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PARTIAL PURIFICATION AND PROPERTIES OF A K^+- AND Mg^{2+}-DEPENDENT PHOSPHODIESTERASE FROM *SALMONELLA TYPHIMURIUM*

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SUMMARY

A phosphodiesterase (orthophosphoric diester phosphohydrolase, EC 3.1.4.1) which has been partially purified from the lysate of P22(C_1)-infected spheroplasts of *Salmonella typhimurium* is dependent on either K^+ or Mg^{2+} for its activity and displays maximal activity in the presence of both. The enzyme is practically free from interfering activities and specifically hydrolyses single-stranded polyribonucleotides leading to the production of 5'-nucleotides. The homopolymers are hydrolysed in the following sequence: poly A > poly U > poly C. Poly C and tRNA are poorly hydrolysed, and poly I is not hydrolysed at all. The enzyme is most active at neutral or slightly alkaline pH. It is thermolabile but could be protected against heat denaturation by native and denatured DNA's as well as by poly A. During the hydrolysis of homopolymers no oligonucleotides could be detected as intermediate products, which indicates the exonucleolytic nature of the enzyme. Though there are some differences in properties, the behaviour of this phosphodiesterase is similar to that of ribonuclease II of *Escherichia coli*.

INTRODUCTION

Of the nucleases present in *E. coli*, three or four classes have been well characterized: ribonuclease I (ref. 1) (EC 2.7.7.16), ribonuclease II (refs. 2–4) (EC 3.1.4.1) and ribonuclease III (refs. 5, 6). Recently another new endonuclease has been designated as ribonuclease IV (ref. 7). The first two enzymes are specific for single-stranded structures whereas ribonuclease III preferentially hydrolyses double-stranded RNA. Ribonuclease IV cleaves phage R17 RNA into two fragments.

In a search for enzyme(s) concerned in the degradation of mRNA, the enzymes of *S. typhimurium* responsible for the degradation of single-stranded RNA's are being studied in this laboratory. On chromatography of the extracts on DEAE-cellulose,

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four single-stranded RNA hydrolysing activities appeared in the eluate as four distinct peaks[8]. Of these, the ribonuclease I type of activity has been well characterized and found to be the major nuclease activity[9]. On conversion of the cells to spheroplasts this activity, which is probably located in the cell wall, is mostly lost and the chromatography of the spheroplast lysate on DEAE-cellulose indicated that a phosphodiesterase dependent on K^+ and Mg^{2+}, similar to K^+-activated phosphodiesterase from *E. coli*, is the major activity present. It has also been observed that the spheroplasts of *S. typhimurium* can be infected with phage P22(C^+) or its mutants, probably owing to the residual cell wall material[10,11]. Surprisingly, the amount of ribonuclease I in the spheroplasts becomes further lowered on infection with C_1. Therefore C_1-infected spheroplasts have been used as the starting material for the preparation of K^+ and Mg^{2+}-activated phosphodiesterase, though this enzyme is actually the host enzyme and not phage-induced.

MATERIALS AND METHODS

Bacteria, phage and chemicals

S. typhimurium LT2 and the clear plaque-forming C_1 mutant of temperate phage P22(C^+) were obtained through the courtesy of Dr. M. LEVINE of the University of Michigan, Ann Arbor, Mich., U.S.A. Potassium penicillin G was the product of Pfizer Private Ltd., Bombay, India. Calf thymus DNA and crystalline pancreatic deoxyribonuclease were obtained from Sigma Chemical Co., U.S.A. Denatured DNA was prepared by heating a solution of calf thymus DNA (1.5 mg/ml) containing 0.15 M NaCl and 0.015 M sodium citrate at 100° for 15 min followed by quick cooling to 0°. Partially purified 5'-nucleotidase (EC 3.1.3.5) was obtained from snake venom through DEAE-cellulose chromatography. The sources of other materials used have been described in an earlier publication[9].

Assay of enzyme

The incubation mixture contained, in a volume of 0.25 ml: Tris–HCl (pH 7.0) 25 μmoles; KCl, 37.5 μmoles; $MgCl_2$, 0.375 μmole; poly A, 50 μg and a suitable amount of enzyme. The incubation was carried out at 37° for 10 min, at the end of which 0.05 ml of 50% trichloroacetic acid containing 0.75% uranyl acetate was added, and the mixture was cooled to 0°. After removal of the precipitate the amount of AMP released was measured at 260 mμ, in a Zeiss spectrophotometer. One unit of enzyme has been defined as that amount which catalyses the release of 1 μmole of acid-soluble nucleotide under the assay conditions described above. The assay was proportional to the amount of enzyme added, even up to 90% hydrolysis of poly A.

The protein was measured by Folin reagent as described by LOWRY *et al.*[12]. The protein content of the dialysed DEAE-cellulose eluate (to be described later) was low and was measured after precipitation of the protein with trichloroacetic acid.

Preparation of penicillin spheroplasts and their infection with C_1

The spheroplasts were prepared from LT2 by the penicillin method of SPIZIZEN[13] as described earlier[11]. Usually 100 ml spheroplast suspension in penassey broth medium were infected with C_1 at multiplicity of infection of 10 at 35°. An adsorption time of 5 min was allowed, after aeration for 20 min the mixture was chilled to 0°, and

the phage-infected spheroplasts were collected by centrifugation at 10 000 × *g* for 10 min.

Chromatographic identification of the product of hydrolysis of poly A

Incubation was carried at 37° for 1 h (to ensure complete hydrolysis) in a total volume of 0.27 ml containing: Tris–HCl (pH 7.0), 20 μmoles; $MgCl_2$, 0.375 μmole; KCl 37.5 μmoles; poly A, 250 μg; and dialysed DEAE-cellulose eluate, 0.1 ml. The incubation mixture was applied to the paper chromatogram without further treatment as the presence of salts and the small amount of protein did not interfere with the chromatographic separation. The chromatogram was developed with isobutyric acid–0.5 M NH_4OH (10:3, by vol.)[14].

RESULTS

Purification of enzyme

All operations were carried out at 0–5°.

Lysate of C_1-infected spheroplasts. The C_1-infected spheroplasts obtained from 180 ml of penassey broth medium were lysed by treatment with 90 ml of ice-cold 0.05 M Tris–HCl (pH 7.4) containing 0.2 mM mercaptoethanol for 25–30 min. The clear lysate was obtained by centrifugation at 25 000 × *g* for 10 min.

$(NH_4)_2SO_4$ fractionation. To the lysate (95 ml), the protein content of which was adjusted to 1.8 mg/ml, 37.8 g of $(NH_4)_2SO_4$ were added over a period of 15 min to make it 65% saturated. After a further 15 min had been allowed for complete precipitation, the precipitate was collected by centrifugation at 30 000 × *g* for 10 min and dissolved in 10 ml of 0.05 M Tris–HCl (pH 7.4) containing 0.2 μM mercaptoethanol. This was dialysed for 3 h against 1 l of 2.5 mM Tris buffer (pH 7.4) containing 0.2 mM mercaptoethanol with 3 changes. There was slight loss of enzyme activity due to dialysis.

DEAE-cellulose. Treatment with DEAE-cellulose was carried out in small batches. Usually 3 ml of the dialysed solution was put on a 12 cm × 1.4 cm column. The washing procedure was as described by PETERSON AND SOBER[15]. The column was eluted (flow rate, 1 ml/min) in stepwise fashion with increasing concentrations of

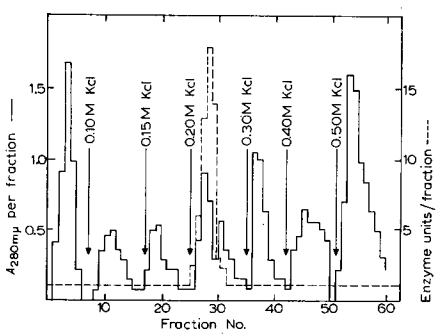


Fig. 1. Elution of the LT2 phosphodiesterase from the DEAE-cellulose column. The chromatographic separation is described in the text.

TABLE I

PARTIAL PURIFICATION OF THE PHOSPHODIESTERASE

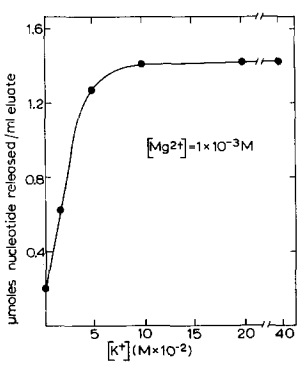
Fraction	*Vol. (ml)*	*Total units*	*Specific activity (units/mg protein)*
Spheroplast lysate	95	430	2.5
$(NH_4)_2SO_4$ (0–65%)	10	370	5.9
Dialysed $(NH_4)_2SO_4$ (0–65%)	10	320	5.7
Dialysed DEAE-cellulose eluate*	120	180	60.0

* Though this step was carried out batchwise, the values presented are on the basis of the total starting volume of lysate.

KCl buffered with 0.05 M Tris–HCl (pH 7.4) containing 0.2 mM mercaptoethanol, and 8-ml fractions were collected. The 0.2 M KCl eluate containing the phosphodiesterase (Fig. 1) was dialysed for 3 h against 2.5 mM Tris–HCl (pH 7.4) containing 0.2 mM mercaptoethanol. About 45% of the units present in the lysate were recovered at the final stage (Table I). Though little purification was achieved (24-fold), the enzyme preparation was found to be free from some of the interfering activities such as ribonuclease I, nucleotidase and deoxyribonuclease.

Properties of the enzyme

Effects of K^+ and Mg^{2+}. The rate of hydrolysis of poly A as catalysed by the dialysed eluate in the presence of 0.001 M Mg^{2+} increased linearly with increasing K^+ concentration up to about 0.05 M (Fig. 2). Maximal stimulation (7-fold) was obtained at a concentration of 0.10 M. In the presence of 0.1 M K^+ there was a linear relationship between the nucleotide released from poly A and the Mg^{2+} concentration, but the amount of Mg^{2+} required to produce maximal stimulation (4–5-fold) was much less



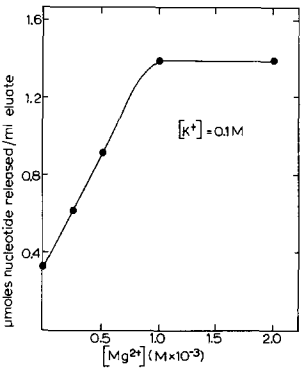


Fig. 2. Variation of the rate of hydrolysis of poly A with various concentrations of K^+. The concentration of Mg^{2+} was kept constant at 0.001 M, and an increasing amount of KCl was added. Other conditions were described under *Assay of enzyme*.

Fig. 3. Variation of the rate of hydrolysis of poly A with the various concentrations of Mg^{2+}. The concentration of K^+ was kept constant at 0.1 M, and an increasing amount of Mg^{2+} was added. Other conditions were described under *Assay of enzyme*.

TABLE II

EFFECT OF PREINCUBATION IN PRESENCE OF POLY A ON PHOSPHODIESTERASE

An aliquot of the DEAE-cellulose eluate (0.5 ml) was mixed with 0.04 ml of poly A (100 μg) and incubated for 10 min. An aliquot (0.1 ml) of the incubated eluate was used in the standard method of assay after the addition of a further 30 μg of poly A. A zero min control was also run to check the amount of poly A hydrolysed during preincubation

Condition of preincubation	*Nucleotide released (μmole)*
Control (no preincubation)	0.13
Without poly A	0.05
With poly A	0.11*

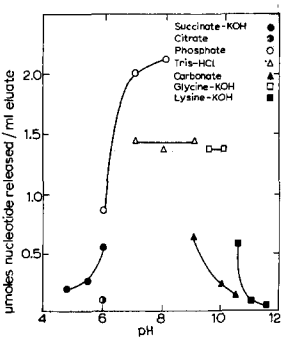
* The blank value in this assay was < 0.01 (about 0.004).

(Fig. 3). In the absence of both K^+ and Mg^{2+} the activity became insignificant. Two activities in the preparation, one K^+-dependent and the other Mg^{2+}-dependent, could not be detected.

Optimal pH. The enzyme appeared to have a broad pH optimum and was dependent on the type of buffer used (Fig. 4). No sharp pH optimum was discernible as the activity varied considerably not only with pH but also with the nature of the buffer used.

Stability of the enzymes

The enzyme was rapidly inactivated even at temperatures close to room temperature. Incubation at 45° for 5 min led to about 90% loss of activity and at 37° about 75% of the activity disappeared. This extreme lability raises doubts about the stability of the enzyme under the assay conditions though the optimal temperature for assay is 37°. To test whether the substrate was stabilizing the enzyme, the latter was



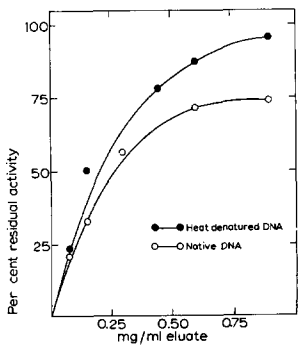
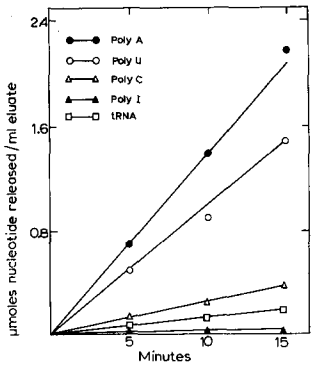


Fig. 4. Effect of pH of the incubation mixture on the rate of hydrolysis of poly A. Various buffers (25 μmoles each) were used as indicated. The incubation conditions were described in the text.

Fig. 5. Protection of the phosphodiesterase by native and denatured DNA's against heat inactivation. The standard assay conditions were used and the preincubation was carried out at 45° for 10 min in the presence of the indicated amount of native or denatured calf thymus DNA. The method of denaturation is described under MATERIALS AND METHODS.



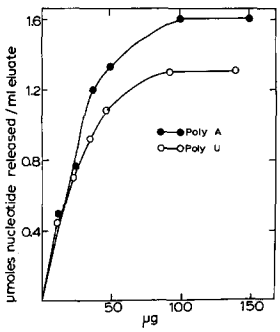


Fig. 6. Time-course of hydrolytic breakdown of various homopolymers and tRNA. The incubation conditions were as described under *Assay of enzyme*. The amount of each homopolymer used in the incubation was equivalent to 0.15 μmole nucleotide content.

Fig. 7. Dependence of the rates of hydrolysis of polyribonucleotides on their concentrations. The incubation conditions were as described under *Assay of enzyme* with the exception that various amounts of the homopolymers were used.

preincubated with poly A and then assayed as shown in Table II. Since the preincubation was carried out in absence of K^+ and Mg^{2+} the hydrolysis of poly A was negligible. An aliquot of the DEAE-cellulose eluate incubated in the absence of poly A, and assayed under standard conditions, lost about 60% of its activity during the preincubation whereas there was only 15% loss when the eluate was incubated in the presence of poly A. Native and denatured DNA's (Fig. 5) were also able to stabilize the enzyme. Heat-denatured DNA seemed to be somewhat more efficient in protecting the enzyme.

Specificity

Single-stranded substrates were hydrolysed at different rates by the phosphodiesterase (Fig. 6). Poly A was hydrolysed at the maximal rate whereas poly I was practically unaffected under these conditions. There is a probability that poly I exists in a hydrogen-bonded structure under the conditions of assay[4,16]. The rate with poly U

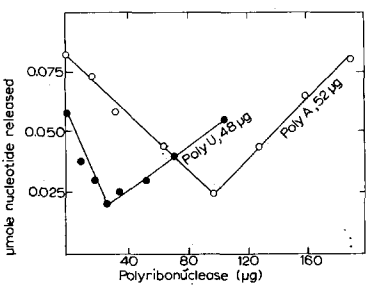


Fig. 8. Rates of hydrolysis of mixtures of poly A and poly U present in various proportions. The incubation conditions and other methods were as described under *Assay of enzyme* with the only differences that poly A and poly U were present in various proportions as indicated. In one set of experiments 48 μg of poly U and various amounts of poly A were used. In the other set 52 μg of poly A and various amounts of poly U were used.

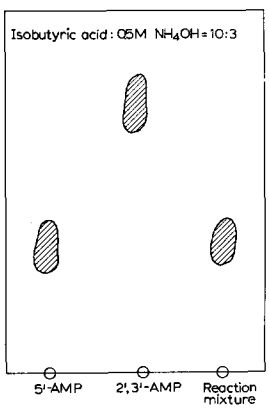
was less than that with poly A. Though poly C and tRNA were hydrolysed at significant rates, the rates were rather slow in comparison with those with poly A and poly U. The low rate of hydrolysis of poly C is not clear. The rate of hydrolysis gradually increased with increasing concentration of poly A or poly U, and the maximal rate was attained with 100 μg of each polymer present in the incubation mixture (Fig. 7).

Effect of secondary structure

In order to test that the enzyme preferentially hydrolyses single-stranded polyribonucleotides, mixtures of poly A and poly U in various proportions were used as substrates (Fig. 8). With an increasing amount of poly U added to the incubation mixture containing a fixed amount of poly A the rate decreased and became minimal when the amount of poly U was approximately double that of poly A. Addition of more poly U beyond double the amount of poly A resulted in an increase in the rate of hydrolysis. The same was true when a fixed amount of poly U and various amounts of poly A were used. It is well known that poly A and poly U produce maximal secondary interaction when they are present in the ratio 1:2 under the above-mentioned conditions[17].

Identification of product

It is evident from the chromatogram in Fig. 9 that 5'-nucleotide is the product of hydrolysis of poly A as catalysed by the phosphodiesterase. There was no detectable amount of 2',3'-nucleotide. Checking with other solvents showed the absence of adenosine, even after incubation for 2 h, clearly indicating the absence of any nucleotidase in the partially purified preparation. The treatment of the incubation mixture with 5'-nucleotidase and complete conversion of AMP to adenosine indicate that the product is 5'-nucleotide (Fig. 10). The products of hydrolysis for short periods were analysed on paper chromatograms developed with either 95% ethanol–1 M ammo-



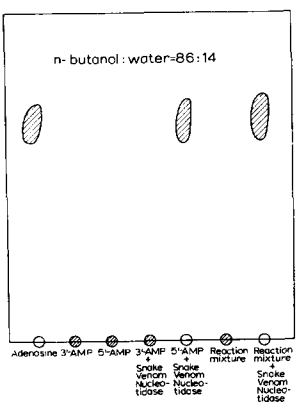


Fig. 9. Chromatographic identification of the product formed as a result of hydrolysis of poly A. The method of identification is described under MATERIALS AND METHODS.

Fig. 10. Identification of the product of hydrolysis of poly A with the help of snake venom 5'-nucleotidase. An incubation was carried out as described under MATERIALS AND METHODS. The incubation mixture was heated in a boiling-water bath for 2 min and then incubated for 30 min at 37° with 210 μg of 5'-nucleotidase. The incubation mixture was applied to the chromatogram which was developed with *n*-butanol–water (86:14, by vol.).

nium acetate (4:6, by vol.)[18] or isobutyric acid–NH_4OH–0.1 M EDTA (disodium salt)-water (66:1:1·5:31.5, by vol.)[19] which are known to separate the oligonucleotides efficiently. Oligonucleotides, however, were never detected, and AMP was found to be the only product formed. Thus it is most likely that the enzyme under study is an exonucleolytic phosphodiesterase.

DISCUSSION

The LT2 phosphodiesterase is similar in many respects to the K^+-activated phosphodiesterase from *E. coli*[3,4]. Both behave as exonucleases. The specificity in both is towards the single-stranded structure of the substrate. The sequence in which the homopolymers are hydrolysed by *E. coli* phosphodiesterase is poly A > poly U > poly C. The same sequence was observed with the enzyme under study. Poly C, however, was poorly attacked in present conditions. As expected, poly I was not hydrolysed by either enzyme and tRNA acted as a poor substrate. Protection against heat denaturation by DNA was evident for both enzymes. Though heat-denatured DNA was found to be most effective for the *E. coli* enzyme, native DNA was only slightly less active than denatured DNA for the LT2 enzyme. Substrates such as poly A were also found to protect the enzyme under study during incubation.

Though their products of catalysis are different, the ribonuclease I reported from LT2 (ref. 9) and the K^+ and Mg^{2+}-dependent phosphodiesterase behave similarly in respect to specificity for single-stranded structures. The rates of hydrolysis of the homopolymers by the former enzyme are, however, in different sequence (poly U > poly C > poly A). Another striking difference is in respect to the rates in presence of higher concentrations of homopolymers. With ribonuclease I, poly A and poly U added to the incubation mixture beyond 50 μg resulted in the inhibition of hydrolysis whereas with phosphodiesterase, the maximal velocity was attained in presence of 100 μg of homopolymer in the incubation mixture, and the addition of even 50 μg more of the homopolymer did not result in inhibition.

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