkat/mg protein (Table 1).

The modification of the procedure (Luhova et al., 1995) was done by a stepwise saturation of ammonium sulphate to remove unwanted proteins, instead of revinol lactate (Luhova et al., 1995). The crude homogenate was saturated to 40% of the protein with ammonium sulphate, at 4°. Insoluble protein did not show any significant DAO activity. On further saturation of the supernatant to 60%, the pellet when redissolved in 20 ml of 50 mM potassium phosphate (pH 7.0) contained 75.7 μkat of total DAO activity and 12.01 nkat/mg protein of specific activity having total protein contents of 4.3 mg. Further saturation to 60% instead of 70% was done because there is very little DAO activity in 60–80% saturation of the homogenate. The ammonium sulphate fractionation was followed by heating of the homogenate at 80° in a hot water bath for 5 min and immediately transferring it to 4° (Luhova et al., 1995). After the heat-shock treatment, the protein was loaded to hydroxylapatite column for further purification of DAO. A series of varying [50, 100, 150 and 200 mM potassium phosphate buffer (pH 7)] discontinuous gradient elution of the proteins was done until A_{260} became 0.01. The fractions collected in 200 mM phosphate buffer showed maximum DAO activity as compared to the other elutes (Luhova et al., 1995), so these were pooled and concentrated by ultrafiltration to 10 ml. This resulted in a 22-fold purification of the enzyme (Table 1). The large reduction in the enzyme yield in 200 mM buffer is possibly because the fractions eluted with 50–150 mM buffer had low DAO activity but were discarded.

2.2. Thermal properties of mungbean DAO

The effect of temperature in the range 25 to 85° was studied on purified DAO. The enzyme activity increased as a function of temperature up to 60° while at 85° only 35% of the enzyme activity could be recorded. At lower temperatures (25°) only 60% of the DAO activity could be detected. The duration of the enzyme incubation at various temperatures was increased up to 1 h to assess the DAO's thermal stablity. The enzyme was stable up to 30 min at 85° and for 1 h at 60° without loss of activity. Zajoncova and coworkers (1997) have reported the DAO from *Onobrychis viciifolia* seedling DAO to be stable at 60° for 5 min, without loss of the activity. The *Lathyrus sativus* seedlings DAO retained about 45% activity at 60° as compared to 30° (100%) (Suresh, Ramakrishna & Adiga, 1976) and *Cicer arietinum* seedling DAO retained about 50% activity at 70° for 20 min of as that at 37° (100%) (Angelini, DiLisi & Federico, 1985).

The energy of activation of *V. radita* DAO was 657.42 joules/mol (2.748 kcal/mol) over the range 25–60° for 1 h. A straight line was obtained in the Arrhenius plot. The energy of activation (E_a) calculated by Arrhenius plot has been reported to be 11.22 kcal/mol over the range 6 to 40° for *Trifolium subterraneum* leaf DAO (Delheize & Webb, 1987). The E_a of 10.03–11.94 kcal/mol over the range 20 to 50° under standard assay condition in *Euphorbia characias* latex DAO (Rinaldi, Floris & Finazzi-Agro, 1982) and pea DAO showed $E_a = 14$ kcal/mol for the range 4–26° (Smith, 1977).

The DAO from mungbean is a thermostable enzyme having an optimum activity at 60° and at physiological temperature (28°) the enzyme activity is 72% as compared to that at 60°. The relative stability of the enzyme up to 85° and its evolutionary importance needs to be further studied. The activation energy of the enzyme, i.e. 657.42 joules/mol as calculated over the range 25–85°, is lower as compared to those reported in the literature. A lower activation energy denotes that the enzyme is highly reactive to the substrate.

2.3. Properties of DAO

Gel chromatography revealed the M_r to be 139 ± 4 kDa and native PAGE was 139 kDa of DAO from *V. radiata* seedlings. The SDS–PAGE revealed a single band of M_r 68 kDa. This shows that the DAO is a dimer with M_r under non-denaturing conditions as 139 kDa and under denaturing conditions as 68 kDa.

Table 1
Purification of *Vigna radiata* cv. P105 Cu^{2+} diamine oxidase

Purification step	Total volume (ml)	Protein (mg ml^{-1})	Total protein (mg)	Total DAO activity (μkat)	Specific activity (nkat mg^{-1})	Purification fold
Crude homogenate	107	0.115	12.305	87.15	7.07	1.0
$(NH_4)_2SO_4$ fractionation						
After 40% saturation (supernatant)	95	0.045	4.275	75.66	12.01	1.7
After 60% saturation (pellet)	20	0.152	3.046	50.68	16.64	2.4
Heat shock treatment	18	0.069	1.242	47.93	38.59	5.45
200 mM elute of hydroxylapatite column	10	0.0063	0.063	9.66	153.4	21.7

Table 2
Substrate specificity of diamine oxidase[a]

Substrate DAO	Activity (pkat)	Relative activity (%)
Putrescine	95 ± 0.01	100
Cadaverine	95 ± 0.03	99
Spermidine	36 ± 0.05	38
Spermine	8.3 ± 0.08	16

[a] Standard assay conditions were used except for the substitution of putrescine with other substrates as indicated above. Since putrescine showed the maximum activity, it was taken as reference. Relative activity denotes activity with reference to putrescine taken as 100%.

The copper content was one mole atom per mole of the enzyme.

The optimum time of incubation of DAO was determined from 2 to 60 min where the generation of Δ′-pyrroline in the enzyme reaction increased up to 30 min and thereafter showed no further increase. Therefore, in all the subsequent assays, the reaction mixture was incubated for 30 min. The optimum pH of DAO from *V. radiata* seedlings was determined in the range of pH 4.0 to 10 at an interval of 0.5 pH. The enzyme was found to be most active at pH 7.0 under standard assay conditions.

2.4. Substrate specificity and K_m for putrescine

Putrescine, cadaverine, spermidine and spermine were tested for their ability to be utilized as substrate by mungbean DAO. The enzyme showed 99% activity with cadaverine with respect to putrescine. With spermine the activity was reduced to 16% of that with putrescine whereas with spermidine the purified DAO showed 38% activity as compared to the putrescine (Table 2). The results show that the DAO from mungbean shows highest affinity with putrescine as the substrate.

The effect of varying putrescine concentration on the activity of DAO was studied in the concentration range of 0.015 mM to 0.055 mM. A hyperbolic curve between Put concentration and DAO activity was obtained, from which 1/S and 1/V was plotted to calculate K_m and V_{max} value of Put as the substrate, 0.15 mM and 0.065 μmol of Δ′-pyrroline formed per minute respectively. The K_m values of many other plant DAO that have been summarized by Medda and coworkers (1995) shows the values to be more than 0.2 mM, indicating that the mungbean DAO has higher affinity for the substrate as compared to other legume DAO.

2.5. Effect of inhibitors

The purified DAO from 1-day-old *V. radiata* DAO was tested for various possible inhibitors which binds to the prosthetic group (i.e. known inhibitors of DAO), nutrient salts and heavy metal ions (Table 3). The DAO was inhibited up to 100% by copper binding (*o*-phenanthroline and α, α′-bipyridyl) and carbonyl group (aminogunidine, isonicotinic acid and phenylhydrazine) inhibitors. The heavy metals seems to be more toxic to the enzyme probably due to direct

Table 3
Inhibitory effect of the mono- and divalent cations and inhibitors of DAO carbonyl group and cofactor on the DAO activity under standard assay conditions

Inhibitors	Soluble DAO activity (nkat)	Relative inhibition (%)
Control	91.6 ± 0.01	0
Inorganic salts		
1. NaCl (10 mM)	82.6 ± 0.07	10
2. $MgCl_2$ (10 mM)	84.3 ± 0.07	8
3. $CaCl_2$ (10 mM)	92.1 ± 0.02	0
4. $CuSO_4$ (10 mM)	18.9 ± 0.05	80
5. $FeCl_3$ (10 mM)	26.1 ± 0.05	71
6. $MnCl_2$ (10 mM)	56.1 ± 0.05	38
7. $HgCl_2$ (1 mM)	10.1 ± 0.04	89
8. $CdCl_2$ (0.1 mM)	26.2 ± 0.07	73
9. $Pb(COO)_2$ (1 mM)	32.1 ± 0.01	66
10. $NH_4(SO_4)_2$ (10 mM)	64.3 ± 0.01	30
Inhibitors of carbonyl group and cofactor		
11. Phenylhydrazine	ND[a]	100
12. Sodium azide	6 ± 0.05	93
13. Isonicotinic acid	6 ± 0.05	93
14. O-phenanthroline	ND	100
15. α,α′-Dipyridyl	ND	100
16. Aminoguanidine	12 ± 0.04	87

[a] ND—nondetectable.

binding to the active sites of the enzyme (Parsad & Parsad, 1987). The $CaCl_2$ had negligible effect and NaCl could inhibit only up to 10% of the enzyme activity.

2.6. DAO activity from 7-day-old etiolated seedlings

A partially purified prepration of DAO from 7-day-old etiolated mungbean seedling was compared for some of the properties with 1-day-old seedlings DAO from the same cultivar. The results show that the enzymes isolated from 1- and 7-day-old seedlings showed many similar properties, viz. time, temperature and pH optima, substrate specificity, and the thermal stability along with the molecular weight in the SDS–PAGE corresponding to 68 kDa under denaturing conditions. These data indicate that the same isoform of the DAO might approximately appear again 7 days after seed germination. The physiological significance of the enzyme expression at the 7th day is not clear and further studies are required to elucidate it.

3. Experimental

3.1. Plant material and planting

Certified Seeds of *V. radiata* (L.) Wilczek cv. P105 were obtained from the Scientist-in-change Pulse Research Laboratory, Indian Agricultural Research Institute, New Delhi. The seeds were surface sterilized with 90% (v/v) ethyl alcohol for 5 min and then washed thoroughly with distilled water before planting. The seeds were planted on small petri dishes (90 mm diameter) containing two layers of moist filters paper (Whatman No. 1) for 1 day and 7 days total darkness, in growth chambers at $25 \pm 2^{\circ}$. Our previously published work states that the DAO activity is at its maximum in 1-day-old light-grown mungbean seedlings and the enzyme activity again gives a peak on 7 days after seed sowing in dark-grown seedlings. The seedlings grown in light conditions do not show this second peak on the 7th day.

3.2. Estimation of DAO activity and protein

The DAO activity was estimated colorimetrically with the method of Cona, Federico, Niglio, Shephard and Dey (1993) and also by a slight modification of the method given by Naik, Goswami and Srivastava (1981) by estimating the amount of H_2O_2 and Δ'-pyrroline produced. The reaction mixture for the assay of DAO contained 20 μmol of Put as the substrate in a total volume of 3 ml of 0.1 mM potassium phosphate buffer (pH 7) for 30 min and the reaction was terminated with trichloroacetic acid. The aliquot was made to react with ninhydrin reagent (ninhydrin and hydrindantin solution) as described by Naik et al. (1981). The pink colour developed was measured at 510 nm with spectrophotometer (Hitachi U-2000). Standard curves were made by using commercial DAO and putrescine was used as substrate in the method of Naik et al. (1981) and H_2O_2 as the substrate was used to prepare the standard curve for the method of Cona et al. (1993). The total soluble protein was estimated by the method given by Bradford (1976), using B.S.A. to estimate the protein concentration.

3.3. Isolation and Purification of DAO

The enzyme was isolated by homogenization of whole seedlings and purified following slight modification of the method given Luhova et al. (1995). The gel chromatography by Sephadex G200 was performed as given by Andrews (1970).

3.4. Electrophoresis of the enzyme

Polyacrylamide gel electrophoresis (PAGE) was performed using a vertical slab 10 cm × 10 cm × 2 mm slab gel electrophoresis chamber by the method given by Lammelli (1980). SDS–PAGE was performed at 8% gel concentration with and without 2-mercaptoethanol (ME) after treatment of samples at 90° for 2 min and native PAGE on 7% gel concentration. Silver staining of gels was performed by the method given by Morrissey (1981). For native PAGE, all reagents were prepared without SDS.

3.5. Estimation of copper contents

The copper content in the enzyme was estimated by the method given by Klinman, Krueger, Brenner and Edmondson (1984).

Acknowledgements

Financial assistance from the CSIR, New Delhi (Research project ♯9/328/91, EMR-II) to RPS and IS is thankfully acknowledged along with the financial assistance to AC by UGC, New Delhi.

References

Andrews, U. K. (1970). *Meth. Biochem. Anal*, *18*, 1.
Angelini, R., DiLisi, F., & Federico, R. (1985). *Phytochemistry*, *24*, 2511.
Bradford, M. M. (1976). *Anal. Biochem*, *72*, 248.
Choudhary, A., & Singh, R. P. (1997). *Biol. Plant*, *30*, 504.
Cona, A., Federico, R., Niglio, A., Shephard, Z., & Dey, P. M. (1993). *Biotechnol. Appl. Biochem*, *17*, 57.

Delheize, E., & Webb, J. (1987). *Phytochemistry*, *26*, 641.
Klinman, J. P., Krueger, M., Brenner, M., & Edmondson, D. E. (1984). *J. Biol. Chem*, *239*, 2399.
Lammelli, U. K. (1980). *Nature*, *227*, 680.
Luhova, L., Slavik, L., Fredort, I., Halata, M., & Pec, P. (1995). *Phytochemistry*, *38*, 23.
Mann, P. J. G. (1955). *Biochem. J*, *79*, 623.
McIntire, W. S., & Hartmann, C. (1992). In V. L. Davidson, *Principles and applications of quinoproteins* (p. 97). New York: Marcel Dekker.
Medda, R., Padiglia, A., & Floris, G. (1995). *Phytochemistry*, *39*, 1.
Morrissey, J. M. (1981). *Anal. Biochem*, *117*, 307.
Naik, B. I., Goswami, R. G., & Srivastava, S. K. (1981). *Anal. Biochem*, *111*, 146.
Parsad, P. D. K., & Parsad, A. R. K. (1987). *Phytochemistry*, *26*, 881.
Rinaldi, A., Floris, G., & Finazzi-Agro, A. (1982). *Eur. J. Biochem*, *127*, 417.
Sharma, M. L. (1997). *Physiol. Mol. Biol. Plant*, *3*, 3.
Smith, T. A. (1977). *Prog. Phytochem*, *4*, 27.
Smith, T. A. (1985). *Biochem. Soc. Trans*, *13*, 319.
Suresh, M. R., Ramakrishna, S., & Adiga, P. R. (1976). *Phytochemistry*, *15*, 483.
Zajoncova, L., Sebela, M., Frebort, I., Faulhammer, H. G., Navratil, M., & Pec, P. (1997). *Phytochemistry*, *45*, 239.