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PURIFICATION AND CHARACTERIZATION OF DIAMINE OXIDASE FROM RICE EMBRYOS

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Key Word Index—*Oryza sativa*; Gramineae; rice; diamine oxidase; FAD.

Abstract—Diamine oxidase of rice seedlings has been purified 1800-fold to homogeneity. The MW of the enzyme as determined by Sephadex G-100 gel filtration was 12.3×10^4 and the enzyme contained two identical subunits each with a MW of 6.12×10^4. The optimal temperature and pH for the enzyme were 30° and 7.5 respectively and the enzyme followed typical Michaelis kinetics with a K_m of 10^{-5} M. Each enzyme molecule contained four molecules of FAD.

INTRODUCTION

Werle and his co-workers [1, 2] first reported the occurrence of amine oxidase activity in legumes and other plants and since then, plant diamine oxidases have been characterized mainly from various peas [3–6]. It has been also partially purified and characterized from tobacco [7], soybean [8], *Lathyrus sativus* [9] and broad bean [10] but only the enzyme from pea epicotyls [4], *Vicia faba* leaves [11] and *Euphorbia* latex [12] have been purified to homogeneity, though the enzymes have been purified from animals [13, 14] and fungi [15]. The importance of diamine oxidase, which oxidises putrescine to γ-amino-butyric acid thereby changing the levels of polyamine concentration, cannot be overlooked. We have previously reported the presence of the diamines, putrescine and cadaverine, in rice seeds [16] and arginine decarboxylase, a key enzyme in polyamine biosynthesis, in higher plants [17, 18] has been purified to homogeneity from rice embryos [19]. However the polyamine levels depend not only on the biosynthetic enzymes but also on the oxidizing enzymes. Diamine oxidase has been found in ageing rice seeds [20], and in the present paper we describe the purification and partial characterization of diamine oxidase (DAO) from rice seedlings.

RESULTS AND DISCUSSION

Purification

With the procedure described in the Experimental 1800-fold purification of the enzyme with a specific activity of 277 pkats/mg protein and a yield of 21% was achieved (Table 1). The purity of the enzyme was judged by observing a sharp single band of protein in polyacrylamide disc gel electrophoresis. The MW of the enzyme determined by Sephadex G-100 gel filtration was estimated to be 12.3×10^4, whereas the MW of DAO from *Vicia faba* [11] is 12.6×10^4, from pea [4] 18.4×10^4 and that of *Euphorbia* latex 14.4×10^4 [12]. However with SDS gel electrophoresis only one protein band with a MW of 6.12×10^4 was detected, suggesting that the rice diamine oxidase consisted of two identical subunits. Other amine oxidases also have been reported to have two identical subunits [12, 14, 15].

Table 1. Purification of diamine oxidase from *Oryza sativa*

Steps	Total protein (mg)	Sp. activity (pkat/mg protein)	Purification factor	Yield (%)
Crude extract	1572	0.154	1	100
$(NH_4)_2SO_4$ ppt	360	0.61	4	87
Acetone ppt	54	33.8	22	79
DEAE-cellulose	1.8	61.6	400	46
DEAE-sephadex	0.62	143.6	933	38
Hydroxylapatite	0.27	277	1800	21

The enzyme exhibited a pH optimum at 7.5 with putrescine as substrate and the optimal temperature was found to be 30°. The enzyme remained stable for 15 days at 4°, but the activity of the enzyme was completely lost by boiling for 10 min.

Substrate specificity

DAO was investigated for its specificity towards several amines considered to be of possible biological importance. With cadaverine about 60% activity of the enzyme was obtained in comparison to that of putrescine as substrate. Spermidine and spermine were also observed as substrate but in decreasing order (only 20% and 12% respectively). Using putrescine as the substrate the enzyme exhibited typical Michaelis–Menten kinetics with a K_m value of 10^{-5} M whereas Smith [21] reported a K_m of 4×10^{-5} M for pea enzyme and McGowan [4], a K_m value of 8×10^{-5} M for pea epicotyls enzyme. However, Srivastava *et al.* [5] showed that the K_m for cotyledon and embryo of pea was 1.6×10^{-4} M, and 9×10^{-5} M respectively, suggesting the presence of two different enzymes.

Identification of FAD and determination of its content

FAD of the enzyme was identified by comparison with standard FAD on a TLC plate under UV-light. The flavin

extracted from the enzyme migrated to the same distance as standard FAD in the TLC system. The FAD of rice DAO was distinguished from FMN and riboflavin. Furthermore the absorption spectrum of the purified enzyme showed a typical three banded spectrum for flavoprotein; one absorption maximum in the UV-range at 275 nm and two maxima in the visible range at 385 and 455 nm were observed for the native enzyme, which is almost identical to that reported by Isobe *et al.* [22].

FAD is reported to be present in amine oxidase of other systems [12, 23] though DAO is usually a copper containing enzyme [10, 12]. The flavin concentration was calculated from the molar absorbance index at 450 nm ($E_{450} = 1.13 \times 10^4$ cm M^{-}). Each molecule (dimer) of the rice DAO was found to contain four molecules of FAD whereas the FAD content was 2 moles per molecule of polyamine oxidase from *Aspergillus terrens* [24]. We wonder if one subunit of rice DAO contains two molecules of FAD.

Effect of inhibitors

Metal ions Ca^{2+}, Mg^{2+}, sulphydryl reagent such as Hg^{2+} significantly inhibited the enzyme activity and the extent of the inhibition depended on the concentration. At 10 mM Hg^{2+} inhibited the enzyme activity by 80%, but at the same concentration, Ca^{2+} and Mg^{2+} inhibited the enzyme activity to a lesser extent (37%). Previous reports also indicate that Hg^{2+} inhibited the DAO activity strongly though Mg^{2+} and Ca^{2+} were ineffective [9]. Addition of pyridoxine–HCl at 10 mM concentration inhibited the enzyme activity by 72%. EDTA also strongly suppressed the enzyme activity and at 10 mM concentration inhibition was about 80% in contrast to the marginal inhibition at high concentration of DAO activity in *Lathyrus sativus* seedlings [9].

EXPERIMENTAL

Chemicals. DEAE-cellulose, DEAE-sephadex (A-25), FAD, FMN and riboflavin were purchased from Sigma. All other chemicals used were of analytical reagent grade.

Materials. Rice seeds were obtained from the experimental garden of Bose Institute and germinated in the dark at $37 \pm 1°$ as detailed elsewhere [16].

Enzyme assay. DAO activity was measured following the method of ref. [25]. The assay mixture consisted of 2.7 ml of 0.1 M NaPi buffer (pH 7.5), 0.2 ml peroxidase (1 mg/ml), 0.5 ml hydroquinone (1 mg/ml) and protein (0.5–2.0 mg) in a total vol. of 3.9 ml. After 2 min 0.1 ml of 10 mM putrescine was added, incubated at 30° and the *A* was measured at 470 nm.

For the determination of kinetics, a different assay method was used [26]. Here the assay system consisted of NaPi buffer (pH 7.5), 50 μM; pyridoxal 5-phosphate, 0.1 μM; and an appropriate amount of purified enzyme protein and putrescine in a total vol. of 3.5 ml. After incubation at 30° for 30 min the reaction was terminated by adding 0.5 ml of 20% TCA. It was then centrifuged and to the supernatant 1 ml ninhydrin reagent (250 mg ninhydrin in 6 ml HOAc and 4 ml of 6 M H_3PO_4 was added which was then kept at 100° for 30 min to develop the colour. Readings were taken at 510 nm. Protein was determined by the method of ref. [27] after precipitation with TCA and solubilization in (1 M) NaOH.

Flavin extraction and analysis. FAD was extracted following the method of ref. [28]. The purified enzyme was subjected to heat treatment for 5 min at 100° followed by precipitation with 5% TCA. The mixture was then centrifuged at 10000 *g* for 20 min. The protein was precipitated leaving a clear supernatant. Prior to chromatography TCA was removed by three Et_2O extractions in a separating funnel and the aq. fraction containing protein-free FAD together with standard FAD, FMN and riboflavin was subjected to TLC using the following solvent system: 2-BuOH–85% HCO_2H–H_2O (14:3:3). When the run was complete, the TLC plate was dried and the spot of FAD was detected under UV-light. The FAD content of the enzyme was determined according to the method of ref. [23] which is based on the *A* decrease at 460 nm upon addition of Na-dithionite to the enzyme soln.

Polyacrylamide gel electrophoresis. This was carried out in 7.5% gel at pH 8.5 using Tris–glycine buffer according to the method of ref. [29]. Gels were stained with coomassie brilliant blue and destained with 7.5% HOAc. SDS gel electrophoresis was carried out according to ref. [30].

Enzyme purification. A six step purification procedure has been adopted in the present investigation for purification of DAO from 72 hr germinated rice seedlings. All the operations were carried out at 4°.

Step 1: rice seedlings were homogenized in NaPi buffer (10 mM, pH 7.5). The homogenate was filtered through cheese cloth and centrifuged at 10000 *g* for 20 min. Step 2: The crude extract was subjected to $(NH_4)_2SO_4$ fractionation and the protein precipitating at 35–65% satn. was dispersed in NaPi buffer (10 mM, pH 7.5) and dialysed against the same buffer for 24 hr [$(NH_4)_2SO_4$ fraction]. Step 3: The dialysed fraction was then precipitated with precooled (−20°) Me_2CO (50 ml/100 ml enzyme soln) with stirring and centrifuged at 15000 *g* for 20 min. Further Me_2CO at −20° was added (150 ml/100 ml enzyme soln) in the same manner and the ppt recovered by centrifugation at 20000 *g* for 30 min was dissolved in NaPi buffer (10 mM, pH 7.5), dialysed and centrifuged at 5000 *g* for 5 min (Me_2CO fraction). Step 4: The Me_2CO fraction was applied to a DEAE-cellulose column (25 cm × 2.2 cm) previously equilibrated with NaPi buffer (0.1 M, pH 7.5) and the column was washed with 200 ml of the same buffer. The enzyme fractions were eluted from the column with 0.1 M NaPi buffer containing 0.2 M NaCl. The active fractions were pooled, brought to 70% $(NH_4)_2SO_4$ saturation, centrifuged at 10000 *g* for 20 min, the ppt dissolved in 10 mM NaPi buffer and dialysed. Step 5: The dialysed enzyme fraction (10 ml) was passed through the DEAE-Sephadex (A-25) column (33 cm × 1.8 cm) equilibrated with NaPi buffer (0.1 M, pH 7.5) and inactive protein was washed from the column with NaPi buffer containing 0.1 M NaCl. The enzyme was then eluted from the column with 0.3 M NaCl in NaPi buffer (0.1 M, pH 7.5), precipitated with 70% $(NH_4)_2SO_4$, centrifuged at 10000 *g* for 20 min, dissolved in 10 mM NaPi buffer and dialysed overnight. Step 6: The column (4 cm × 2 cm) of hydroxylapatite was equilibrated with NaPi buffer (0.1 M, pH 7.5). The enzyme fraction obtained in step 5 was passed through the column and the column was washed with the same buffer and then the enzyme was eluted with NaPi buffer (0.3 M, pH 7.5).

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