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PHOSPHODIESTERASE I IN CULTURED CELLS OF *MENTHA ARVENSIS*

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Key Word Index—*Mentha arvensis*; Labiatae; Japanese mint; cultured cells; alkaline phosphodiesterase I; purification; substrate specificity.

Abstract—Alkaline phosphodiesterase I (5′-nucleotide phosphodiesterase, EC 3.1.4.1) in cultured cells of *Mentha arvensis*, was purified about 75-fold by ammonium sulphate fractionation and three chromatographic steps. The optimum pH of the enzyme was 9.5. Its M_r was estimated by gel filtration to be *ca* 105 000. The enzyme was strongly inhibited by SH reagents and $HgCl_2$. It did not require divalent cations such as Mg^{2+} or Ca^{2+}. It hydrolysed thymidine 5′-*p*-nitrophenylphosphate but did not act on DNA or RNA. These properties, such as divalent cation requirement and substrate specificity, were different from those of phosphodiesterase I obtained from carrot.

INTRODUCTION

Alkaline phosphodiesterase I in the plant kingdom was first identified by Razzell [1]. This enzyme from animal sources has been extensively studied [2–4]. It is found in the mammalian plasma membrane fraction [4, 5]. In kidney and intestine cells, this enzyme is a glycosyl-phosphatidylinositol anchor protein [6, 7]. In contrast to the type from animal sources, little is known about the enzyme from plant sources [8, 9]. In the present study, we examined the properties of alkaline phosphodiesterase I from cultured cells of *Mentha arvensis* in order to compare them with those from carrot [8, 9].

RESULTS AND DISCUSSION

Solubilization

Since mammalian alkaline phosphodiesterase I is a membrane-bound enzyme, Triton X-100 was used for solubilization. To extract the enzyme in *Mentha arvensis* by sonication, we used four kinds of detergents, Triton X-100, *n*-heptyl-β-D-thioglucopyranoside, *n*-octyl-β-D-glucopyranoside and octa-ethyleneglycol mono *n*-dodecyl ether in 10 mM Tris–HCl buffer, pH 7.5 (buffer A). These detergents did not affect the extent of solubilization. The extraction method in the presence or absence of detergents did not affect the enzyme elution profiles from Q Sepharose Fast Flow ion exchange chromatography or Superose 12 gel filtration. Additionally, this enzyme could not be solubilized by phosphatidylinositol-specific phospholipase C obtained from *Bacillus thuringiensis* (Funakoshi), a potent tool for solubilization of glycosyl-phosphatidylinositol anchor protein [10]. These data indicated that alkaline phosphodiesterase I in *Mentha arvensis* is not a membrane-bound enzyme. To extract the enzyme, cells were sonicated in buffer A.

Enzyme distribution

In the screening test of alkaline phosphodiesterase I activity in the crude extract of 11 plant cells, we found that *Mentha arvensis* showed the highest specific activity value (Table 1). Other cultured cells, such as *Citrullus battich*, *Lycopersicon esculentum* and *Gardenia jasmoides* A, showed relatively higher specific activity.

Table 1. Specific activities of alkaline phosphodiesterase I in various cultured plant cells

Family	Species	Specific activity (units mg^{-1})
Labiatae	*Mentha arvensis*	600
	Isodon japonicus	65.8
Cucurbitaceae	*Citrullus battich*	197
	Luffa cylindrica	68.8
Solanaceae	*Lycopersicon esculentum*	157
	Datura inoxia	19.7
	Scopolia japonica	7.5
Rubiaceae	*Gardenia jasmoides* A	114
	G. jasmoides H	50.2
Moraceae	*Ficus elastica*	65.4
Liliaceae	*Asparagus officinalis*	3.4

Values are means of five determinations. s.d. were within 5% of the mean values.

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Table 2. Purification of alkaline phosphodiesterase I from cultured Japanese mint cells

Step	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1})	Purification (fold)	Yield (%)
Crude extract	185	105 000	570	1	100
$(NH_4)_2SO_4$ fractionation (30–80% satn)	33.8	90 000	2650	4.7	85
Q Sepharose Fast Flow	6.0	69 100	11 500	20.2	66
Superose 12	2.0	45 100	22 600	39.6	43
5′-AMP Sepharose 4B	0.25	10 600	42 300	74.2	10

Purification

The purification procedure is summarized in Table 2. The purified enzyme preparation showed a sp. act. of 42 300 units mg^{-1}. As one unit is defined as that amount of enzyme which releases 1 μmol *p*-nitrophenol hr^{-1} in carrot phosphodiesterase [8, 9], this value is *ca* 150 times higher than that of the carrot enzyme. In addition to the main band, p*I* 4.7, which showed the enzyme activity, there were other bands revealed by Coomassie Brilliant Blue staining of IEF–PAGE. The overall fold-purification was only *ca* 75-fold, although there were four separate purification steps. The final 5′-AMP Sepharose 4B step offers only very low recovery (23%) but is needed to remove the DNA hydrolysing activity. There are two possible explanations for the low recovery. First, the enzyme becomes unstable during the purification, and second, the enzyme has a high affinity for 5′-AMP Sepharose 4B and cannot be easily eluted. Further work is necessary to elucidate and improve the procedure.

Properties

The optimum pH of the enzyme was observed at *ca* 9.5. The thermal stability was examined by incubation at various temperatures for 10 min prior to use of the standard assay conditions. The enzyme was heat-stable and the temperature for half maximal activity was *ca* 70°. The M_r of the enzyme was determined to be *ca* 105 000 by gel filtration.

In contrast to the carrot enzyme [8, 9], divalent cations such as Mg^{2+}, Ca^{2+} or Zn^{2+} were not without effect on the enzyme activity, whereas Co^{2+} or Hg^{2+} were inhibitory (Table 3). The enzyme activity was markedly inactivated by sulphidyl reagents such as dithiothreitol, suggesting that the active site of the enzyme contained the SH residue.

Among the compounds tested, thymidine 5′-*p*-nitrophenylphosphate, a synthetic substrate for phosphodiesterase I, was the best substrate having a K_m value of 0.9 mM (Table 4). Bis(*p*-nitrophenyl)phosphate and nucleoside tri- or diphosphate, such as ATP or ADP, were also hydrolysed, but hydrolysis of these substrates was less than 1% of thymidine 5′-*p*-nitrophenylphosphate. Unlike carrot phosphodiesterase [8, 9], the enzyme did not hydrolyse native, denatured DNA or RNA.

Table 3. Effects of various compounds on alkaline phosphodiesterase I activity

Compound*	Relative activity (%)
None	100
NaCl	113
$MgCl_2$	100
$CoCl_2$	64
$CaCl_2$	95
$ZnCl_2$	95
$HgCl_2$	17
EDTA	65
KH_2PO_4	81
N-Ethylmaleimide	93
Dithiothreitol	12
L-Cysteine	3
Iodoacetic acid	22
Adenosine	59
5′-AMP	47

The enzyme was preincubated at 37° for 3 min, then activity was assayed by the standard procedure. Values are means of three determinations. s.d. are within 5% of the mean values.

*Final concentration: 1 mM.

EXPERIMENTAL

Cell culture. Cell suspension cultures of *Mentha arvensis*, which were originally derived from seedlings, were maintained in Linsmaier–Skoog medium [11] containing 10^{-6} M 2,4-dichlorophenoxyacetic acid and 10^{-6} M kinetin in 100 ml conical flasks over a period of 60 months by subculturing at 15-day intervals. The culture flasks were agitated on a reciprocating shaker (97 rpm) at 27° in the dark.

Enzyme assay. Alkaline phosphodiesterase I was measured by the method of ref. [8] with slight modification. The reaction mixt. (1 ml) contained 2 mM thymidine 5′-*p*-nitrophenylphosphate (Sigma) and enzyme in 0.1 M carbonate buffer (pH 9.5). Liberated *p*-nitrophenol at 37° was monitored spectrophotometrically at 410 nm, using a Shimadzu UV-160 spectrophotometer. One unit was defined as that amount of

Table 4. Substrate specificity of alkaline phosphodiesterase I from *Mentha arvensis*

Substrate	Concentration (mM)	Relative activity (%)
Thymidine 5′-*p*-nitrophenylphosphate	2	100
Thymidine 3′-*p*-nitrophenylphosphate	2	0
Bis(*p*-nitrophenyl)phosphate	2	1
p-Nitrophenyl phosphate	2	0
Pyrophosphate	2	0
ATP	4	>1
ADP	4	>1
5′-AMP	4	0
3′-AMP	4	0

No hydrolysis: DNA (double strand, 0.64 mg ml^{-1}), DNA (single strand, 0.64 mg ml^{-1}), RNA (0.64 mg ml^{-1}).

Values are means of three determinations.

enzyme which released 1 nmol *p*-nitrophenol min^{-1}. Sp. act. was expressed as units mg^{-1} protein. The liberated Pi from Pi-containing compounds such as ATP, ADP and PPi was assayed by the method of ref. [12] and modified as in ref. [13]. The hydrolysis of DNA or RNA was measured by the method of ref. [9]. Protein was determined by the method of ref. [14] with BSA as a standard.

Solubilization. Frozen cells were suspended in buffer A, in the ratio of 1 g cells vs 10 ml buffer, in the presence or absence of detergent. Next, the enzyme was extracted by sonication with a Tomy UR-200P Ultrasonifier for 3 min, with a 1-min intermission every 30 sec to avoid overheating. The homogenate was centrifuged at 40 000 *g* for 20 min. The supernatant was used as the crude extract enzyme preparation.

Purification. Mentha arvensis frozen cells (40 g) were suspended in buffer A (400 ml) and crude extract was prepd as described above. The supernatant was enriched by $(NH_4)_2SO_4$ (20–80% satn) and the pellet obtained (40 000 *g*, 20 min) was dissolved in 20 mM Tris–HCl buffer (pH 7.5, buffer B) and desalted by Pharmacia PD-10 column. The desalted soln was applied to a column of Q Sepharose Fast Flow (16 × 300 mm) equilibrated with buffer B. The enzyme was eluted with a linear gradient from buffer B to buffer B containing 0.5 M NaCl at a flow rate of 1 ml min^{-1}, using a Pharmacia FPLC system. The active frs were collected and concd by Amicon Centriflo CF 25. The concd enzyme soln was applied to a Superose 12 column (16 × 500 mm) equilibrated with 50 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl and eluted with the same buffer at a flow rate of 1 ml min^{-1}. The active frs were concd by ultrafiltration (Centriflo CF 25) and desalted by PD-10 column equilibrated with buffer B. The concd enzyme soln was then loaded on 5′-AMP Sepharose 4B column (16 × 95 mm) equilibrated with buffer B. The enzyme was eluted with a linear gradient of 0–2 M NaCl in buffer B at a flow rate of 0.5 ml min^{-1}. The active frs were collected and concd by Centriflo CF 25.

pI determination. The p*I* of alkaline phosphodiesterase I was estimated by IEF–PAGE with Pharmacia Ampholine PAGplate (pH 3.5–9.5) on a Pharmacia Multiphor II system according to the manufacturer's instructions. Gels were stained for protein using Coomassie Brilliant Blue and for alkaline phosphodiesterase I activity by incubating the gel in 0.1 M carbonate buffer (pH 9.5) containing 2 mM thymidine 5′-*p*-nitrophenylphosphate. The standard proteins used were from Pharmacia p*I* calibration kit 3–10.

M_r *determination.* The M_r of alkaline phosphodiesterase I was estimated by Superose 12 gel filtration. The separation conditions were as described for the purification. The standard proteins used were ferritin (450 000), aldolase (150 000), transferrin (90 000), ovalbumin (45 000), chymotrypsinogen A (25 000) and cytochrome *c* (12 500).

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