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PURIFICATION AND PROPERTIES OF DIAMINE OXIDASE FROM PEA EPICOTYLS

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Key Word Index—*Pisum sativum*; Leguminosae; pea epicotyl; amino acid composition; diamine oxidase.

Abstract—Diamine oxidase (DAO) (EC 1.4.3.6) was purified from pea epicotyls to homogeneity by the criterion of polyacrylamide gel electrophoresis (PAGE). The purified enzyme showed absorption maxima at 280 and 500 nm and ornithine was absent from the component amino acids. The MW estimated by gel filtration was *ca* 180 000 and the enzyme contained 1 mol of carbonyl group per mol. Sodium dodecylsulphate (SDS) gel electrophoresis yielded a single band at a MW of 85 000. The isoelectric point was pI 7.4. Methylglyoxalbis(guanylhydrazone) (MGBG) was inhibitory, and MGBG-Sepharose was used as a purification step.

INTRODUCTION

Preparations of pea seedling diamine oxidase (DAO) partially purified separately from cotyledon and embryo have been shown to differ in respect to K_m, thermal stability and electrophoretic mobility [1]. The enzyme is very active in the cotyledons, but less so in the epicotyl [2], and many workers have not segregated these organs prior to extraction and purification [3–5]. McGowan and Muir, however, have studied the properties of homogeneous DAO isolated from pea epicotyls [6]. Pea DAO, like other DAOs from animals [7–9] and bacteria [10], contains copper and unidentified carbonyl group [11]. In the present work, we describe a simple procedure for the purification of DAO from pea epicotyls to homogeneity by the use of phosphocellulose and MGBG-Sepharose 4B.

RESULTS AND DISCUSSION

Enzyme purification

The DAO was purified by column chromatography on phosphocellulose and MGBG-Sepharose. The procedures were developed using DAO which had been treated with 5% protamine and then concentrated with ammonium sulphate of 65% saturation [6]. The results of purification are summarized in Table 1. The enzyme could be purified to homogeneity by the MGBG-Sepharose procedure (Fig. 1). Our procedure for the enzyme preparation was simpler and gave higher yields than that of ref. [6]. The recovery of the enzyme in these experiments was 35%, but the purification resulted in only 32-fold increase in specific activity. The absorption spectrum of the purified enzyme is characterized by the maxima at 280 and 500 nm.

Bio-gel P-200 chromatography

The apparent MW of native enzyme, estimated by Bio-gel P-200 chromatography, was 180 000. This value agreed with results reported earlier [5, 6].

Electrophoretic behaviour

Disc gel electrophoresis of the purified enzyme yielded a single protein band at pH 4.3 and 9.4 and the enzyme activity on each gel was associated with this band. Using the SDS gel electrophoresis system, a single band of MW of 85 000 appeared. These results suggest that the enzyme consists of two identical subunits as in other copper containing amine oxidases [9, 10, 12]. Isoelectric focusing of purified enzyme showed a peak of activity with pI 7.4.

Inhibition by MGBG

Williams-Ashman and Schenone [13] reported that MGBG strongly inhibits putrescine-activated *S*-

Table 1. Purification of pea epicotyl DAO

Step	Fraction	Total vol. (ml)	Total act. (μkat)	Protein (mg/ml)	Sp. act. (nkat/mg)	Recovery (%)
1.	Crude extract	1240	103	2.3	36.1	100
2.	$(NH_4)_2SO_4$ ppt.	80	56.2	15.5	45.2	54
3.	P-cellulose column eluate	56	41.3	1.6	460.9	40
4.	MGBG-Sepharose 4B column eluate	24	36.2	1.3	1160.3	35

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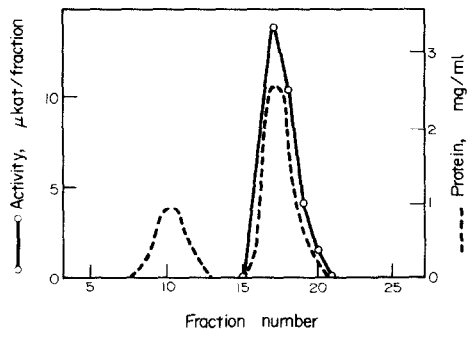

Fig. 1. Gel chromatography of pea epicotyl DAO on MGBG-Sepharose 4B.

adenosylmethionine decarboxylases of rat ventral prostate and baker's yeast. MGBG was also a strong inhibitor of *S*-adenosylmethionine decarboxylases of maize shoots [14] and of pea epicotyl (unpublished work). The injection of MGBG into the rat markedly decreased the activity of DAO in thymus and it was also a potent inhibitor of DAO *in vivo* [15]. MGBG was also an inhibitor of pea DAO [16]. The inhibition of pea DAO by MGBG was dependent on the preincubation time. The inhibition type in the case of non-preincubation appears to be competitive with a K_i of 2.6×10^{-4} M. However, when the enzyme was preincubated with MGBG for 1 hr at 30°, the inhibition was non-competitive and its K_i value was 1.7×10^{-5} M (Fig. 2). Aminoguanidine, an integral part of the MGBG molecule, is a powerful inhibitor of DAO [17], but it is somewhat unlikely that MGBG acts solely as a carbonyl reagent, since MGBG did not influence the absorption spectrum of an equimolar pyridoxal phosphate (PLP) or pyruvate solution in the range of about 330–550 nm. In addition, MGBG-inhibited enzyme could not be reactivated by the addition of PLP or pyruvate which is 10-fold higher as molar concentrations, but about 50% of the enzyme activity was recovered after dialysis overnight against 10 mM Tris–HCl buffer, pH 7.5. The effect of MGBG on polyamine metabolism in pea seedlings appears to be complicated because the reagent is not only a potent inhibitor of putrescine degradation by DAO but it is also a powerful inhibitor of *S*-adenosylmethionine decarboxylase which produces decarboxylated-*S*-adenosylmethionine as a substrate for the spermidine synthetase [18].

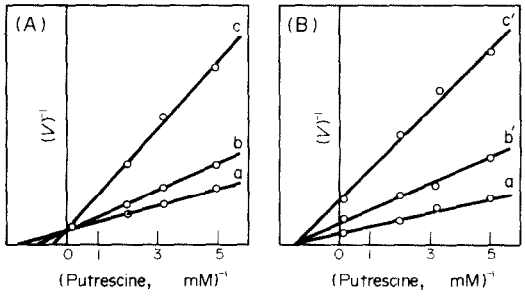

Fig. 2. Competitive and non-competitive inhibitions of pea epicotyl DAO by MGBG. A: No preincubation, B: preincubation. Additions of MGBG: a, none; b, 3.3×10^{-4} M; c, 10^{-3} M; b′, 2×10^{-5} M; c′, 5×10^{-5} M. Enzyme of 0.14 μg was used. Standard assay conditions were used, except for 0.5 M Tris–maleate buffer, pH 7.

Carbonyl radical requirement of pea diamine oxidase

It is well known that amine oxidases containing copper are strongly inhibited by phenylhydrazine, hydrazine and semicarbazide [17], implying that a carbonyl group is involved in the active state. PLP or pyruvate do not seem to be constituents of the carbonyl group in pea DAO, since the peak at 410 or 323 nm which is characteristic of phenylhydrazone of PLP [19] or pyruvate [20, 21], respectively, was not found in the spectrum after the addition of phenylhydrazine. However, a new peak of absorption at 450 nm was observed. This position of the peak is similar to those found for amine oxidase from *Aspergillus niger* [22], but it differs somewhat from the peak at 435 nm of ref. [23]. According to Pegg [24], the enzyme which had been treated with NaB^3H_4 was hydrolysed and then the hydrolysate was subjected to PC using a solvent system: PrOH–18 M NH_4OH (4:1). However, no radioactive spot was found for the R_f value of lactate. The increase in the A at 450 nm and the corresponding decrease in the enzyme activity were linear at pH 7.5 with the amount of phenylhydrazine added until the molar ratio of it to the DAO (assuming a MW of 180 000) reached 1.1 and no more change was observed at higher molar ratio. Therefore, 1 mol of pea DAO contains 1 mol of carbonyl group which is essential for its activity. This value is similar to that found for pig plasma amine oxidase [25], but differs from those of *Aspergillus niger* [10]. The peak at 450 nm is probably due to absorption of a chromophore by an enzyme–phenylhydrazine complex and its millimolar extinction coefficient calculated from the spectrophotometric titration data was 48.3/mM per cm. The nature of the chromophore, as in other amine oxidases containing copper, remains unsolved.

Amino acid composition

The amino acid composition of our purified enzyme is shown in Table 2. In contrast to the results of Nylén and Szybek [23], our preparation does not contain ornithine but it does contain a half-cystine molecule. In addition, the enzyme contained 13% sugar as glucose determined according to the method of ref. [26]. Similar results were obtained for the amino acid composition of DAO from *Vicia faba* (unpublished work).

EXPERIMENTAL

Plant. Pea (*Pisum sativum* cv Alaska) seeds were germinated and grown for 8 days in moist vermiculite in plastic trays at 25° in total darkness.

Chemicals. The following were used: catalase, aldolase, trypsin inhibitor (chicken egg white) and putrescine. 2 HCl (Sigma); trypsin inhibitor (soybean), BSA and *E. coli* RNA polymerases (α, β and β′) (Boehringer); MGBG (Aldrich); CH-Sepharose 4B and Pharmalyte (Pharmacia); Bio-gel P-200 (Bio-Rad); phosphocellulose (Serva); and NaB^3H_4 (New England Nuclear). MGBG-Sepharose-4B was prepared as in ref. [14].

Enzyme purification. The initial 65% $(NH_4)_2SO_4$ fraction obtained from *ca* 700 g of fresh epicotyls of pea seedlings [6] was suspended in 80 ml of 50 mM KPi buffer, pH 6.4 and dialysed against 2 l. of 20 mM KPi buffer, pH 6.4, with one change. After centrifugation (10 000 *g*, 15 min), the supernatant (80 ml) was applied to a phosphocellulose column (2 × 25 cm) equilibrated

Table 2. Amino acid composition of pea DAO

Amino acid residue	
Aspartic acid	63.2
Threonine	37.0
Serine	36.9
Glutamic acid	51.8
Proline	12.6
Glycine	34.1
Alanine	28.1
Half-cystine	3.5
Valine	54.8
Methionine	1.0
Isoleucine	48.1
Leucine	41.7
Tyrosine	20.5
Phenylalanine	29.0
Histidine	21.5
Lysine	32.5
Arginine	23.0

Average values of hydrolysis for 24 and 48 hr. Values are expressed as mol of amino acid per mol of methionine found in the hydrolysate. Tryptophan was not determined.

with 20 mM KPi buffer, pH 6.4. After washing the column with 0.5 l. of equilibrating buffer until the eluate had no further *A* at 280 nm, the enzyme was eluted with 0.1 M KPi buffer, pH 7 (flow rate 14 ml/hr; 6-ml fractions). Active fractions (56 ml) were concd with an ultrafilter (Toyo UK-10) to 5 ml (*ca* 0.9 g protein), and then dialysed against 2 l. of 10 mM Tris–HCl buffer, pH 7.5. The dialysate was applied to a MGBG column (1.5 × 23 cm) [14], and the enzyme was eluted with 10 mM Tris–HCl buffer, pH 7.5 (flow rate 30 ml/hr; 6-ml fractions) (Fig. 1). The active fractions (tubes 16–19: 24 ml) was used as the purified enzyme.

The activity of DAO was determined according to the method of ref. [27]. The incubation mixture consisted of 0.2 ml of 50 mM putrescine, 0.6 ml of 0.5 M KPi buffer, pH 7, 0.2 ml of 0.1% *o*-aminobenzaldehyde (in EtOH) and appropriate amounts of enzyme in a total vol of 3 ml. The reaction was initiated by adding the putrescine, and the reaction was stopped by adding 0.2 ml of 50% TCA. After centrifugation, *A* was estimated at 435 nm.

The MW of the enzyme was determined with a molecular sieve according to ref. [28]. Catalase (232 000), aldolase (158 000), BSA (136 000) and trypsin inhibitor (chicken egg white: 28 000) were used to calibrate the Bio-gel P-200 column.

Gel electrophoresis was carried out by the method of ref. [29]. The DAO (*ca* 40 μg) on 7.5% polyacrylamide gel (0.5 × 6.5 cm) was separated at 3 mA/tube (3 hr for pH 4.3; 4 hr for pH 9.4). The gels were stained for 1 hr with 0.1% Coomassie brilliant blue in 7% HOAc to locate protein. Enzyme on gels was detected in a similar manner as in ref. [30]. SDS gel electrophoresis was performed according to ref. [31]. The enzyme (20 μg) was previously treated with 1% SDS and 3% 2-mercaptoethanol at 100° for 5 min. BSA (68 000), trypsin inhibitor (soybean: 21 500), *E. coli* RNA polymerase α (39 000), β (155 000) and β′(165 000) were used as the marker protein.

Isoelectric point was determined by gel electrofocusing technique [32].

Amino acid composition was determined according to the method of ref. [33] for the two independently purified enzymes. Amounts of threonine (4.8%), serine (12%), methionine (14.3%) and tyrosine (9%) were corrected for loss during hydrolysis for 24 hr. Valine (3.9%) was corrected for incomplete release. Half-cystine was determined after performic acid oxidation [34]. Protein was determined according to ref. [35].

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