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A Phosphodiesterase from the Carrot*

C. Harvey, L. Malsman, and A. L. Nussbaum

ABSTRACT: A method is given for the purification of a phosphodiesterase from carrots. The characteristics of the partially purified enzyme have been studied. The enzyme possesses exonucleolytic activity which degrades both oligodeoxyribonucleotides and oligoribonucleotides, starting from a free hydroxyl on the 3′ end and producing 5′-mononucleotides. Native deoxyribonucleic acid (DNA) is resistant while denatured DNA and transfer ribonucleic acid (tRNA) are hydrolyzed. Divalent ions (Mg^{2+}, Mn^{2+}, Ca^{2+}, and Zn^{2+}) increase activity while EDTA inhibits. The pH optimum for assay is 9.0–9.5 and the enzyme is most heat stable at a pH of 8.5–9.0. Some phosphatase activity remains after purification, but does not seem to be associated with phosphodiesterase. The similarity of this enzyme to phosphodiesterase I of animal origin is noted.

We recently required fairly large amounts of a phosphotransferase (Tunis and Chargaff, 1960) for the phosphorylation of 5-fluoropyrimidine arabinosides (Strider *et al.*, 1967). During the course of this work, we noticed the presence of appreciable quantities of phosphodiesterase activity in the carrot (*Daucus carota sativa*) extracts which were being used as a source of enzyme.

In the present paper, we describe a purification procedure leading to a 300-fold enrichment of this phosphodiesterase activity, and some of its properties. Enzymes of this type are potentially useful in nucleotide sequence studies (Lehman *et al.*, 1965), once their specificities have been established. The present carrot phosphodiesterase appears to be an exonuclease which degrades small oligonucleotides and nucleic acids lacking secondary structure, while double-stranded DNA is hardly attacked. Model oligonucleotides are degraded completely to 5′-mononucleotides, the mode of attack being 3′→5′. In this respect, the enzyme resembles such phosphodiesterases which are found in snake venom (Razzell and Khorana, 1959), hog kidney (Razzell, 1961), *Escherichia coli* (Lehman, 1963), and an enzyme recently discovered in malt (Holbrook *et al.*, 1966). Similar activities seem to have been encountered, in peas, corn, and potatoes (Razzell, 1966), but the active principles have not been purified.

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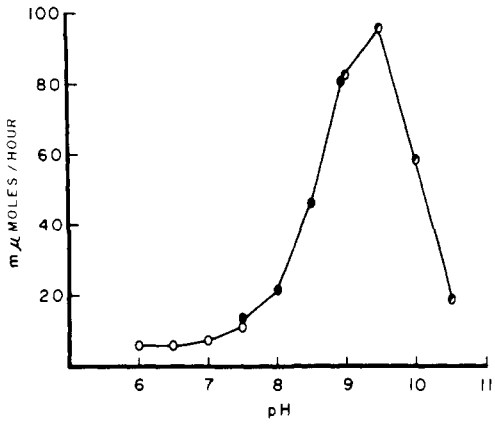

FIGURE 1: pH optimum with PNP-pT. Assay is described in the Experimental Section. The pH was taken before assay and after assay on a second sample. Buffers used: *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (○—○), Tris-HCl (●—●), and sodium bicarbonate (◐—◐).

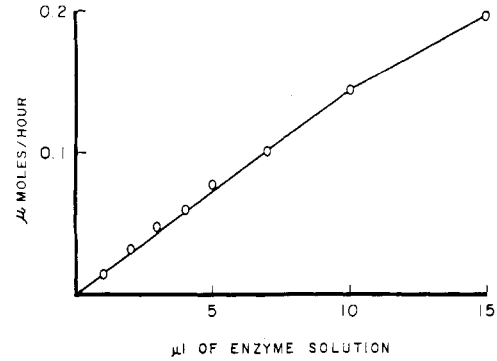

FIGURE 2: Assay linearity. Assay conditions as described in the Experimental Section.

Such enzymes occur widely throughout the plant kingdom.

Experimental Section

Materials. The following items were purchased from commercial sources: sodium *p*-nitrophenyl 5′-thymidylate (Calbiochem), calcium bis(*p*-nitrophenyl) phosphate (Mann), calf thymus DNA (Calbiochem), tRNA (yeast) (General Biochemicals), diribonucleoside monophosphates CpU[1] and CpA (Miles), Up(2′,3′) (Pabst), purified bovine albumin (Pentex), crude *Crotalus adamanteus* venom (Ross Allen's Reptile Institute, Silver Springs, Fla.), Sephadex G-200 (Pharmacia), carboxymethylcellulose Whatman CM-11 (Reeve Angel), and DEAE-cellulose Whatman DE-23 (also Reeve Angel). Oligodeoxyribonucleotides were prepared according to published procedures (Khorana and Vizsolyi, 1961; Kössel *et al.*, 1967; Schaller and Khorana, 1963; Jacob and Khorana, 1965).

Methods. The standard diesterase assay was carried out in the following manner. Tris-HCl buffer (50 μmoles) (pH 8.9), 0.25 μmole of PNP-pT, 10 μmoles of $MgCl_2$, and the enzyme solution to be assayed were made up to 0.5 ml with distilled water and incubated at 37° for 15 min. The enzymatic reaction was stopped by addition of 2.5 ml of 0.05 N NaOH, and the optical density of the liberated *p*-nitrophenol was determined at 410 mμ by means of a Zeiss PMQ-II spectrophotometer. A molecular extinction coefficient of 15,600 was used in the calculations. A blank containing the same ingredients, but lacking the enzyme, was also carried. A unit of enzyme activity was defined as that quantity which hydrolyzed 1 μmole of PNP-pT/hr under assay conditions.

Susceptibility to 5′-nucleotidase (Heppel and Hillmoe, 1964) was established as follows. A total volume of 200 μl containing 20 μmoles of glycine (pH 9.0, NaOH), 2 μmoles of $MgCl_2$, 0.5 mg of lyophilized venom, and 1 μmole of suspected 5′-mononucleotide was incubated at 37° for 1 hr. A 50-μl aliquot was then spotted on Whatman No. 1 paper and the chromatogram was developed in ethanol–1 M ammonium acetate, pH 7.5 (70:30). Undegraded nucleotide and the corresponding nucleoside could be clearly distinguished. Under these conditions, Up(2′,3′) was not dephosphorylated, while pT was completely converted to thymidine.

Ion-exchange materials were prepared according to manufacturers' recommendations. The cellulose exchangers were packed under a hydrostatic pressure head of approximately 36 in. The Sephadex columns were prepared according to Pharmacia technical data sheet no. 6. Protein was determined according to the procedure of Lowry (Lowry *et al.*, 1951).

The digestion of oligodeoxyribonucleotides was followed by paper chromatography (system as above), appropriate deoxymononucleotide spots being defined under a Mineralight short-wave lamp. These spots were cut out, eluted with distilled water, and measured in a Zeiss PMQ-II spectrophotometer at appropriate wavelengths; known quantities of reference compounds treated in an identical manner served as quantitative controls.

DNA was denatured as described by Lehman and Nussbaum (1964). Susceptibility to enzymatic digestion of both the native and denatured species was determined by examining the amount of acid-soluble nucleotides. The enzymatic reaction (for details, see Results) was stopped by addition of 0.5 ml of cold 0.7 N perchloric acid, followed by 2.5 ml of cold distilled water. The resulting suspension was kept in an ice bath for 5 min and the precipitate was removed by centrifugation. The optical density of the supernatant was then determined at 260 mμ.

tRNA was prepared by the procedure of Nathans

[1] Abbreviations used: PNP-pT, *p*-nitrophenyl-5′-thymidylic acid. Nucleosides and nucleotides are abbreviated according to the IUPC–IUB Revised Tentative Rules, *Arch. Biochem. Biophys. 115*, 1 (1966).

TABLE I: Results of a Typical Purification Procedure.

Step	Vol. (ml)	Concn (units/ml)	Total Units ($\times 10^{-3}$)	Protein (mg/ml)	Sp Act. (units/mg of protein)	Yield (%)	Purifcn
1. Crude extract	7400	125	925	7.1	17.6	100	
2. pH 4 precipitation	6690	72	482	2.4	30	52.2	1.7
3. 80% ammonium sulfate precipitation	300	1000	300	12.6	79.4	31.5	4.5
4. 30–70% acetone precipitation, dialysis	125[a]	2464	309	12.6	195.5	33.4	11.2
5. CM-cellulose, lyophilization	25	5980	150	10	598	16.2	34.0
6. Sephadex G-200	220	355	78	0.24	1480	8.4	84
7. DEAE-cellulose	415	196	81	0.037	5300	8.5	300

[a] From step 4, purification was carried out on 20% aliquots. Figures in table have been prorated.

and Lipmann (1961). The acid-soluble material was determined as described by Holbrook *et al.* (1966).

Results

Assay. The presence of the enzyme was first recognized by the ability of the extract to generate *p*-nitrophenol from PNP-pT. From this and subsequently cited data, it was concluded that this enzyme degraded oligonucleotides to the 5′-phosphate monomers. A well-defined pH optimum near 9.5 was demonstrated when the substrate was assayed with PNP-pT (Figure 1). The reaction assay was proportional to time up to an optical density of 0.6, and linear over a wide range of enzyme concentrations (Figure 2).

Purification. Table I summarizes the several steps employed in a typical purification procedure. Carrots (38 lb) purchased through commerical channels and stored at 10° were homogenized with 4 l. of crushed ice for 5 min, in a commercial vegetable shredder (Hobart high-speed cutter Model VCM40E). All subsequent steps were carried out in the cold room at 2°. Filtration of the homogenate on a Büchner funnel, through two layers of cheesecloth, gave a crude extract, which was carefully adjusted with glacial acetic acid to pH 4. The resulting precipitate, which contained most of the nonspecific phosphatase activity, was removed by centrifugation at 10,000*g*. The supernatant was subjected to ammonium sulfate precipitation (525 g/l.) and the insoluble protein was collected again by centrifugation (10,000*g*). The residue was dissolved in 300 ml of 0.1 M sodium acetate (pH 5.2) and 129 ml of acetone was added at −20°. The resulting precipitate was discarded after centrifugation at 8000*g*. Further addition of 570 ml of acetone at −20° resulting in precipitation of the active fraction, which was collected by centrifugation as before and dissolved in 100 ml of distilled water. Additional purification was carried out on an aliquot; 20 ml was dialyzed against 0.01 M sodium acetate (pH 4.5) until the dialysate gave a negative test with barium chloride. The dialyzed sample was subjected to chromatography on a column (2 × 15 cm) of carboxymethylcellulose previously equilibrated with 0.01 M sodium acetate (pH 4.5). Elution was carried out with a linear gradient (mixing chamber, 500 ml of equilibrating buffer; reservoir, an equal volume of the same buffer containing 0.2 M sodium chloride) at a flow rate of 30 ml/hr, 5-ml fractions being collected. The activity emerged as a single peak throughout tubes 60–90. The combined active fractions were lyophilized, dissolved in 5 ml of distilled water, and charged into a second column of Sephadex G-200 (2.5 × 40 cm), previously equilibrated with 0.01 M Tris-HCl (pH 7.8). Elution with the same buffer (flow rate, 8 ml/hr) and collection of 2-ml fractions resulted in the emergence of the activity at an elution volume twice the void volume. Fractions having an activity greater than 200 units/ml (fractions 39–61) were combined and applied to a DEAE-cellulose column (1 × 15 cm) previously equilibrated with the same buffer as the Sephadex column. A linear gradient (mixing chamber, 200 ml of equilibrating buffer; reservoir, 200 ml of the same buffer, 0.5 M in sodium chloride) was applied at flow rate of 18 ml/hr, and a single peak of activity was eluted from 0.20 to 0.26 M sodium chloride. The active fractions were combined, lyophilyzed, and stored at −20°.

Stability. The enzyme shows remarkable heat stability at high pH (Figure 3). Heat inactivation studies indicated that activity was not lost when the enzyme was precincubated for 1 hr at 60° in 0.1 N Tris-HCl buffer (pH 8.9). When the temperature was raised to 95° under these conditions, all activity had disappeared after 10 min. Repeated freezing and thawing tends to inactivate the crude enzyme; this sensitivity seemed to decrease with increasing purity. The enzyme is quite sensitive to dialysis at low pH (about 50% of the activity is lost after overnight dialysis against

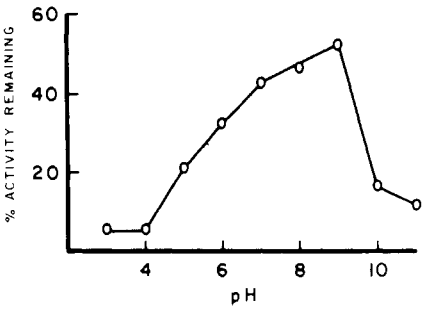

FIGURE 3: Heat stability of phosphodiesterase at different pH. A 0.1 M Tris-acetate solution was heated to 80°, the enzyme was added, and heating was continued for 10 min. An aliquot was assayed as described in the Experimental Section.

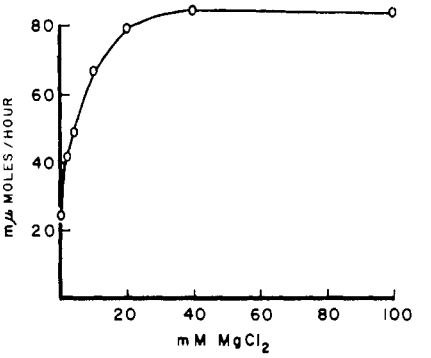

FIGURE 4: Optimum concentration of Mg^{2+}. The $MgCl_2$ concentration was varied and assay run as described in the Experimental Section.

0.01 M sodium acetate, pH 4.5), though no such sensitivity was observed at pH 7.8 (Tris-HCl, 0.01 M).

Activators and Inhibitors. The enzyme benefits from, but does not require, the addition of certain divalent metal ions (Mg^{2+}, Mn^{2+}, Zn^{2+}, and Ca^{2+}), as shown in Table II. The enzyme seems to be tightly bound to metal: chelating (EDTA) and complexing (cyanide) agents decrease activity, though they did not abolish it. Thus, a 0.002 M solution of EDTA still permitted 30% of control levels of activity. Addition of Cu^{2+} and Co^{2+} (0.002 M) had little or no effect. The stimulating effect of Mg^{2+} is shown in Figure 4; maximum effect was observed at 0.04 M.

Substrate Specificity and Direction of Cleavage. The synthetic assay substrate PNP-pT was readily degraded to *p*-nitrophenol and thymidylate. From a Lineweaver–Burk plot (Figure 5), $K_m = 2.0 \times 10^{-5}$ M and $V_{max} = 3200$ μmoles/hr per mg of protein were calculated. Similar data for bis(*p*-nitrophenyl) phosphate, a less susceptible substrate, gave $K_m = 1.5 \times 10^{-3}$ and a $V_{max} = 100$ μmoles/hr per mg. Tables III and IV list a variety of substrates and their respective rates of hydrolysis. It will be seen that native DNA is attacked only very slowly, if at all, while denatured DNA is degraded quite extensively. Possibly due to its partial helical structure, RNA falls somewhere in between. The enzyme efficiently degrades small synthetic deoxyribooligonucleotides and ribooligonucleotides in precisely the same manner as snake venom phosphodiesterase (Razzell and Khorana, 1959) and an exonuclease from *E. coli* (Lehman and Richardson, 1964). Thus, TpT was degraded to T and pT (structure of the latter being ascertained by susceptibility to 5′-nucleotidase), pTpT was converted to pT (at a more rapid rate than the aforementioned dinucleoside phosphate), and CpA and CpU were degraded to C and pA or pU, respectively (the latter faster than the former by a factor of about two). The direction of cleavage can be ascertained from the following experiment: d-TpTpC gave initially TpT and d-pC; the former was subsequently degraded to T and pT (Figure 6). This further points to the direction of nucleolysis as being from 3′ to 5′, as already suggested by the fact that

TABLE II: Effect of Activators and Inhibitors on Phosphodiesterase Activity.

Addn (2×10^{-3} M)	% of Control
None	100
$MgCl_2$	223
$CuSO_4$	128
$ZnCl_2$	195
$HgCl_2$	20
$CaCl_2$	218
$CoCl_2$	116
EDTA	30
NaF	125
Sodium arsenate	120
NaCN	57

[a] Assay used is described in Experimental Section.

TABLE III: Hydrolysis of DNA and tRNA by Carrot Phosphodiesterase.[a]

Substrate	% Acid Soluble (24 hr)
Native DNA (calf thymus)	0.24
Denatured DNA (calf thymus)	40
RNA (stripped)	24

[a] The reaction mixture contained in 1 ml: 100 μmoles of Tris-HCl (pH 8.9), 20 μmoles of $MgCl_2$, 1 mg of substrate, and 345 units of enzyme. The mixture contained 50 mg of ethyl mercurithiosalicylate to prevent bacterial growth. After incubation at 37° for 24 hr, the enzyme reaction was stopped and the per cent acid soluble was determined as given in the Experimental Section.

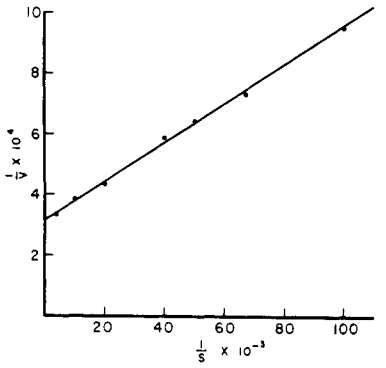

FIGURE 5: Lineweaver–Burke plot of PNP-pT hydrolysis. The increase in optical density at 400 mμ was measured in a Gilford spectrophotometer at 37°. Total volume (1 ml) containing twice the standard assay components was used. Velocity in micromoles per hour; substrate concentration in moles per liter.

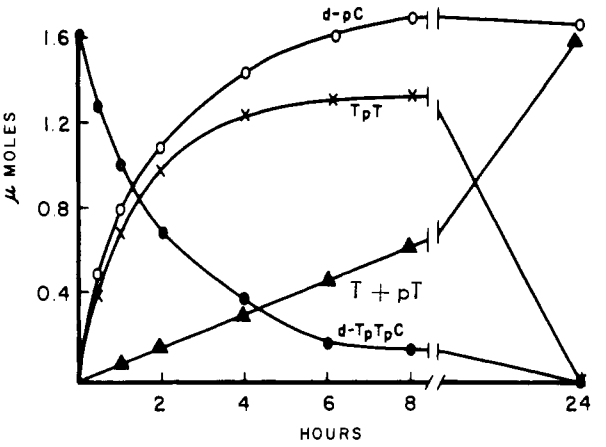

FIGURE 6: Kinetic study of d-TpTpC hydrolysis. The reaction mixture contained, in 0.5-ml total volume: 1.6 μmoles of d-TpTpC, 50 μmoles of Tris-HCl buffer (pH 8.9), 10 μmoles of $MgCl_2$, and 15 units of enzyme. The reaction mixture was incubated at 37° and 50-μl samples were withdrawn at indicated times. The separation of hydrolysis products is described in the Experimental Section. (The system used does not distinguish between d-TpTpC and pT; the latter is assumed to be equal to T, as confirmed by an independent study with TpT.)

PNP-pT is a substrate. Furthermore, pTpT-3′-acetate was not a substrate. Thus, approximately 5 μmoles of the latter compound was exposed to 20 units of enzyme for several hours without generating any smaller fragments. A model cyclic nucleotide, the trimer pTpTpT, was likewise not attacked. All these data suggest that the carrot nuclease is an exonuclease with a specific direction of hydrolic attack.

Discussion

Enzymes, whose mode of attack is clearly defined, are possible tools in the elucidation of biopolymer structure. This has been especially true in the nucleic acid field, for instance in the clarification of the primary structure of various RNA species. Several exonucleases have been characterized sufficiently to permit their use in structural analysis (Lehman *et al.*, 1965), the carrot phosphodiesterase herein described being a conveniently available addition to the existing armamentarium. Phosphodiesterases possessing exonuclease activity are widely distributed in nature, although such enzymes of higher plant origin have perhaps not been as extensively studied as those of microbiological (Lehman, 1963) or animal provenance (Razzell, 1967; Khorana, 1961). An exception is the phosphodiesterase of malt (Holbrook *et al.*, 1966) which has been extensively purified.

Carrot phosphodiesterase is a phosphodiesterase I insofar as it degrades polynucleotides to 5′-mononucleotides, commencing from the 3′ end exclusively. This end must be accessible as a free hydroxyl group. The nature of the opposite (5′) end is less critical; it may be either free hydroxyl or esterified with either free or further esterified phosphate. The enzyme resembles several other known phosphodiesterases, notably that from venom (Razzell and Khorana, 1959) and malt (Holbrook *et al.*, 1966). It differs from the former in its inability to attack a model cyclic trinucleotide and its relative sluggishness in degrading denatured DNA. From the present data, it is difficult to state whether the carrot diesterase differs from the malt enzyme; we did not observe the ammonium sulfate activation observed for the latter. The question of whether the remaining phosphomonoesterase activity is a contaminant or an integral part of the enzyme

TABLE IV: Hydrolysis of Oligonucleotide Substrates by Carrot Ph osphodiesterase.[a]

Substrate	μmoles	Initial Rate (μmoles/hr)
TpT	5	5.5
pTpT	5	8.5
Cyclic $(pT)_3$	2	0[b]
pTpTo-acetate	4	0[b]
CpU	1	1.4
CpA	1	0.8

[a] The reaction mixture contained in 0.25 ml: 25 μmoles of Tris-HCl (pH 8.9), 5 μmoles of $MgCl_2$, substrate, and 10 units of enzyme. The reaction mixture was incubated at 37°; 40-μl samples were removed at different time intervals and hydrolysis products were separated by paper chromatography as described in the Experimental Section. [b] No hydrolysis products were found after 4-hr incubation with pTpTo-acetate or 24 hr with cyclic 5′–3′$(pT)_3$.

remains moot, although we feel that the two activities are separate, since their maxima are partially resolved in several columns. At any rate, this activity at the current stage of purification represents only $^1/_{5000}$ of the phosphodiesterase activity.

Studies concerning the homogeneity of the enzyme are underway. A preliminary estimate of the average molecular weight by the method of gel filtration (Andrews, 1965) indicates a value of *ca.* 75,000–100,000. The ready availability of the source of this enzyme may be of interest.

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