AN *ESCHERICHIA COLI* ACID PHOSPHATASE WHICH HYDROLYZES PREFERENTIALLY NUCLEOSIDE 3′,5′-DIPHOSPHATES

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1. Introduction

During the isolation of RNA and DNA ligase from extracts of phage T4 infected *Escherichia coli* [1,2], I observed phosphatase activity which attacked 5′-phosphates of the oligonucleotide substrates. Since a characterized phosphatase would be useful in the structural studies of nucleotides and nucleic acids, purification of the phosphatase activity was undertaken. The phosphatase activity is also present in uninfected *Escherichia coli* A19 extracts and the enzyme has unique substrate specificity.

2. Materials and methods

The standard assay for the phosphatase contained 1 mM substrate, 10 mM $MgCl_2$, 50 mM sodium acetate buffer (pH 4.1), in 0.2 ml total vol. After incubation at 37°C for 10 min, the phosphorus released was measured as in [3]. One unit of activity is defined as the amount of enzyme which catalyzes the release of 1 μmol phosphorus from adenosine 3′,5′-diphosphate (3′,5′-ADP) under the above conditions. For simplicity, the assay used for the enzyme purification measured the increase in A_{420} using 1 mM *p*-nitrophenyl phosphate as substrate in 1.0 ml 50 mM sodium acetate (pH 4.1) and 10 mM $MgCl_2$ at 37°C for 30 min.

Escherichia coli A19 (RNase I^-) [4] was grown in M9 medium supplemented with 0.3% polypeptone at 37°C and harvested at late-log phase. Cells were washed once with 50 mM Tris–HCl (pH 7.2). All operations were carried out at 0–4°C. Frozen cells (60 g) were ground with 120 g quartz sand in a mortar for 5 min and suspended in 300 ml 50 mM Tris–HCl (pH 7.2). The mixture was centrifuged at 10 000 × *g* for 10 min and the supernatant was further centrifuged for 1 h at 30 000 rev./min in a Beckman 35 rotor. The high speed supernatant (260 ml) was treated with 65 ml 5% streptomycin sulfate with gentle stirring, and the suspension was centrifuged at 10 000 × *g* for 10 min. The supernatant (315 ml) was dialyzed overnight against 2 changes of 2 liters each of 20 mM Tris–HCl (pH 7.2) containing 20 mM KCl. After centrifugation, the dialyzed supernatant (300 ml) was applied to a DEAE-cellulose column (Whatman DE-52, 4 × 12 cm) equilibrated with 20 mM Tris–HCl (pH 7.2) containing 20 mM KCl. The column was washed with 400 ml above buffer, and the phosphatase was then eluted with a linear gradient (1 liter) from 20–120 mM KCl in 20 mM Tris–HCl (pH 7.2). Fractions (17 ml) were collected (fig.1A). Active fractions were pooled and dialyzed overnight against 2 changes of 2 liters each of 5 mM Tris–HCl (pH 7.2). The dialyzed fraction (200 ml) was applied to a phosphocellulose column (Whatman P11, 1 × 7 cm) equilibrated with 5 mM Tris-HCl (pH 7.2). The column was washed with 40 ml 5 mM Tris–HCl (pH 7.2), and the enzyme was eluted with a 20–200 mM KCl gradient (140 ml) in 5 mM Tris–HCl (pH 7.2). Fractions of 2.2 ml were collected (fig.1B). Active fractions were pooled and concentrated in a collodion bag at reduced pressure. The concentrated sample (2 ml) was applied to a Sephadex G-100 column (1.6 × 40 cm) in 5 mM Tris–HCl (pH 7.2) containing 10% glycerol. The enzyme was eluted with the above buffer. Fractions (1 ml) were collected (fig.1C). Active fractions were pooled (7 ml) and concentrated as above (1 ml). Glycerol was added to 20% by vol. final conc. and the enzyme was stored at –20°C. About 200 units of the enzyme was obtained. The preparation was not homogeneous as judged by electrophoresis on SDS–polyacrylamide gel.

3. Results and discussion

The phosphatase was estimated to have $M_r \sim$ 35 000 by gel filtration as shown in fig.1C. The enzyme could be stored for ⩾1 year without appreciable loss of the activity. The optimum pH was found to be 4.1 in sodium acetate buffer with *p*-nitrophenyl phosphate and 3′,5′-ADP as substrates. Magnesium and cobalt ions (10 mM) enhanced the activity and EDTA inhibited it. No sulfhydryl reagents were required.

As shown in table 1, the phosphatase hydrolyzed 3′,5′-ADP and the reaction continued until the release of 1 mol phosphate/mol 3′,5′-ADP. The end products

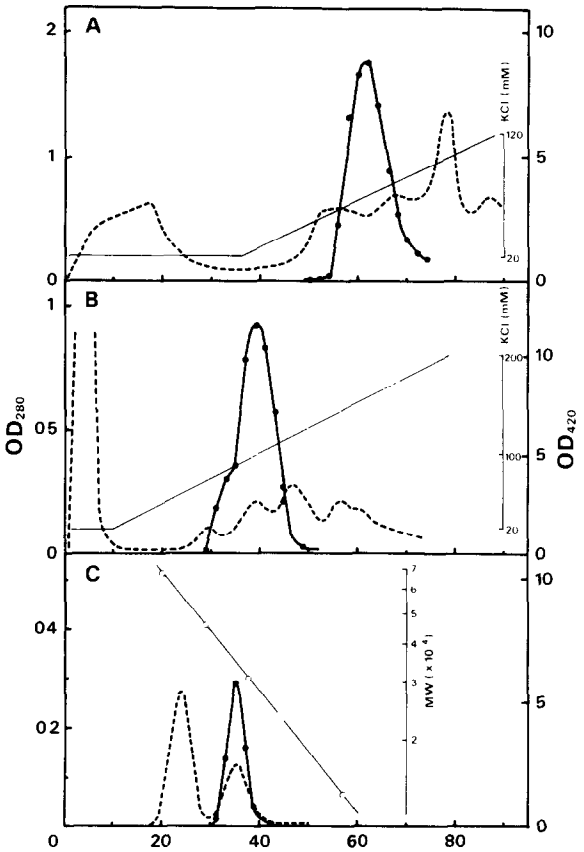

Fig.1. Purification of the acid phosphatase: (A) chromatography on DEAE-cellulose – 30 μl of each fraction was used to measure enzyme activities; (B) chromatography on phosphocellulose – 10 μl of each fraction was used to measure enzyme activities; (C) gel filtration on Sephadex G-100 – 3 μl of each fractions was used to measure enzyme activities. The M_r standards (○) were bovine serum albumin (67 000), ovalbumin (45 000), DNase I (31 000) and cytochrome *c* (13 500) in the order of elution: (——) phosphatase activity (OD_{420}); (– –) protein content (A_{280}); (— —) [KCl].

Table 1
Substrate specificity

Substrate	P_i released (μmol)	
	10 min	30 min
3′,5′-ADP	0.13	0.19
3′,5′-GDP	–	0.21
5′-ADP	0.05	0.16
5′-ATP	0.07	0.30
2′-AMP	0.02	0.11
3′-AMP	<0.01	<0.01
5′-AMP	<0.01	0.03

Reaction mixtures (0.2 ml) contained 1 mM (0.2 μmol) nucleotide, 0.13 unit of enzyme, 50 mM sodium acetate buffer (pH 4.1) and 10 mM $MgCl_2$. Incubations were at 37°C for 10 and 30 min

were analyzed by cellulose thin-layer chromatography developed in 66% isobutyric acid: 0.2 mM EDTA (pH 3.7) and were found to be a mixture of 5′-AMP and 3′-AMP, present in about equal amounts. The phosphatase also hydrolyzed 5′-ADP and 5′-ATP, and 2′-AMP at a very slow rate. 5′-AMP, 3′-AMP and bis *p*-nitrophenyl phosphate were not practically hydrolyzed by the enzyme.

The phosphatase could remove quantitatively 3′-phosphates of oligoribonucleotides, and 3′- and 5′-phosphates of RNA molecules (not shown). Therefore, substitution of position 3′- or 5′- of nucleotides is necessary for the enzyme activity. This enzyme resembles the phosphatase I in [5] and the pH 4, *p*-nitrophenyl phosphatase in [6] with respect of its elution profile of DEAE-cellulose chromatography and its optimum pH, but the latter 2 enzymes hydrolyzed 3′-AMP. As 3′,5′-ADP and 3′,-5′-GDP were the most efficient substrates, I prefer to designate this enzyme as 'acid nucleoside diphosphate phosphatase'. Since this phosphatase has unique substrate specificity, it may be useful for the structural analysis of nucleotides.

References

[1] Sugiura, M., Suzuki, M., Ohtsuka, E., Nishikawa, S., Uemura, H. and Ikehara, M. (1979) FEBS Lett. 97, 73–76.
[2] Sugiura, M. (1980) Anal. Biochem. 108, 227–229.
[3] Fiske, C. H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400.
[4] Gesteland, R. F. (1966) J. Mol. Biol. 16, 67–84.
[5] Rogers, D. and Reithel, F. J. (1960) Archiv. Biochem. Biophys. 89, 97–104.
[6] Neu, H. C. (1967) J. Biol. Chem. 242, 3896–3904.