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AMINOALDEHYDE DEHYDROGENASE OF PEA EPICOTYLS

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Key Word Index—*Pisum sativum*; Leguminosae; pea epicotyl; aminoaldehyde dehydrogenase; 1,5-diazabicyclo[4.3.0]nonane.

Abstract—NAD-dependent aminoaldehyde dehydrogenase was partially purified *ca* 110-fold from pea epicotyls. The purified enzyme (M_r *ca* 82 000) catalysed the oxidation of 1,5-diazabicyclo[4.3.0]nonane (1,5-DBN) to *N*-(3-aminopropyl)-2-pyrrolidinone. The K_m values were 1.0×10^{-4} M for 1,5-DBN, 2.6×10^{-5} M for 3-aminopropionaldehyde (3-APA), 1.2×10^{-4} M for 4-aminobutyraldehyde, 2.2×10^{-4} M for indole-3-acetaldehyde and 3.7×10^{-5} M for NAD (in the case of 3-APA). The enzyme had some activity for an aldehyde from spermine oxidized by pea diamine oxidase. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Plant diamine oxidases (DAOs, EC 1.4.3.6) are of widespread occurrence in leguminous plants. They oxidatively deaminate putrescine (Put), cadaverine and spermidine (Spd) to 4-aminobutyraldehyde (4-ABA), 5-aminopentyraldehyde and 1,5-diazabicyclo [4.3.0]nonane (1,5-DBN), respectively [1, 2]. 1,3-Diaminopropane (1,3-DAP) was a suicide substrate for pea DAO [3], while the enzymes from *Setaria italica* [4] and *Triticum aestivum* [5] oxidized 1,3-DAP, the best substrate tested, to 3-aminopropionaldehyde (3-APA).

In our previous paper, we showed that 3-APA and 4-ABA were further oxidized to β-alanine and γ-aminobutyric acid (GABA) by 3-APA dehydrogenase from *S. italica* [6]. The same enzymes as 3-APA dehydrogenase, γ-guanidinobutyraldehyde dehydrogenase from *Vicia faba* [7] and 1-pyrroline dehydrogenase from *Pisum sativum* and *Avena sativa* [8] were found. This paper describes the partial purification and properties of pea aminoaldehyde dehydrogenase (AAD), which we named on the basis of its substrate specificity.

RESULTS AND DISCUSSION

The purification of pea AAD using $(NH_4)_2SO_4$ fractionation, and DEAE-cellulose, hydroxyapatite and Sephacryl S-200 column chromatography is summarized in Table 1. The apparent M_r of the enzyme estimated by Sephacryl S-200 gel filtration is *ca* 82 000. The enzyme was most stable at pH 6.0, and could not be further purified due to its lability after elution from the Sephacryl column. When we examined the purity of the enzyme purified *ca* 110-fold, four bands were detected in the gel. The optimum pH of the enzyme for 3-APA and 1,5-DBN was 9.0.

AAD had some activity for indole-3-acetaldehyde (IAAld), 1,5-DBN and an aldehyde formed in oxidation of spermine (Spm) with pea DAO (Table 2). An amine oxidase from *Lens culinaris* catalyses the oxidation of Spm to a dialdehyde [9]. In the case of pea DAO, 1 mol of H_2O_2 was produced from 1 mol of each of the following substrates: Put, Spd and Spm (data not shown). The results suggested that pea DAO catalysed the oxidation of Spm to *N*-(3-aminopropyl)-1,4-butanediamine-*N'*-propionaldehyde, and pea AAD catalysed the oxidation of the monoaldehyde to *N*-(3-aminopropyl)-1,4-butanediamine-*N'*-propionic acid. An aldehyde dehydrogenase (NAD-dependent) in extracts from *Phaseolus aureus* catalyses the oxidation of IAAld to indole-3-acetic acid [10]. The enzyme may be AAD. The primary amine group in 3-APA and 4-ABA, the secondary amine group in 1,5-DBN and nitrogen in IAAld seem to be essential for the activity of AAD.

The stoichiometry of AAD was obtained by measuring the disappearance of 3-APA or 4-ABA, and the appearance of β-alanine or GABA and NADH, respectively. About 1 mol each of β-alanine or GABA and NADH were produced from 1 mol of 3-APA or 4-ABA, respectively. *N*-(3-Aminopropyl)-2-pyrrolidinone (*N*-APN) was detected when reacted

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Table 1. Purification of pea AAD

Step	Volume (ml)	Total protein (mg)	Total activity (nkat)	Specific activity (nkat per mg protein)	Recovery (%)
Crude extract	310	558	112	0.201	100
45–65% $(NH_4)_2SO_4$ precipitate	60	86.4	272	3.15	243
DEAE-cellulose chromatography	30	15.3	152	9.93	136
Hydroxyapatite chromatography	45	7.2	143	19.9	128
Sephacryl S-200 chromatography	3.3	1.85	41	22.2	37

Table 2. Substrate specificity

Substrate	Relative reaction rate (%)
3-APA	100 (12)†
4-ABA	57
5-Aminopentyraldehyde*	33
1,5-DBN	21
1,5-Diazabicyclo[4.3.0]non-5-ene	0
N-APN	0
N-(3-aminopropyl)-1,4-butane-diamine-*N'*-propionaldehyde*	18
Indole-3-acetaldehyde	54
Aminoacetaldehyde	1
Glutaraldehyde	27
Succinaldehyde	8
Propionaldehyde	0
Acetaldehyde	0
Succinic semialdehyde	0

The assay mixture contained 670 μM NAD and 800 μM aldehyde or analogue.

*Pea DAO soln (1 ml of 0.5 nkat ml^{-1}) in 0.1 M K-Pi buffer (pH 7) was added to 0.1 ml of 20 mM amine and incubated for 10 min at 37°. The incubated mixture was then added to the assay mixture for AAD.

†NADP (670 μM) was used instead of NAD.

mixture using 1,5-DBN replaced 3-APA as the substrate in the reaction mixture. After PAGE, A clear band was detected in the gel by the AAD-staining method using 3-APA. A weak band appeared in the gel when 1,5-DBN was used. When 3-APA was added to gel in which the weak band had been detected using 1,5-DBN, the clear band overlapped the weak band in the gel. The results suggested that oxidation of both 3-APA and 1,5-DBN is due to the same enzyme, namely AAD. 1,5-DBN was detected in oats, maize, barley and wheat [1]. In mouse, *N*-APN is a product of Spm catabolism *in vivo* [11]. The K_m values were 1.0×10^{-4} M for 1,5-DBN, 2.6×10^{-5} M for 3-APA, 1.2×10^{-4} M for 4-ABA, 2.2×10^{-4} M for IAAld and 3.7×10^{-5} M for NAD (in the case of 3-APA). When pea DAO was added to a mixture containing pea AAD, NAD, 1,3-DAP and glycine–NaOH (pH 9.0), the A_{340} nm increased linearly for 15 min at 37° (data not shown). Pea DAO is inhibited irreversibly by 3-APA produced from 1,3-DAP by DAO [2]. The results suggested that 3-APA was oxidized immediately by AAD due to its low K_m value for 3-APA.

EXPERIMENTAL

Plant. Pea (*P. sativum* cv. Alaska) seeds were germinated and grown for 5 days in moist vermiculite in plastic trays at 25° in total darkness.

Chemicals. 3-APA diethylacetal, succinaldehyde bis(dimethylacetal) and PVP pyrrolidone were

obtained from Tokyo Kasei, *N*-ethyl-*N*-(2-hydroxy-3-sulphopropyl)-3-methylaniline from Dojin, 1,5-diazabicyclo [4.3.0]non-5-ene and *N*-APN from Aldrich, Sephacryl S-200 HR from Pharmacia, aminoacetaldehyde diethylacetal, 4-ABA diethylacetal and other chemicals (pure grade) from Wako. Aminoacetaldehyde, 3-APA, 4-ABA and succinaldehyde were prepd from their acetals, respectively, by heating to 80° with 0.1 M HCl in a plugged test tube for 10 min. 1,5-DBN was prepd according to ref. [1].

Activity of AAD. This was routinely assayed by measuring the increase in A at 340 nm of the reaction mixt. at 37°. The standard assay mixture consisted of 0.5 ml 0.5 M glycine–NaOH buffer (pH 9.0), 24 μl 0.1 M 3-APA, 0.1 ml 20 mM NAD and enzyme soln in a total vol. of 3 ml. The assay was initiated by addition of substrate and the changes in A_{340} monitored. Enzyme activity is presented as the amounts of NADH formed (mol s^{-1}) in the standard assay system.

Pea AAD. All the operation were carried out at 4°. The washed epicotyls (150 g) were ground in a Waring blender with 300 ml 0.1 M K-Pi buffer (pH 6) containing 15 mM 2-mercaptoethanol, 10% glycerol and 1% PVP (insoluble). The resulting slurry was filtered through a layer of cotton cloth, then centrifuged at 10 000 *g* for 15 min. The supernatant was fractionated stepwise with solid $(NH_4)_2SO_4$, and the fr. obtained between 40 and 65% satn was collected by centrifugation. The ppt. was dissolved in 40 ml 0.1 M K-Pi buffer (pH 6) containing 15 mM 2-mercaptoethanol and 10% glycerol (buffer A) and centrifuged at 10 000 *g* for 10 min. The supernatant was freed from $(NH_4)_2SO_4$ by passing the soln through a Sephadex G-25 column (2.2 × 50 cm) equilibrated with buffer A. The active fr. (60 ml) was applied to a DEAE cellulose column (1.5 × 24 cm) equilibrated with buffer A. The enzyme was eluted with 100 ml of a linear gradient of buffer A to buffer A containing 0.5 M NaCl. The active frs (30 ml) were applied to a hydroxyapatite column (1.4 × 5 cm) equilibrated with buffer A. The column was washed with 150 ml buffer A containing 1 M NaCl and then washed with 150 ml of buffer A. The enzyme was eluted with 100 ml of a linear gradient of buffer A to 0.5 M K-Pi buffer (pH 6) containing 15 mM 2-mercaptoethanol and 10% glycerol. The active frs (45 ml) were collected and concd in a collodion bag. The concd sample (1 ml) was applied to a Sephacryl S-200 HR column (1.5 × 117 cm) equilibrated with buffer A. The active frs (3.3 ml) were collected and used as the purified enzyme.

Determination of H_2O_2 produced from amines by DAO. A modification of the quinoneimine dye method [12] was used. The assay mixt. consisted of 0.1 ml 1 mM amine, 1 ml 0.1 M K-Pi buffer (pH 7), 0.1 ml 4-aminoantipyrine, 10 mM *N*-ethyl-*N*-(2-hydroxy-3-sulphopropyl)-3-methylaniline, horseradish peroxidase (1 mg ml^{-1}) and pea DAO soln (0.5 nkat ml^{-1} with Put) in a total vol. of 3 ml. The reaction was initiated on adding the amine. Incubation was for 30 min at 37° after which the A_{555} was measured. The dye produced in the mixt. was stable between pH 5.5 and 9.0.

Stoichiometry. After incubation of the standard assay mixt. containing either 3-APA or 4-ABA, the mixt. was applied to an Ultrafree C3 10 000 NMWL-PLGC membrane (Millipore) and centrifuged. The filtrate was then analysed (Hitachi Amino Acid Analyser L-8500, elution buffer Mitsubishi-MCI L-8500 kit) for the presence and amounts of 3-APA and β-alanine, or 4-ABA and GABA.

In the case of the incubation containing 1,5-DBN, the post Ultrafree filtrate was analysed by HPLC [Shimadzu LC system equipped with LC-10A pump, SPD-10 Vis–UV HPLC monitor (A = 210 nm); column: Shim-pack CLC-ODC (M) 4.6 × 250 mm; solvent: 10 mM $NaClO_4$ containing 1 mM 1-octanesulphonic acid and 0.2% $HClO_4$ in H_2O–MeCN (9:1); flow rate 0.8 ml min^{-1}; *N*-APN and 5-DBN were eluted at *ca* 10 and 20 min, respectively].

PAGE. This was carried out by the method of ref. [13]. The purified enzyme (0.5 nkat) on 7.5% polyacrylamide gel (0.5 × 6.5 cm) pH 9.4, was sepd at 1.5 mA/tube for 6 hr in a cold room. Protein in the gels was stained with 0.1% Coomassie brilliant blue. To stain for AAD, the gels were incubated at 30° in a soln consisting of 1 ml glycine–NaOH buffer (pH 9), 0.15 ml 20 mM NAD, 3.5 mg nitro blue tetrazolium, 0.1 ml phenazine methosulphate (20 mg ml^{-1}) and 0.06 ml 0.1 M 3-APA or 1,5-DBN in a total vol. of 10 ml.

Pea DAO. DAO was prepd from pea epicotyls using P-cellulose and hydroxyapatite columns according to refs. [3, 14].

Protein. This was estimated according to ref. [15] with BSA as standard.

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