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PURIFICATION AND PROPERTIES OF ORNITHINE DECARBOXYLASE FROM GERMINATED BARLEY SEEDS

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Abstract—Chromatin-bound ornithine decarboxylase of germinated barley seeds has been purified to homogeneity. The M_r is 55000 with pI 5 2 and K_m for L-ornithine 0.36 mM An ornithine decarboxylase–antizyme inactive complex was also co-purified.

INTRODUCTION

In animals, ornithine decarboxylase, a pyridoxal phosphate dependent enzyme, (ODC, EC 4.1.1.17) is the first and rate limiting enzyme in polyamine biosynthesis This enzyme responds rapidly to various agents such as hormones, drugs, growth factors and plays an important role in the control of growth, proliferation and differentiation of cells [1, 2].

In microorganisms and mammalian cells the properties and the regulation of this enzyme have been extensively studied in the last decade [1–3]. Although ODC was initially characterized as a cytosolic enzyme, recent reports indicate the presence of this enzyme also in the nucleus [2, 4]

In plant cells putrescine is formed either from L-arginine decarboxylase (ADC, EC 4.1.1.19) or from ODC [4]. Although ODC in plant cells was thought to be less important than ADC, recently it has been shown that considerable ODC activity exists in several plant tissues [4] In germinated barley seeds the cytosolic and the chromatin-bound ODC are induced by gibberellic acid (GA_3) and superinduced by actinomycin D [5]. Both activities are inhibited by diamines or polyamines *in vivo* through the non-competitive inhibitory protein, antizyme [6]

The induction and the properties of antizyme have been described in a variety of normal or cancer cells and in *E. coli* [1]. Two antizymes of ODC have been extracted from germinated barley seeds, one from the cytosol with M_r 16000 and another from chromatin with M_r 9000 [7, 8]

In this paper the purification to homogeneity of ODC from plant tissue is reported for the first time. A partial purification of cytosolic and chromatin-bound ODC has previously communicated [9]. The physical and catalytic properties of chromatin-bound ODC are described and evidence is presented for the existence of an ODC-antizyme complex in the germinated barley seeds

RESULTS AND DISCUSSION

Extraction of ODC from chromatin

The extraction of ODC activity was achieved by freezing and thawing the chromatin three times, a procedure which releases 80–90% of ODC activity When the three extracts were combined, ODC activity represented 80% of total ODC activity extracted from chromatin This result suggested that an inhibitor exists in the initial extract that can inhibit ODC activity and that this inhibitor can be removed during the purification. A variety of reagents such as salts, non ionic detergents, EDTA, and polyamines were unsuccessful in extracting ODC from chromatin Autoincubation or incubation with pancreatic DNase I also proved ineffective [5] Therefore, the most advantageous procedure remained the extraction of ODC by freezing and thawing

Purification of ornithine decarboxylase from chromatin

The purification procedure which was developed for the chromatin-bound ODC is summarized in Table 1 Fractionation of the extracted ODC by ammonium sulphate or by increasing the temperature of the solution were unsuccessful as steps of purification A calculation of the yield and purification factor of ODC at the step of hydroxylapatite column shows a 16% recovery with *ca* 700-fold purification Analysis on SDS-polyacrylamide gel electrophoresis of the hydroxylapatite fraction showed two major protein bands one of 55000 which corresponds to ODC and another of around 9000 which corresponds to chromatin-bound antizyme (Fig 1). When the hydroxylapatite column fraction was treated with 10% w/v ammonium sulphate and then applied on a Sephadex G-200 column, ODC was separated from antizyme (Fig. 2) The peak of ODC from Sephadex G-200 column analysed on SDS-polyacrylamide gel presented a homogeneous preparation with M_r of 55000 (Fig 3). The purified preparation (*ca* 2700 fold purification) has a specific activity of 2400 units per mg protein and the final purification factor is raised to 9300 when it is expressed on the total cell protein

It should be noted that the specific activity of our preparation is two to three orders of magnitude lower than those of *E coli*, *Lactobacillus sp* 30*a* and *Physarum polycephalm*, respectively and very close to that reported for rat prostate [10]

Barley seeds variety Beca were used in our initial experiments, with a specific activity of ODC extracted from chromatin of 49 units per mg protein [9]. Finally,

Table 1 Purification of chromatin-bound ornithine decarboxylase

Purification step	Total activity (units)	Protein (mg)	Specific activity (units/mg protein)	Purification factor	Yield (%)
1 Grude chromatin	1190	1200	0 9	1	100
2 Chromatin extract	952	422	2 2	2	80
3 DEAE-Biogel A	880	35 7	24 6	27	74
4 TEAE-cellulose	380	6 8	55 9	62	32
5 Phenyl-Sepharose CL 4B	350	1 7	206	229	29
6 Hydroxylapatite	190	0 3	633	704	16
7 Sephadex G-200	240	0 1	2400	2670	20

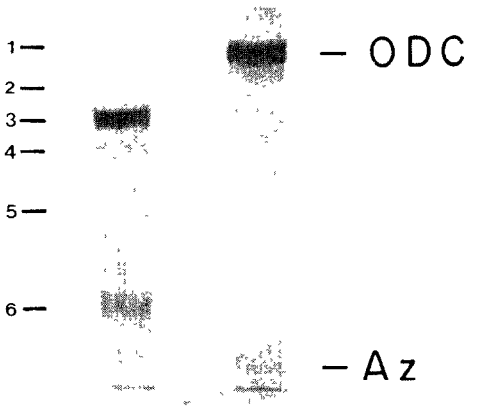

Fig 1 SDS-Polyacrylamide gel electrophoresis of the hydroxylapatite fraction *Ca* 5 μg of enzyme (step 6) was electrophorized on SDS-polyacrylamide gel electrophoresis, 12% in polyacrylamide, as described in the Experimental The standard protein markers used were 1 Bovine serum albumin, 66 000, 2 egg ovalbumin, 45 000, 3 pepsin, 34 700, 4 trypsinogen, 24 000, 5 β-lactalbumin, 18 400 and 6 lysozyme, 14 300

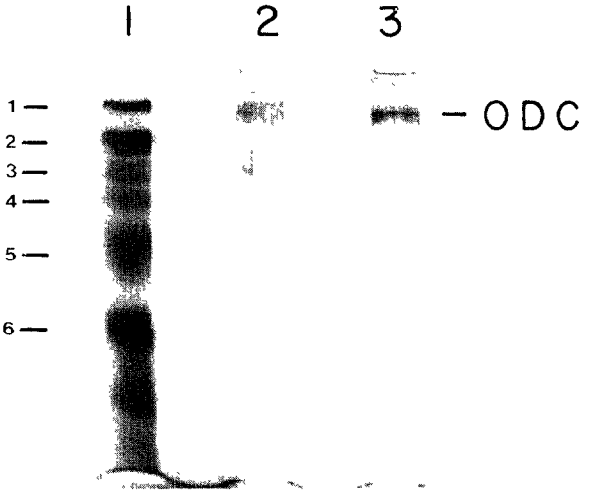

Fig 3 SDS–Polyacrylamide gel electrophoresis of purified ODC *Ca* 5 μg of the purified enzyme from the last step of Sephadex G-200 (line 3) and the TEAE-cellulose step (line 2) were electrophoresed on SDS-polyacrylamide gel, 12% in polyacrylamide Standard protein markers (line 1) are the same as described at Fig 1

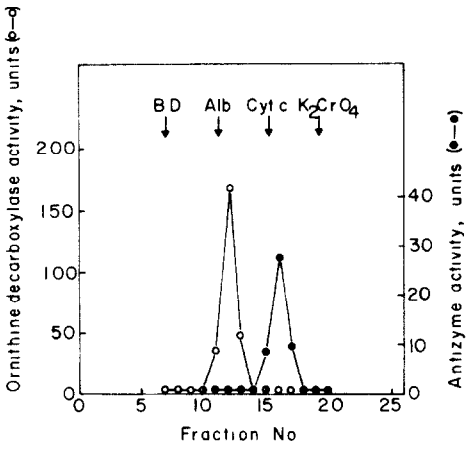

Fig 2. Chromatography of the hydroxylapatite ODC preparation on a Sephadex G-200 column *Ca* 300 μg protein (Table 2, step 6) containing 190 units of ODC was treated with 10% ammonium sulphate and fractionated on a Sephadex G-200 column as described in Experimental Fractions of 3 ml were collected B D , Blue dextran, Alb , bovine serum albumin, Cyt *c*, cytochrome *c*

we used the variety Georgia for this purification, whose extracts with ODC activity were more stable

Physical and catalytic properties of ornithine decarboxylase The M_r of the enzyme estimated either by the Sephadex G-200 column or more accurately by SDS-polyacrylamide gel electrophoresis was found to be 55 000 These findings lead to the conclusion that the enzyme exists in monomeric form The isoelectric point of ODC is 5.2 and the pH optimum is 8 5 The K_m for L-ornithine determined from the Lineweaver–Burk plot is 0.36 mM The enzyme does not present any L-lysine decarboxylase activity

Sensitivity to α-difluoromethylornithine (DFMO) The purified chromatin-bound ODC is sensitive to DFMO The DFMO concentration for 50% inhibition of ODC was 0.05 mM and 0 38 mM when the concentration of L-ornithine was 0 08 mM and 0 35 mM, respectively

Inhibition of ODC activity by metals and polyamines. The inhibitory effects of some compounds on ODC activity are presented in Table 2 Among the metals and polyamines tested, Co^{2+} and spermine were the more effective in inhibiting ODC activity, respectively

Stability. ODC activity is stable for 24 hr at 0–4 in the

Table 2 Inhibition of ornithine decarboxylase

Compound	Concentration (mM) required for 50% inhibition
Mg^{2+}	15
Ca^{2+}	0 9
Zn^{2+}	0 8
Mn^{2+}	1 0
Co^{2+}	0 5
Ba^{2+}	0 8
Na^{+}	300
K^{+}	220
Putrescine	0.65
Spermidine	0.50
Spermine	0.10

Assay conditions were as described in Experimental except that enzyme was incubated with inhibitors 10 min before the addition of substrate.

presence of 50 μM pyridoxal phosphate, 2 5 mM dithiothreitol or 5 mM β-mercaptoethanol and 10% w/v glycerol at 8.5. Freezing and thawing of purified enzyme decreases the activity to 50% unless crystallized bovine serum albumin in added at a final concentration of 0.5–1 mg/ml.

EXPERIMENTAL

Chemicals L-[1-^{14}C]-Ornithine (sp act 50 mCi/mmol) were purchased from Amersham Searle and L(U-^{14}C) lysine (sp. act 300 mCi/mmol) from New England Nuclear Putrescine, spermidine and spermine were products of Aldrich. α-Difluoromethylornithine (DFMO) was a generous gift of Merrell Research Center DEAE-Biogel A and hydroxylapatite (Bio-Gel HTP) were from BioRad All other chemicals were obtained from Sigma

Plant material Seeds of barley cv. Georgia were used throughout this work The seeds were sterilized according to ref. [5] and germinated in 50 × 30 cm dishes, in the dark at 26 ± 1° on filter papers which were kept moist by underlying cotton soaked in H_2O containing 5×10^{-5} M gibberellic (GA_3) The barley seeds were grown for 90 hr and then the growth was continued for 10 more hr in a medium containing GA_3 5×10^{-5} M and 2 μg/ml actinomycin D

Preparation of chromatin. The method of Bonner was employed for the isolation of chromatin [11]

Enzyme assays Ornithine decarboxylase assay was performed in buffer A (50 mM Tris–HCl pH 8.5, 0 3 EDTA, 50 μM pyridoxal phosphate, 5 mM NaF, 50 μM phenylmethylsulphonylfluoride and 2 5 mM dithiothreitol) in 55 μl final vol. as described [12]. One unit of ODC activity is defined as the amount of enzyme that releases 1 nmol of $^{14}CO_2$ in 1 hr at 37° under the conditions of the experiment. Sp act is defined as units per mg protein Lysine decarboxylase assay was performed under the same conditions as those used for ODC activity except that L-[U-^{14}C]-lysine was employed as substrate

DFMO binding assay. One unit of ODC was incubated at 37° for 1 hr with increasing concn of DFMO in buffer A in a final vol 55 μl Each sample was dialysed against buffer A and assayed for ODC activity

ODC antizyme assay Antizyme was assayed as previously described [13]. One unit of antizyme activity is defined as the amount of antizyme which inhibits one unit of ODC activity

Protein determination. Protein was determined according to the method of ref. [14] using bovine serum albumin as a standard.

SDS-polyacrylamide gel electrophoresis Purified ODC was subjected to SDS–polyacrylamide gel electrophoresis in 12% polyacrylamide gels [15] Gels were stained with $AgNO_3$ as described [16]

Isoelectric focusing Polyacrylamide gel electrofocusing of ODC was performed in 5% polyacrylamide tube gels (10 × 0 4 cm) with ampholytes of pH range 3–10 [17] The gel was cut in 0 3 cm slices and the enzyme activity was extracted for 1 hr at 4° with 200 μl of buffer A. Radioactive substrate (0.69 μmol) was added and the assay of ODC was performed as described above.

Purification of ornithine decarboxylase Step 1 preparation of chromatin Chromatin from 300 g of germinated barley seeds was suspended in 100 ml of buffer A

Step 2: extraction of chromatin The mixture from step 1 was frozen and thawed, stirred for 45 min at 4° and centrifuged at 100 000 *g* for 1 hr This treatment was repeated × 3 and the 100 000 *g* supernatants were combined

Step 3 DEAE-Biogel A chromatography A DEAE-Biogel A column (2 × 18 cm) was equilibrated with buffer A and 10% v/v glycerol. The preparation of the step 2 was applied to the column The column was thoroughly washed with buffer A plus 10% v/v glycerol and the enzyme was eluted with a 700 ml linear gradient containing 0–0 3 M NaCl in the above buffer. Fractions with ODC activity were pooled, diluted four times with buffer A and 10% v/v glycerol and applied to the next column

Step 4 TEAE-cellulose chromatography A TEAE-cellulose column (2 × 12 cm) was equilibrated with buffer A and 10% v/v glycerol. The enzyme soln of step 3 was applied and the column washed with 100 ml of buffer A plus 10% v/v glycerol followed by 100 ml of 0 1 M NaCl in this buffer. The enzyme was eluted with 120 ml of 0.25 M NaCl in buffer A and 10% v/v glycerol The active fractions were pooled and concentrated to 5 ml by Amicon ultrafiltration (PM 10 filter).

Step 5. phenyl–Sepharose CL-4B column The enzyme soln of step 4 was dialysed in buffer B (10 mM phosphate pH 6 8, 0.3 mM EDTA, 50 μm pyridoxal phosphate, 5 mM NaF, 50 μM phenylmethyl sulphonylfluoride, 2 5 mM dithiothreitol and 10% v/v glycerol) The dialysed preparation was chromatographed on a phenyl–Sepharose CL-4B column (1 × 6 5 cm) equilibrated with buffer B The active fractions, which were eluted in the flow-through, were combined and applied to the hydroxylapatite column.

Step 6 Hydroxylapatite chromatography A hydroxylapatite column (0.8 × 6 cm) was equilibrated with buffer B The enzyme fraction of step 5 was applied to the column The enzyme was eluted with a 50 ml linear gradient containing 0 to 0 5 MNa phosphate pH 6.8 The active fractions were concentrated to 2 3 ml by Amicon ultra-filtration (PM 10 filter)

Step 7· Sephadex G-200 chromatography The enzyme soln of step 6 was dialysed in buffer A, made 10% w/v with $(NH_4)_2SO_4$ and then applied on a Sephadex G-200 (1 2 × 60 cm) column previously equilibrated with buffer A plus 10% v/v glycerol The active fractions were pooled and concentrated to 2.5 ml by Amicon ultrafiltration (PM-10 filter). The enzyme was stored at −25°.

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