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IMMUNOAFFINITY PURIFICATION AND CHARACTERIZATION OF DIAMINE OXIDASE FROM *CICER*

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Key Word Index—*Cicer arietinum*; Leguminosae; diamine oxidase; immunoaffinity purification.

Abstract—Antiserum specific for diamine oxidase (DAO; EC 1.4.3.6) from *Lens culinaris* cross-reacted with DAO from several other members of the Leguminosae when tested by agar double diffusion. Antibodies purified by affinity chromatography were used to make an immunoadsorbent for the one-step purification of DAO from various species of the Leguminosae. This technique has made it possible to purify in one step the already characterized DAO from pea and lentil, and the unknown diamine oxidase from *Cicer arietinum*. This enzyme was partially characterized; it showed a pH optimum of 7.5 with putrescine as substrate and followed typical Michaelis–Menten kinetics with a K_m of 2.4×10^{-4} M. Copper ligands and carbonyl group-directed reagents inhibited the enzyme.

INTRODUCTION

Plant diamine oxidases, which catalyse the following reaction:

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

are important in several cellular functions such as regulation of intracellular polyamine concentration [1], initial nutrition of the seedling [2], and IAA biosynthesis [3]. Plant DAOs contain copper [1] and it has been suggested that their coenzyme could be FAD [4] or a pyrroquinoline quinone [5]. In the plant kingdom DAO has been purified to homogeneity from pea epicotyls [6], *Vicia faba* leaves [7], *Euphorbia characias* latex [8] and *Lens esculenta* [9] and has been partially purified and characterized from tobacco [10], soybean [11], *Lathyrus sativus* [12], broad bean [13] and rice [4].

Cross-reactivity of antibodies raised against pure lentil DAO with diamine oxidases present in other species of the Leguminosae suggested to us the possible use of immunoaffinity techniques for their purification [14, 15]. The present paper describes a rapid and simple method for one-step purification of DAO from Leguminosae and also some properties of the enzyme from *Cicer arietinum* shoots.

RESULTS AND DISCUSSION

Immunodiffusion

Analysis by Ouchterlony double diffusion in agar gel revealed that antiserum specific for the lentil DAO cross-reacts with the same enzymes present in crude homogenates from shoots or cotyledons of pea or *Cicer arietinum* seedlings. These enzymes showed partial identity with the lentil DAO indicating that they share some, but not all, antigenic determinants. Immunoprecipitation lines were active when stained for DAO activity.

Purification

With the immunoaffinity procedure described in Experimental a one-step purification of diamine oxidase from lentil, pea and *Cicer arietinum* shoots and cotyledons was achieved. The procedure was repeated at least five times and the yield of protein obtained was almost the same for each experiment. In Table 1 is reported a typical purification procedure. For all the enzymes only one band was observed in polyacrylamide gel electrophoresis (PAGE) whether the gel was stained for protein or for enzymatic activity. The enzymes purified from both the cotyledons and the shoots of the different species used showed the same electrophoretic mobility in PAGE. Chemicophysical characteristics of immunoaffinity-purified lentil DAO were similar to those of the same enzyme prepared as described by Floris *et al.* [9].

Partial characterization of DAO from Cicer arietinum *shoots*

The general characteristics of DAO from *Cicer arietinum* are very similar to those of the lentil and pea enzymes. Using putrescine as substrate the enzyme exhibited typical Michaelis–Menten kinetics with a K_m of 2.4×10^{-4} M. The effect of pH on the enzyme was tested in the pH range 4.5–8.5 with putrescine as substrate. Buffers (all at 0.1 M) were: sodium acetate (pH 4.5–5.9), potassium phosphate (pH 6.0–7.5) and Tris–HCl (pH 7.6–8.5). The pH optimum was 7.5 in 0.1 M potassium phosphate buffer. The enzyme was stable for 20 days at 4° but its activity was reduced to 50% of maximum by heating for 20 min at 70°.

Substrate specificity and inhibitors

The substrate specificity of DAO from *Cicer arietinum* shoots towards several amines was studied. The enzymatic activity is high for putrescine and cadaverine (80% compared to that of putrescine as substrate) and very low

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Table 1. Purification of DAO from shoots and cotyledons of lentil, pea and *Cicer*

	Total volume (ml)	Total activity (μkat)	Total protein (mg)	Specific activity (μkat/mg protein)	Purification (fold)	Recovery (%)
Lentil shoots						
Crude homogenate	400	12.1	13400	9×10^{-4}	—	100
Immunoadsorbent eluate	15	8.4	7.1	1.2	1330	70
Lentil cotyledons						
Crude homogenate	300	11.6	21500	5.4×10^{-4}	—	100
Immunoadsorbent eluate	15	8.8	7.3	1.2	2220	76
Pea shoots						
Crude homogenate	400	11.8	10100	1.2×10^{-3}	—	100
Immunoadsorbent eluate	15	8.5	7.2	1.2	1000	72
Pea cotyledons						
Crude homogenate	250	10.7	21100	5.1×10^{-4}	—	100
Immunoadsorbent eluate	15	8.1	7.0	1.2	2353	76
Cicer shoots						
Crude homogenate	400	9.5	10600	8.9×10^{-4}	—	100
Immunoadsorbent eluate	15	7.0	7.1	0.99	1112	74
Cicer cotyledons						
Crude homogenate	300	9.2	24700	3.7×10^{-4}	—	100
Immunoadsorbent eluate	15	6.9	6.8	1.0	2700	75

for spermidine (15%). No activity was found with spermine, 1,3-diaminopropane, ornithine and 4-aminobutyric acid. DAO from *Cicer arietinum* shoots, like the *Euphorbia characias* and the lentil enzyme, does not oxidize histamine [8, 9]. Copper ligands (sodium azide and sodium cyanide) and carbonyl group-directed reagents (phenylhydrazine and semicarbazide) inhibit the enzyme, as already found for other amine oxidases [9].

EXPERIMENTAL

Chemicals. Peroxidase and benzidine were purchased from Sigma; CNBr-activated Sepharose 4B was from Pharmacia. All other chemicals were obtained as pure commercial products.

Plant. Commercial seeds of lentil (*Lens culinaris* L.), pea (*Pisum sativum* L.) and *Cicer arietinum* L. were soaked for 12 hr in aerated tap water and grown in moistened vermiculite for 10 days in the dark at 25°.

Homogenates. The crude homogenates were obtained by grinding the plant tissues in a Waring Blendor with 3 vols of 0.01 M KPi buffer, pH 7, for 2 min. The suspension was pressed through a cotton sack, and centrifuged at 20000 rpm for 30 min. The ppt was discarded and the supernatant used both for the immunodiffusion and the immunoaffinity purification procedure described below.

Antibody preparation and immunological methods. Anti-lentil DAO antiserum and specific antibodies were prepared as previously described [16]. Double diffusion in agar gel was performed according to ref. [17]. Immunoprecipitation lines with DAO activity were detected by staining the agar gel in 0.1 M KPi buffer, pH 7, containing 100 μg peroxidase, 1 mg benzidine and 17 mM cadaverine. The immunoadsorbent, containing 3 mg of specific anti-DAO-antibodies per ml of swollen CNBr-activated Sepharose 4B, was prepared as described in ref. [16].

Enzyme assay. DAO activity was estimated in a Gilson oxigraph equipped with a Clark electrode. The reaction was carried out according to ref. [8] and was started by addition of a small vol of amine substrate (as the hydrochloride salt) after at least 10 min preincubation. Protein bands with DAO activity were detected by PAGE by staining the gels, after the run, as reported for the immunodiffusion analysis.

Purification of diamine oxidase. The different crude extracts (vols in Table 1) were applied to 4 ml of anti-lentil DAO-immunoadsorbent equilibrated with 0.01 M phosphate-buffered saline, pH 7, at flow rate of 80 ml hr. The column was then washed with 0.5 M NaCl in 15 mM KPi, until the A_{280} had fallen to background level. The elution conditions were different for the different enzymes according to their differing stabilities to the elution media. For the pea and lentil enzymes elution was performed with 0.1 M KPi–glycerol (1:1), p_aH 11.5 (the pH in mixed hydro-organic media is termed p_aH, $_aH$ being the protonic activity in mixed solvent [18]), followed by neutralization of the eluates with 1 M KPi [16]. The enzyme from shoots or cotyledons of *Cicer arietinum* was eluted with 3 M KI. For both procedures the collected fractions were pooled, dialysed against 50 mM KPi, pH 7, and concd by ultradialysis. The elution yield was 75% calculated in activity units. The specific activities of the eluted lentil and pea enzymes were of the same order of magnitude as those of the same enzymes purified with conventional procedures [6, 9].

Regeneration of the immunoadsorbent. Regeneration of the immunoadsorbent was easily achieved by washing it thoroughly with 0.01 M phosphate-buffered saline, pH 7. The immunoadsorbent was stored for subsequent use at 4° in 0.01 M phosphate-buffered saline, pH 7 containing 0.2% NaN_3 and it was reutilized several times without any lack of efficiency.

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