Further Purification and Properties of Phosphodiesterase from the Carrot*

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ABSTRACT: Carrot phosphodiesterase has been purified by a modified procedure until homogeneous, using disc gel electrophoresis, immunoelectrophoresis, and sedimentation in the ultracentrifuge as criteria. The preparation is 1300 times as active as the crude extract representing a threefold increase in specific activity over the material previously described. The overall yield has also been improved to 30%. The enzyme is free from nonspecific phosphatases and 5′-nucleotidase. Endonuclease activity has been reduced to barely detectable levels. The protein appears to have a molecular weight of approximately 10^5 daltons, and can be dissociated into subunits, themselves inactive, in 6 M guanidine HCl. Among its more useful properties is the ability to survive prolonged incubation at 60° without loss of activity. Absolute dependence on divalent cations has been demonstrated after preincubation with ethylenediaminetetraacetate. The enzyme hydrolyzes single-stranded deoxyribonucleic acid, but has no activity toward double-stranded molecules.

The ubiquity of nucleoside phosphodiesterases in the plant kingdom has been discussed by Razzell (1966). In a previous paper (Harvey *et al.*, 1967) we had occasion to describe a phosphodiesterase of type I occurring in the carrot. The activity resembled in many ways the widely used snake venom diesterase (Razzell and Khorana, 1959); that is, it was an exonuclease which hydrolyzed oligonucleotides to 5′-mononucleotides with a 3′→5′ direction of cleavage, and possessed an alkaline pH optimum.

In the present paper we describe a modified purification procedure resulting in a protein homogeneous by several criteria and endowed with properties making it a useful tool in the manipulation of nucleic acid fragments.

Experimental Section

Materials. The following enzymes were purchased from Worthington Biochemical Corp.: snake venom phosphodiesterase, chromatographic grade BAP,[1] pancreatic DNase I (crystallized IX), and micrococcal nuclease. Gd·HCl was purchased from Mann Research Laboratories, Carbowax 20,000, from Fisher Scientific Co.; NAD, from Calbiochem; and NaEDTA from Mallinckrodt. Poly I and poly C were obtained from Miles Laboratories and the complex poly(I·C) was prepared by adding equimolar amounts to 0.01 M sodium phosphate buffer (pH 7.0)–0.1 M NaCl. Poly d(A-[^{14}C]T) was made by the method of Schachman *et al.* (1960) using [^{14}C]dTTP obtained from Schwartz BioResearch. The tetramer d(T-A)$_2$ was obtained by digesting [^{14}C]poly d(A-T) with pancreatic DNase and separating the oligomers by DEAE chromatography (Tomlinson and Tener, 1963; Elson and Jovin, 1969). Oligomers terminated with a 3′-phosphate were obtained by digesting salmon sperm DNA (Calbiochem) with micrococcal nuclease and separating the digest according to chain length on a DEAE column (Tomlinson and Tener, 1963). The compound T(5′)pppT was donated by Mr. M. Holman, Materials for gel and exchange columns were purchased from Reeve Angel (DEAE-cellulose DE-23); Pharmacia Fine Chemicals, Inc. (Sephadex G-100); and Bio-Rad Laboratories (Agarose, Bio-Gel A 5m and hydroxylapatite, Bio-Gel HT).

Methods. Assays for carrot phosphodiesterase were made in a Gilford spectrophotometer with a Haake constant-temperature circulator at 37°. A total volume of 1 ml containing twice the standard assay components (Harvey *et al.*, 1967) was used. Initial rates of *p*-nitrophenol release were determined at 400 mμ using an extinction coefficient of 15,600. One unit of activity is that amount of enzyme which hydrolyzes 1 μmole of PNP-*p*T/hr.

The assay mixture for nonspecific phosphatase was the same as for the standard assay except 1 mM *p*-nitrophenylphosphate was substituted for PNP-*p*T. The reactions were run for 6 hr at 37°. 5′-Nucleotidase activity was determined in an assay medium containing 100 mM Tris-HCl (pH 8.9), 10 mM $MgCl_2$, and 0.1 μmole of [^{14}C]AMP (50,000 cpm), in a final volume of 0.31 ml. The mixture containing enzyme was incubated for 2 hr at 37° and subjected to paper chromatography (1 M ammonium acetate (pH 7)–ethanol, 3:7). Spots corresponding to adenosine and adenylic acid were cut out and counted in a toluene-based scintillation cocktail.

Antisera for immunoelectrophoresis was made by intravenous injection of a rabbit with 2.1 mg of fraction IV (Harvey *et al.*, 1967) which wasre peated twice at 2-week intervals. The immunoelectrophoresis apparatus was obtained from National Instrument Labs (Rockville, Md.) and the procedure used was a modification of that of Graber and Williams (1953). Melted 1% Agarose (3 ml) (Sea Kem, Bausch and Lomb) containing the buffer (5.4 g of sodium diethyl barbiturate, 2.5 g of sodium acetate, and 58.2 ml of 0.1 N

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[1] Abbreviations used are: BAP, bacteriol alkaline phosphatase; Gd·HCl, guanidine hydrochloride; PNP-*p*T, *p*-nitrophenyl-5′-thymidylic acid; poly I and poly C, homopolymers of inosinic and cytidylic acids; poly (I·C), the hydrogen-bonded complex of the same; poly d(A-T) the alternating polymer of deoxyadenylic and thymidylic acid. Nucleosides and nucleotides are abbreviated according to the IUPAC–IUB Revised Tentative Rules, *Arch. Biochem. Biophys. 115*, 1 (1966).

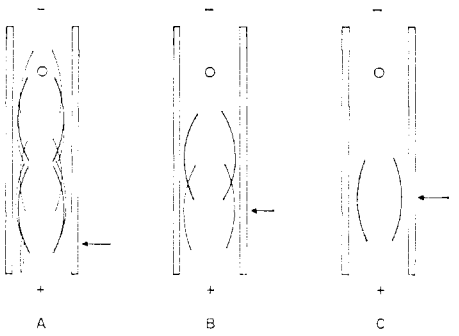

FIGURE 1: Immunoelectrophoresis on Agarose at pH 8.6. The arrows indicate where phosphodiesterase activity was found: (A) step IV, former procedure; (B) step VI; (C) step VII. The antisera was made with protein from step IV, former procedure.

TABLE I: Summary of Purification.

Step	Vol (ml)	Total Act. (units)	Sp Act. (units/mg)
I. Crude filtrate	1,100	48,400	11.5
II. DEAE-cellulose (pH 5.0)	390	35,900	191
III. Heat at 60°	390	34,800	970
IV. Agarose	95	29,100	1,200
V. DEAE-cellulose (pH 8.9)	58	21,500	4,800
VI. Hydroxylapatite	30	18,700	8,200
VII. Sephadex G-100	36	15,000	15,200

HCl for 100 ml) was pipetted to a 1 × 3 in. glass slide. A 0.02-ml sample containing 60 mg of protein was added to the well and 20 V was applied per slide. After 1.5 hr, the activity was located with a strip of Whatman No. 1 paper saturated with 5 mM PNP-*p*T. Antisera were added to troughs cut on each side of the well (1 and 0.75 cm) and developed for 18 hr in the cold.

Polyacrylamide disc electrophoresis was run in a Canalco apparatus using the procedure recommended by the manufacturer. Their standard gel (7%) which stacks at pH 8.9 and runs at 9.5 was used. Samples of 0.06 ml containing 60 μg of protein were applied. After electrophoresis, the activity was located as described above for immunoelectrophoresis and the gels were stained with aniline black.

Columns were packed and equilibrated as described in the earlier paper. Protein was determined by the method of Lowry (Lowry *et al.*, 1951). Radioactivity was monitored in a Packard Tri-Carb scintillation counter.

Results

Purification. Table I summarizes the purification scheme now used. The batch illustrated represents a 6-lb aliquot. This particular batch contained only about one-third of the activity found in other lots. This appeared to be correlated with the length of time the carrots had been stored.

The carrots were macerated and filtered through cheesecloth (step I) as previously described (Harvey *et al.*, 1967), and the filtrate was frozen until use. All purification steps were carried out at room temperature. A DEAE-cellulose column was substituted for the three precipitation steps in the earlier procedure. A column (4 × 12) cm was packed with DEAE-cellulose and equilibrated with 0.05 M sodium acetate buffer (pH 5.0). The crude filtrate was thawed and filtered through a Büchner funnel with a Celite precoat just prior to charging the column. After applying filtrate to the column, the latter was washed with 500 ml of the equilibration buffer and eluted with 0.4 M sodium acetate (pH 5.0). The activity was eluted immediately in 390 ml of effluent (step II) and lyophilized. The dried residue was dissolved in 50 ml of distilled water and the pH was adjusted to 7.6 with 1 M Tris-HCl (pH 8.9). This solution was heated for 1 hr at 60° in a water bath and the denatured protein was removed by centrifugation (step III). The supernatant was applied to an Agarose column (2.5 × 95 cm), equilibrated with 0.05 M Tris-HCl (pH 7.6), and fractionated with the same buffer. The diesterase activity emerged after the passage of 250 ml on the leading edge of the main protein peak (absorption at 280 mμ). Tubes containing the activity were pooled (step IV) and added to a second DEAE column (1 × 10 cm), preequilibrated with 0.02 M Tris-HCl (pH 8.9). Development was carried out with a linear gradient of 200 ml of the same buffer containing sodium chloride, reaching a final concentration of 0.5 M. The enzyme was eluted with a salt concentration between 0.2 to 0.3 M in a volume of 58 ml (step V).

After concentration to 2 ml with Carbowax 20,000, the active solution was diluted 20-fold and used to charge a column of hydroxylapatite (0.9 × 20 cm) which had been equilibrated with 0.005 M potassium phosphate buffer (pH 6.5). A linear gradient of the same buffer extending to 0.3 M in a total volume of 400 ml was used to elute the enzyme. The activity was found in the fractions containing 0.075–0.125 M buffer in a total volume of 30 ml (step VI) and concentrated to 5 ml with Carbowax 20,000. The concentrate was applied to a Sephadex G-100 column (2.5 × 37 cm) preequilibrated with 0.01 M Tris-HCl (pH 7.6)–0.5 M KCl. When the column was developed with the same buffer, the diesterase emerged right after the void volume. The active fractions were pooled and stored at −20° (step VII).

Homogeneity. The final specific activity of the purified enzyme was 15,200 units/mg, a threefold increase over that obtained by the former procedure. The enzyme was found to be a single entity by a number of methods, including disc gel electrophoresis, immunoelectrophoresis, and sedimentation by ultracentrifuge.

Immunoelectrophoresis was used as a sensitive technique for examining the homogeneity of the enzyme (Figure 1). The crude enzyme from step IV of the former procedure gave a number of precipitin lines, but after DEAE chromatography (step V of this paper) only two bands were apparent. The final purified protein (Figure 1 C) developed into only one protein band which was superimposable with the diesterase activity. As can be seen in Figure 2, polyacrylamide disc gel electrophoresis at pH 9.5 yielded a single discrete band, which contained the activity.

The purified protein was sedimented in a Spinco Model E

TABLE II: Assay for Contaminating Endonuclease.[a]

Enzyme	Units	$s_{20,w}$ Control	$s_{20,w}$ Plus Enzyme	Estimated Breaks/ Strand
BAP, commercial	0.2	22	4.4	>100
BAP, purified	0.2	19	15	<2
Venom diesterase	60	25	13.5	4
Carrot diesterase, step VI	60	22	10	18
Carrot diesterase, step VII	60	25	22.8	<1

[a] The enzyme was incubated for 1 hr at 37° in the following reaction mixture: 100 mM Tris-HCl, pH 8.9 (or 8.0 for BAP), 20 mM $MgCl_2$, and 0.3 optical density unit of λ phage DNA in a volume of 0.4 ml. After incubation, the mixture was dialyzed at 2° against 2000 volumes of 100 mM Tris-HCl (pH 7.3)–10^{-3} M EDTA. The sample was denatured with 0.1 volume of 1 M NaOH and sedimented in the Model E ultracentrifuge. Breaks per strand were determined from the $s_{20,w}$ by the method of Studier (1965). Units of venom were the same as for carrot diesterase, while BAP is given in international units. The purified BAP was a gift from Dr. C. C. Richardson.

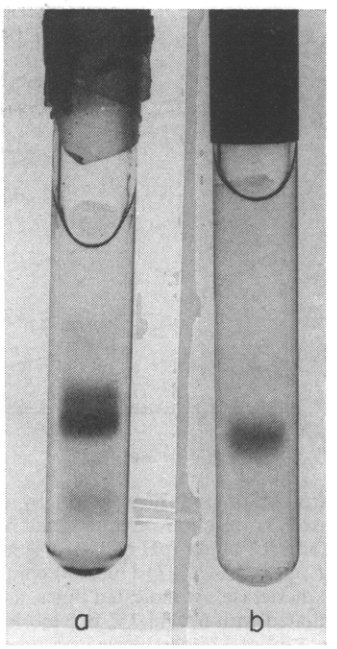

FIGURE 2: Polyacrylamide disc gel electrophoresis at pH 9.5. The direction of electrophoresis was downward: (a) step VI; (b) step VII.

ultracentrifuge in 0.01 M Tris-HCl (pH 7.8)–0.5 M KCl. A single boundary was observed, corresponding to a protein of $s_{20,w} = 5.32$ S. When 0.01 M EDTA was added to the sample, the same result was obtained. However, sedimentation in the same buffer made 6 M in Gd·HCl caused the protein to break into small subunits with a concomitant loss of activity.

The purified enzyme was checked for contaminating activities which would interfere with its use as a tool in analysis of nucleic acid sequence and structure. Nonspecific phosphatase was assayed with *p*-nitrophenylphosphate as described in the Experimental Section. Hydroxylapatite chromatography removed the contaminating phosphatase and none could be detected using 94 units of the final purified diesterase. 5′-Nucleotidase was also absent in the final enzyme preparation; thus, no hydrolysis of deoxyadenosine monophosphate was found with 3 units of the purified enzyme during 2-hr incubation.

The level of endonuclease was barely detectable by a modification of the assay of Weiss *et al.* (1968) (Table II). Controls were run with commercial and purified BAP. The crude preparation gave >100 breaks per strand of λDNA, while the purified preparation yielded <1 such break. Less than one break per strand was also found with purified carrot diesterase.

Properties of the Enzyme. As noted in the previous paper, the properties of the carrot phosphodiesterase closely parallel those of venom diesterase. Thus, the enzyme from carrots was inhibited by reducing agents (Table III) in the same manner as venom diesterase (Razzell and Khorana, 1959). Using cysteine, nearly complete inhibition was obtained with a 0.01 M concentration.

In the earlier publication, it was stated that the enzyme benefits from, but does not require, the addition of certain divalent cations. It has now been possible to demonstrate absolute dependence by preincubation with EDTA. Reversal of EDTA inhibition, either partial or complete, was obtained with Mg^{2+}, Mn^{2+}, Ca^{2+}, and Zn^{2+}. The divalent cations Cu^{2+} and Ba^{2+} were without effect, and Co^{2+} had little effect (10% of the activity in absence of EDTA).

Carrot phosphodiesterase did not hydrolyze dinucleoside diphosphates obtained from micrococcal nuclease digests of calf thymus DNA. These oligomers possess a 3′-phosphate and are known to be resistant to exonucleases initiating hydrolysis from the 3′ end. When 10 units of carrot enzyme was incubated for 3 hr with 5 optical density units of the dimer, no mononucleotides were found by paper chromatography (1 M ammonium acetate (pH 3.8)–ethanol, 30:70).

TABLE III: Effect of Reducing Agents on Phosphodiesterase Activity.

Addition (M)		% of Control
Glutathione	10^{-4}	100
	10^{-3}	71
	10^{-2}	17
2-Mercapto-ethanol	10^{-4}	100
	10^{-3}	76
	10^{-2}	33
Cysteine	10^{-4}	45
	10^{-3}	9
	10^{-2}	2

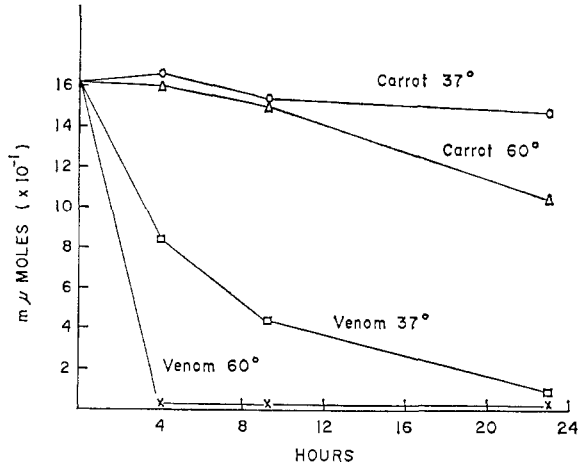

FIGURE 3: Hydrolysis of poly d(A-[^{14}C]T) by carrot and venom phosphodiesterase. The reaction mixture contained in a 1-ml total volume: 0.1 M Tris-HCl buffer (pH 8.9), 0.02 M magnesium acetate, 162 mμmoles of poly d(A-[^{14}C]T) (100,000 cpm), and 7.5 units of carrot or venom diesterase. At indicated times, a 0.2-ml aliquot was removed, precipitated with 0.2 ml 7% perchloric acid, and washed three times with 2 ml of 1% perchloric acid on a glass filter paper (Whatman G F/C, 2.4 cm diameter). The papers were dried and counted in a toluene-based scintillation cocktail.

TABLE IV: Hydrolysis of Compounds Containing Pyrophosphate by Carrot Diesterase.[a]

Substrate	% of PNP-*p*T Activity
ATP	6
ADP	1
NAD	18
T(5′)pppT	50

[a] The assays containing 1 μmole of substrate in the standard assay mixture were incubated with 1 unit of enzyme at 37°. Assays with NAD or T(5′)pppT also included 5 μg of BAP. The ATP assay contained (10^5 cpm) [γ-^{32}P]ATP which was stopped by pipetting a 0.2-ml aliquot into 1 ml of 1 N HCl in ice. To the aliquot was added 0.2 ml of acid-washed Norit (30% packed volume) and 0.2 ml of 0.02 M sodium pyrophosphate–0.025 M NaH_2PO_4 (pH 7.0) buffer–albumin (5 mg/ml). The mixture was shaken and centrifuged. An aliquot of the supernatant was counted in a dioxane-based scintillation cocktail. Aliquots from other substrates were analyzed for P_i by the method of Itaya and Ui (1966).

Spleen phosphodiesterase hydrolyzed the dimers completely to mononucleotides.

Boman (1959) and Razzell (1968) found that venom phosphodiesterase splits the pyrophosphate linkages in ATP, ADP, NAD, and model compounds such as the pyrophosphate derived from 5′,5′-dithymidylic acid. Likewise, carrot diesterase hydrolyzes the pyrophosphate bond in compounds of this type at similar rates (Table IV).

In the preceding paper we postulated that the enzyme had specificity for single-stranded nucleotide polymers. For this reason we attempted to hydrolyze poly d(A-T), which is an alternating polymer of dAMP and dTMP and thus completely hydrogen bonded.

As shown in Figure 3, poly d(A-T) was not hydrolyzed at 37°, while at 60° only partial breakdown of the polymer was observed. Under the same conditions, $d(A\text{-}T)_2$ was completely hydrolyzed, thus proving that the enzyme will break this sequence. Similar results were obtained with the polyribonucleotides poly I and poly C. No detectable breakage of hydrogen-bonded poly(I·C) (1 μmole/ml) with 60 units of enzyme incubated for 24 hr was found. However, with the same quantity of poly C, over 80% had been degraded under these conditions.

Molecular weight determinations by the method of gel filtration (Andrews, 1965) gave values of 0.98–1.15 × 10^5 (five measurements). This agrees with the sedimentation value given earlier, if the assumption is made that the enzyme is a globular protein.

Discussion

Carrot phosphodiesterase has been purified by a revised procedure resulting in considerably better yields than the method published earlier, and a threefold increase of specific activity. Principal changes include a heat-inactivation step and several column chromatographic separations. Incubation of the enzyme at 60° (step III) produced a fivefold increase in the specific activity with almost no loss of diesterase. In fact, it is possible to incubate the enzyme at this temperature for 24 hr with no loss of activity. This thermal stability makes it possible to use the enzyme, which is otherwise completely specific for single-stranded DNA, in the degradation of nucleic acid duplexes dissociated at this temperature.

Chromatographic separations were necessary to remove traces of contaminating enzymes. Thus, hydroxylapatite removed all detectable traces of phosphatase, and Sephadex G-100 removed endonuclease contamination. Although a number of authors have reported venom phosphodiesterase purifications with little or no phosphatase (Richards *et al.*, 1967; Keller, 1964), it has not been possible to remove endonuclease activity. Razzell and Khorana (1959) postulated that venom diesterase may be capable of endonucleolytic scissions.

The resulting protein manifested one precipitin line in immunoelectrophoresis, and was homogeneous in disc gel electrophoresis and sedimentation analysis. Its sedimentation coefficient and behavior in gel filtration permits assignment of a molecular weight of 10^5 daltons. Subunit structure is suspected from its sensitivity toward Gd·HCl.

Most of the properties of carrot diesterase are similar to those of the venom enzyme: both split oligomers of deoxy- and ribonucleotides in a 3′→5′ direction, releasing 5′-mononucleotides. Both are inhibited by the presence of terminal 3′-phosphates, require a divalent cation, and have a pH optimum at 9–9.5. Reducing agents are inhibitory. Carrot diesterase also was found to have pyrophosphatase activity with compounds such as ATP and NAD. The ratio of this activity to the diesterase action remained constant during purification, suggesting that both activities reside in the same protein. A noticeable difference between the carrot and venom enzymes is the specificity of carrot diesterase for

denatured polynucleotides, a property in common with *Escherichia coli* exonuclease I (Lehman and Nussbaum, 1964). However, venom diesterase may show such a complete specificity for single strands when purified to a comparable homogeneous state.

Phosphodiesterases, especially venom diesterases, have been used for characterization of oligonucleotides and as an aid in sequence analysis. The main drawback of venom diesterase has been the difficulty in removing traces of phosphatase and endonuclease. Carrot phosphodiesterase has been purified to a homogeneous state, free from these interfering activities. This should make the enzyme a useful tool in nucleic acid research.

Acknowledgments

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