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A Novel Phosphodiesterase from Cultured Tobacco Cells†

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ABSTRACT: A novel phosphodiesterase was purified from cultured tobacco cells to a state which appeared homogeneous on polyacrylamide gel electrophoresis. The enzyme hydrolyzed various phosphodiester and pyrophosphate bonds, including *p*-nitrophenyl thymidine 5′-phosphate, *p*-nitrophenyl thymidine 3′-phosphate, cyclic nucleotides, ATP, NAD^+, inorganic pyrophosphate, dinucleotides, and poly(adenosine diphosphate ribose), which is a polymer synthesized from NAD^+. However, it did not hydrolyze highly polymerized polynucleotides. The molecular weight of the native enzyme was estimated as 270 000 to 280 000 by gel filtration on Sephadex G-200 and Bio-Gel A-5m. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that the enzyme was composed of subunits with molecular weights calculated to be 75 000. The enzyme did not require divalent cations for activity being fully active in the presence of ethylenediaminetetraacetic acid. The pH optimum for the enzyme was approximately 6 with *p*-nitrophenyl thymidine 5′-phosphate or adenosine cyclic 3′,5′-monophosphate, and 5.3 with NAD^+. Double reciprocal plots of the initial velocity against the concentration of *p*-nitrophenyl thymidine 5′-phosphate gave two apparent K_m values of 0.17 and 1.3 mM, suggesting the presence of at least two active sites.

Although phosphodiesterases that hydrolyze nitrophenyl-pT[1] at acid pH are present in various plant tissues (Razzell, 1966), the enzyme has not so far been purified to determine substrate specificities and other properties.

An enzyme which hydrolyzes nitrophenyl-pT at acid pH is purified to homogeneity on gel electrophoresis from cultured tobacco cells. It is found that many phosphodiesters and pyrophosphate bonds, including cyclic nucleotides, ATP, NAD^+, sodium pyrophosphate, and dinucleotides, are hydrolyzed by the enzyme. Its substrate specificity and enzymological properties are entirely different from those of all other plant and animal phosphodiesterases and pyrophosphatases previously reported. This paper describes the purification and properties of this enzyme.

Experimental Procedure

Plant Material. Cells of tobacco (*Nicotiana tabacum,* cultivar, Bright Yellow 2) were cultured in suspension in the basal medium of Linsmaier and Skoog (1965) supplemented with 1 mg of thiamin-HCl, 0.2 mg of 2,4-dichlorophenoxyacetic acid, and 30 g of sucrose per liter. Cultures were incubated in the dark at 28 °C on a rotary shaker operating at 90

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[1] Abbreviations used are: nitrophenyl-pT, *p*-nitrophenyl thymidine 5′-phosphate; Tp-nitrophenyl, *p*-nitrophenyl thymidine 3′-phosphate; ADP-Rib, adenosine diphosphate ribose; poly(ADP-Rib), polymer of ADP-Rib synthesized from NAD^+; ppGpp, guanosine 3′-diphosphate-5′-diphosphate; NAD, nicotinamide adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-monophosphate; TTP, thymidine 5′-triphosphate; UTP, uridine 5′-triphosphate; CTP, cytidine 5′-triphosphate; GTP, guanosine 5′-triphosphate; NMN, nicotinamide mononucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

rpm. After 7 days the cells were harvested and frozen at −20 °C until enzyme extraction.

Chemicals. Nitrophenyl-pT, Tp-nitrophenyl, bis(*p*-nitrophenyl) phosphate, and *p*-nitrophenyl phosphate were purchased from Calbiochem, La Jolla, Calif. ApA and ApU were obtained from Miles Laboratories, Elkhart, Ind., and ppGpp was a product of Kyowa Hakko Kogyo Co., Tokyo. Calf thymus DNA, yeast RNA, and other nucleotides were products of Sigma Chemical Co., St. Louis, Mo. Poly(ADP-Rib) was prepared and purified as described previously (Sugimura et al., 1971). ^{3}H-Labeled DNA (1.07×10^{6} cpm/mg DNA) was prepared by the procedure of Kay et al. (1952) from Yoshida ascites hepatoma cells labeled with [^{3}H]thymidine. ^{32}P-Labeled tRNA was a gift from Drs. Y. Kuchino and S. Nishimura, National Cancer Center Research Institute, Tokyo.

Alkaline phosphomonoesterase of *Escherichia coli* was a product of Sigma Chemical Co. Protein markers for molecular weight determinations, such as human γ-globulin, bovine serum albumin, and chymotrypsinogen were purchased from Schwarz/Mann, Orangeburg, N.Y., β-galactosidase of *E. coli* from Sigma Chemical Co., and beef liver catalase from Worthington Biochemical Corp., Freehold, N.J.

Methods of Enzyme Assay

Enzyme activities were routinely estimated by Assay 1, and expressed in enzyme units with nitrophenyl-pT as substrate.

Assay 1. This assay was based on the formation of *p*-nitrophenol from nitrophenyl-pT. The standard reaction mixture contained 0.05 M sodium acetate buffer (pH 6.0), 0.5 mM nitrophenyl-pT, and enzyme in a total volume of 1.0 ml. After incubation at 30 °C for 10 min, the reaction was stopped by addition of 2 ml of 0.1 N NaOH and the absorbance at 400 nm was measured against a suitable blank without enzyme. One unit of enzyme was defined as the amount which liberated 1 μmol of *p*-nitrophenol per min. A molar extinction coefficient of 18 000 was used in the calculation.

Assay 2. Enzyme activities toward nucleoside mono-, di-, and triphosphates, sodium pyrophosphate, and other phosphomonoesters were determined by measurement of the amount of inorganic phosphate released. The reaction mixture contained 2 mM substrate, 0.05 M sodium acetate buffer (pH 6.0), and enzyme in a total volume of 0.5 ml, and was incubated at 30 °C for 10 min. The inorganic phosphate liberated was estimated by the procedure of Fiske and SubbaRow (1925) as modified by Josse (1966).

Assay 3. Enzyme activities toward the other substrates shown in Table II were determined by measurement of the amount of inorganic phosphate released by hydrolysis of nucleoside monophosphates by *E. coli* alkaline phosphomonoesterase. The reaction mixture was the same as for assay 2. After incubation at 30 °C for 15 min, the reaction was stopped by heating the mixture in a boiling-water bath for 2 min. Then 0.3 ml of *E. coli* alkaline phosphomonoesterase (0.5 unit) in 0.5 M Tris solution was added, and the mixture was incubated at 30 °C for 10 min. After incubation with the enzyme 0.2 ml of ice-cold 25% trichloroacetic acid was added and the mixture was allowed to stand for 30 min in the cold. Then it was centrifuged at 2000*g* for 10 min and 0.5 ml of the supernatant was taken for determination of inorganic phosphate.

Assay 4. For measurement of enzyme activity toward ^{14}C-labeled poly(ADP-Rib), the decrease in the radioactivity of the acid-insoluble fraction was measured. The reaction mixture, in a final volume of 0.3 ml, contained 25 μM as ADP-Rib residues of poly(ADP-Rib), 50 mM sodium acetate buffer (pH 6.0), 100 μg/ml of bovine serum albumin, and enzyme. After incubation at 30 °C for 10 min, the acid-insoluble radioactivity was determined by the filter paper disk method (Bollum, 1966).

Other Methods

Polyacrylamide Gel Electrophoresis. Gels containing 5% acrylamide at pH 4.3 were prepared as described by Reisfeld et al. (1962). Electrophoresis was carried out at 4 °C at 3 mA/gel for 4.5 h. Gels containing 0.1% sodium dodecyl sulfate were prepared as described by Weber and Osborn (1969). Electrophoresis was carried out at 8 mA/gel for 4 h. Proteins in the gels were stained with Coomassie brilliant blue by the method of Weber and Osborn (1969). To detect the phosphodiesterase activity, gels were cut into 1-mm-thick sections and extracted overnight with 0.01 M Tris-HCl buffer (pH 7.5) containing 10 mM 2-mercaptoethanol, 0.01% (v/v) Triton X-100, and 0.2 M NaCl at 4 °C, and the activity of the extracts was assayed. Alternatively, the following simple procedure was sometimes used. Gels were incubated in the reaction mixture containing 1 mM nitrophenyl-pT and 0.05 M sodium acetate buffer (pH 5.0) for an appropriate time, and then activity was visualized by dipping the gels into 0.1 M Tris-HCl buffer (pH 9.0).

Thin-Layer Chromatography. The following solvents were used for thin-layer chromatography. Solvent A: 2-propanol–saturated ammonium sulfate–water (2:79:19, v/v) was used for cellulose thin-layer chromatography. Solvent B: isobutyric acid–concentrated ammonium hydroxide–water (66:1:33, v/v) was used for silica gel thin-layer chromatography.

Protein Determination. In the first purification steps, protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. To prevent the interference from contaminating phenols, protein was precipitated with 5% trichloroacetic acid, washed with ethanol, and dissolved in 1 N NaOH before color development. From step 4 of purification, protein was estimated spectrophotometrically (Kalckar, 1947).

Results

Purification of Tobacco Phosphodiesterase

All operations were carried out between 0 and 4 °C.

Step 1. Crude Extract. Three hundred grams wet weight of frozen tobacco cells was thawed and suspended in 250 ml of 0.1 M sodium acetate buffer (pH 5.0) containing 0.2 M NaCl, 10 mM 2-mercaptoethanol, and 1 mM EDTA. The cell suspension was homogenized in a Waring blender operating at maximal speed for 4 min. The homogenate was centrifuged at 5000*g* for 20 min to remove cell debris and the resulting supernatant solution (300 ml) was again centrifuged at 20 000*g* for 20 min. The supernatant was collected (fraction 1).

Step 2. Ammonium Sulfate Precipitation. To fraction 1, solid ammonium sulfate was added with stirring over a period of 15 min to give 80% saturation. After stirring for an additional 30 min, the precipitate was collected by centrifugation at 10 000*g* for 20 min and dissolved in 12 ml of 0.01 M Tris-HCl buffer (pH 7.5) containing 10 mM 2-mercaptoethanol, 0.01% (v/v) Triton X-100, and 20% (v/v) glycerol (buffer A). This preparation was desalted by gel filtration on a Sephadex G-25 column (3.5 × 42 cm) equilibrated with buffer A. Active fractions were combined (fraction 2).

Step 3. DEAE-Cellulose Column Chromatography. Fraction 2 (80 ml) was applied to a DEAE-cellulose column (2.4 × 10 cm) previously equilibrated with buffer A. The

TABLE I: Purification of Phosphodiesterase from Cultured Tobacco Cells.

Purification Step	Protein (mg)	Act. (units)	Yield (%)	Sp Act. (units/mg)
I, Crude extract	280	50.0	100	0.178
II, Ammonium sulfate	180	37.0	74	0.212
III, DEAE-cellulose	150	27.0	54	0.172
IV, Phosphocellulose	35	24.4	49	0.800
V, Sephadex G-200	3.9	18.5	37	4.75
VI, 2nd Phosphocellulose	1.0	15.8	32	15.8
VII, Bio-Gel A-5m	0.6	12.6	25	19.8

column was washed with buffer A, and then eluted with 300 ml of a linear gradient of 0.01–0.2 M Tris-HCl buffer (pH 7.5) containing 10 mM 2-mercaptoethanol, 0.01% Triton X-100, and 20% glycerol. The phosphodiesterase activity was separated into two fractions by DEAE-cellulose chromatography. More than 90% of the activity was not adsorbed on the column, and fractions containing this activity were combined (fraction 3). A small amount of activity was adsorbed on the column and eluted with 0.05 M Tris-HCl buffer (pH 7.5), but this was not studied further.

Step 4. Phosphocellulose Column Chromatography. Fraction 3 (100 ml) was adsorbed on a phosphocellulose column (1.4 × 5 cm) previously equilibrated with buffer A. The column was washed with buffer A, and then eluted with 200 ml of a linear gradient of 0–0.4 M NaCl in buffer A. The enzyme was eluted with 0.06–0.1 M NaCl, and these fractions with activity were pooled and concentrated by ultrafiltration on a UM-10 Diaflo membrane (fraction 4).

Step 5. Sephadex G-200 Gel Filtration. Fraction 4 was applied to a Sephadex G-200 column (1.4 × 40 cm) previously equilibrated with 0.01 M Tris-HCl buffer (pH 7.5) containing 0.2 M NaCl, 10 mM 2-mercaptoethanol, and 0.01% Triton X-100 (buffer B). The fractions with activity were pooled (13 ml) and dialyzed against 1000 ml of 0.01 M Tris-HCl buffer (pH 7.5) containing 10 mM 2-mercaptoethanol and 0.01% Triton X-100 (buffer C) to yield fraction 5.

Step 6. Re-chromatography on Phosphocellulose Column. Fraction 5 was applied to a phosphocellulose column (1.0 × 4 cm) previously equilibrated with buffer C. The column was washed with buffer C and then eluted with 100 ml of a linear gradient of 0–0.3 M NaCl in buffer C. The enzyme was eluted with 0.06 M–0.12 M NaCl. The fractions with activity were combined and concentrated by ultrafiltration (fraction 6).

Step 7. Bio-Gel A-5m Gel Filtration. Fraction 6 was applied to a Bio-Gel A-5m column (1.4 × 40 cm) previously equilibrated with buffer B. The fractions with activity were pooled and concentrated by ultrafiltration and used for enzyme characterization.

The results of a typical purification of the enzyme are summarized in Table I. At the final step, the enzyme was purified 120-fold over the crude extract with an overall yield of 25%.

For isolation of the enzyme, tobacco cells were homogenized at a relatively low pH (5.0), because this gave a crude extract with a higher specific activity than that obtained on homogenization at pH 7.5, and because it avoided tanning due to phenols and phenol oxidases (Loomis, 1974) in the cells.

Addition of Triton X-100 to the buffer solution during enzyme purification increased the yield of enzyme activity on subsequent gel filtration on Sephadex G-200 and Bio-Gel A-5m. The effect of this surface activator may be to protect the enzyme from surface inactivation. There are reports that acid phosphatases from various sources lose activity due to surface inactivation, and that their activities can be protected by the presence of a surface activator (Tsuboi et al., 1957; Neumark and Wengner, 1960).

TABLE II: Substrate Specificity of Tobacco Phosphodiesterase.

Substrate	Relative Act.[a] (%)	Assay Method
Nitrophenyl-pT	1410	1
Tp-nitrophenyl	1520	1
Bis(*p*-nitrophenyl) phosphate	3200	1
p-Nitrophenyl phosphate	655	1
Adenosine cyclic 3′,5′-monophosphate	45	3
Adenosine cyclic 2′,3′-monophosphate	120	3
ApA	8	3
ApU	4	3
ATP	100	2
ADP	67	2
AMP	0	2
ppGpp	71	2
NAD^+	28	3
NADH	28	3
UDP-Glucose	29	3
ADP-Rib	21	3
Poly(ADP-Rib)	14	4
Sodium pyrophosphate	55	2

[a] Activities are expressed relative to that obtained with ATP.

Electrophoresis of the Purified Enzyme. The purified enzyme preparation migrated as a single protein band on 5% polyacrylamide disc gel electrophoresis at pH 4.3 for 4.5 h. No other faster moving band was detected on electrophoresis for a shorter time (2 h). All the phosphodiesterase activity was found in the region corresponding to the protein band stained with Coomassie brilliant blue, when the gel was sliced and extracted and extracts were tested for activity toward nitrophenyl-pT or poly(ADP-Rib). The enzyme activity was also found at the same place as the protein band, when the gels were stained in situ for activity with nitrophenyl-pT. A single protein band was also detected on electrophoresis in 6 and 7.5% acrylamide gels.

Properties of Tobacco Phosphodiesterase

Molecular Weight. Molecular Weight of the Native Enzyme. The molecular weight of the native enzyme was estimated by gel filtration on Sephadex G-200 and Bio-Gel A-5m with appropriate molecular weight standards by the method of Andrews (1964). The molecular weights estimated for the enzyme using Sephadex G-200 and Bio-Gel A-5m were 270 000 and 280 000, respectively.

Molecular Weight of Subunits. The enzyme dissociated on treatment with 1% sodium dodecyl sulfate in the presence of 5% 2-mercaptoethanol, giving a single band on electrophoresis in 5% polyacrylamide gel containing sodium dodecyl sulfate. The molecular weight of the subunit was estimated to be 75 000, using standard proteins of known molecular weight.

Substrate Specificity. The activity of the purified enzyme was tested with a number of substrates. The results are summarized in Table II.

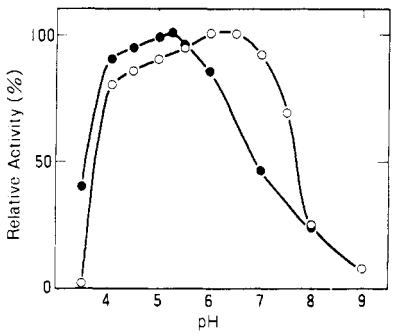

FIGURE 1: Effect of pH on enzyme activity. Activity was estimated with nitrophenyl-pT (○) as substrate by assay 1, and with NAD^+ (●) as substrate by assay 2. The incubation mixtures contained 0.3 and 1.2 μg of purified enzyme and 0.5 mM nitrophenyl-pT and 2.0 mM NAD^+, respectively, in 1.0 ml of 0.05 M Tris-acetate buffer.

Synthetic Substrates. All the synthetic substrates of *p*-nitrophenol esters examined were readily hydrolyzed by the enzyme. Bis(*p*-nitrophenol) phosphate was the most effective substrate, and both nitrophenyl-pT and Tp-nitrophenyl were hydrolyzed. *p*-Nitrophenyl phosphate was also hydrolyzed at a considerable rate, but no activity was found toward naturally occurring nucleoside monophosphates or any of the other phosphomonoesters examined, including 5′-AMP, 3′-AMP, 2′-AMP, glucose 1-phosphate, and glucose 6-phosphate.

Nucleoside Di- and Triphosphates. All the nucleoside triphosphates tested, including ATP, TTP, UTP, CTP, and GTP, were hydrolyzed at a considerable rate. ATP was split to ADP and inorganic phosphate, and ADP to AMP and inorganic phosphate, as revealed by silica gel thin-layer chromatography with solvent A.

Nucleoside Cyclic Monophosphates. Adenosine cyclic 2′,3′-monophosphate and adenosine cyclic 3′,5′-monophosphate were hydrolyzed at comparable rates to ATP and ADP. Adenosine cyclic 3′,5′-monophosphate was hydrolyzed at about 40% of the initial velocity of adenosine cyclic 2′,3′-monophosphate. Cellulose thin-layer chromatography in solvent A revealed that adenosine cyclic 2′,3′-monophosphate was split to 3′-AMP. Adenosine cyclic 3′,5′-monophosphate was hydrolyzed to yield mainly 5′-AMP with a small amount of 3′-AMP.

Dinucleotides. ApA and ApU were hydrolyzed considerably slower than nucleoside triphosphates and nucleoside cyclic monophosphates. ApA was split to 5′-AMP and adenosine, as shown by silica gel thin-layer chromatography with solvent B.

Pyrophosphates. The enzyme also catalyzed the cleavage of pyrophosphate bonds. NAD^+ was hydrolyzed at 28% of the initial velocity of ATP at pH 6.0. NAD^+ was split to 5′-AMP and NMN as shown by silica gel thin-layer chromatography with solvent B. NADH, ADP-Rib, UDP-glucose, ppGpp, and inorganic pyrophosphate were hydrolyzed. This enzyme also hydrolyzed poly(ADP-Rib), a polymer synthesized from NAD^+ by isolated animal cell nuclei (Sugimura, 1973).

Polynucleotides. Hydrolysis of polynucleotides was estimated as described by Heppel and Hilmoe (1955), by measuring the change in absorbance at 260 nm of nucleotides soluble in uranyl reagent. For the reaction 1 mg of yeast RNA, native or denatured calf thymus DNA, or poly(adenylic acid) was incubated with 0.5 unit of the purified enzyme in a final volume of 1 ml at 30 °C for 6 h. No detectable hydrolysis of these polynucleotides was observed. Enzyme activity on polynucleotides was also examined by the method of Weissbach and Korn (1963) using labeled polynucleotides. Thirty nanomoles of ^{32}P-labeled tRNA of *E. coli* or ^3H-labeled native or denatured DNA of Yoshida ascites hepatoma cells was incubated with 0.1 unit of the purified enzyme in a final volume of 0.2 ml at 30 °C for 7 h. The results indicated that the enzyme did not hydrolyze the polynucleotides.

TABLE III: Effect of Nucleotides and Related Compounds.[a]

Addition	Inhibition[b] (%)
5′-ATP	10
5′-ADP	44
5′-AMP	49
3′-AMP	42
2′-AMP	42
Adenosine	0
Adenine	0
NAD^+	14
NADH	9
$NADP^+$	30
NADPH	12
Poly(ADP-Rib)	33
RNA (yeast)	29
DNA (calf thymus)	10

[a] Hydrolysis of nitrophenyl-pT was estimated by assay 1. The compounds, except DNA and RNA, were added at 0.2 mM concentration, and DNA and RNA were added at a concentration of 0.1 mg/ml. [b] Enzyme activity with no addition was taken as 100%.

Other Enzymological Properties. Effect of pH. Results on the effect of pH on enzyme activity are shown in Figure 1. The enzyme had a broad pH optimum of about 6–6.5 for nitrophenyl-pT. With NAD^+ and adenosine cyclic 3′,5′-monophosphate as substrates the enzyme showed maximal activity at around pH 5.3 and pH 6.0, respectively.

Effects of Metal Ions and Other Compounds. Of various divalent metal ions examined (Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+}, Ba^{2+}, Cu^{2+}, and Fe^{2+}), none stimulated the enzyme activity with nitrophenyl-pT as substrate, but Mn^{2+}, Co^{2+}, and Cu^{2+} caused 10–20% inhibition at 1 mM.

Dialysis of the enzyme against the buffer C containing 1 mM EDTA did not cause any inactivation. Addition of 10 mM of EDTA to the reaction mixture stimulated the enzyme activity by 27%.

NaF and NaH_2PO_4 at 1 mM inhibited the activity by 63 and 59%, respectively.

Effect of Nucleotides and Related Compounds. As shown in Table III, hydrolysis of nitrophenyl-pT by the enzyme was inhibited by 5′-AMP, 2′-AMP, 3′-AMP, ADP, $NADP^+$, and NAD^+. Poly(ADP-Rib), RNA, and DNA also inhibited the activity.

Heat Stability. The effects of heat on the enzyme activities toward nitrophenyl-pT, ATP, and NAD^+ were identical, as shown in Figure 2. Preincubation at 80 °C at pH 7.5 for 5 min caused complete loss of activity.

Effects of Sulfhydryl Compounds. When the purified enzyme was dialyzed against 0.05 M Tris-HCl (pH 7.5) to remove 2-mercaptoethanol, it lost 14% of its original activity. Addition of 10 mM 2-mercaptoethanol or cysteine to the dialyzed preparation partially restored this activity. *p*-Chloromercuriphenylsulfonate caused 32% inhibition at a concentration of 1 mM.

Effect of Substrate Concentration. The influence of nitrophenyl-pT concentration on the reaction velocity is shown by double-reciprocal plots in Figure 3. The plots show deviations from the usual kinetics, and two apparent K_m values were obtained, which were calculated to be 0.17 and 1.3 mM. On

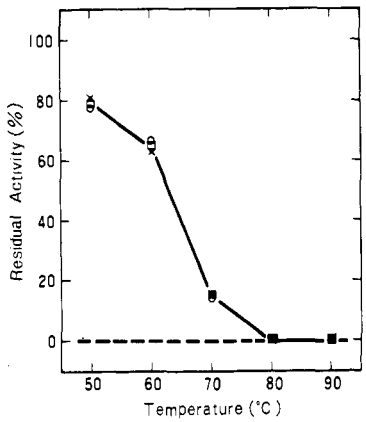

FIGURE 2: Effect of heat on enzyme activity. The enzyme was heated in 0.05 M Tris-HCl buffer (pH 7.5) for 5 min at the indicated temperature, and then the mixture was rapidly chilled in ice. The activities of samples of 1 μg of enzyme protein were assayed immediately, with either 0.5 mM nitrophenyl-pT (○), 2 mM ATP (×), or NAD^+ (□) as substrate by assays 1, 2, and 3, respectively.

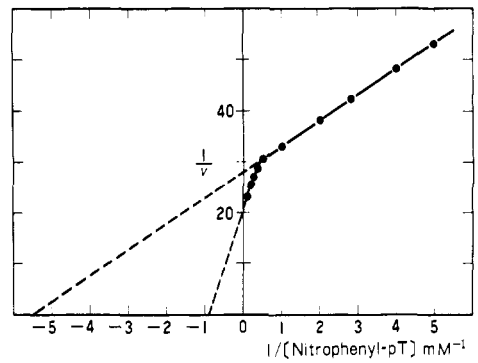

FIGURE 3: Double-reciprocal plot of the reaction velocity with nitrophenyl-pT as substrate. The reaction velocities were measured by assay 1 using substrate concentrations of 0.2, 0.25, 0.35, 0.50, 1.0, 2.0, 3.0, 4.0, 5.0, and 10 mM.

replotting the data in Figure 1 by the equation of Hill (1913), a linear relation was obtained at the concentration below 5 mM nitrophenyl-pT. The Hill coefficient was calculated to be 0.6. The R_s value (Koshland et al., 1966), i.e., the ratio of the substrate concentration at 90% saturation velocities to that at 10% saturation, was calculated to be greater than 81. Thus, the activity shows all the characteristics of negative cooperativity proposed by Levitzki and Koshland (1969). These results suggest the presence of at least two active sites on the enzyme.

Discussion

Although alkaline phosphodiesterases, which are active at alkaline pH and require metal ions for activity, have been purified from carrot (Harvey et al., 1967, 1970), sugar beet (Lerch and Wolf, 1972), and snake venom (Frischauf and Eckstein, 1973; Dolapchiev et al., 1974), the tobacco acid phosphodiesterase appears to be the first purified enzyme which is active at acid pH in the presence of EDTA. The tobacco acid phosphodiesterase appears to be distinct from any other related enzymes in its substrate specificity. This enzyme hydrolyzes most of the phosphodiester bonds of low-molecular-weight substances and the pyrophosphate bonds. However, it does not hydrolyze highly polymerized polynucleotides such as calf thymus DNA or yeast RNA. Poly(ADP-Rib), a biopolymer synthesized from NAD^+ in the cell nuclei, was hydrolyzed by this enzyme. It should be noted that this enzyme is also the first enzyme of plant origin that hydrolyzes poly(ADP-Rib) (Sugimura, 1973). Alkaline phosphodiesterases from plants (Harvey et al., 1970; Lerch and Wolf, 1972), rat liver (Futai and Mizuno, 1967), and snake venom (Dolapchiev et al., 1974) were shown to possess pyrophosphatase activity, but their specificities were more restricted than that of the tobacco acid phosphodiesterase.

Furthermore, the tobacco acid phosphodiesterase purified was shown to be distinct from alkaline phosphodiesterases in many respects: pH optimum, metal ion requirement, molecular weight, heat stability, effect of reducing agents, and kinetic properties. The tobacco acid phosphodiesterase is entirely different from the extracellular nuclease from suspension cultures of tobacco in substrate specificity (Oleson et al., 1974a,b). The tobacco acid phosphodiesterase is also different from potato nucleotide pyrophosphatase (Kornberg and Pricer, 1950) in pH optimum and ability to hydrolyze poly(ADP-Rib) (Hasegawa et al., 1967).

The tobacco acid phosphodiesterase shows considerable activity toward *p*-nitrophenyl phosphate, but no activity toward other phosphomonoesters such as 5′-AMP, 3′-AMP, glucose 6-phosphate, and glucose 1-phosphate. Strictly speaking, the *p*-nitrophenyl esters are not phosphomonoesters or phosphodiesters but rather mixed anhydrides formed by a bond between a phenol and a phosphoryl group, as pointed out by Spahr and Gesteland (1970). The enzyme may have affinity toward this type of bond.

The apparent molecular weight of the native enzyme measured by gel filtration on Sephadex G-200 and Bio-Gel A-5m was 270 000 to 280 000. This value is much higher than those reported for other phosphodiesterases. The molecular weights of plant alkaline phosphodiesterases from carrot and sugar beet are around 100 000 (Harvey et al., 1970; Lerch and Wolf, 1972), and that of snake venom phosphodiesterase is 130 000 (Frischauf and Eckstein, 1973).

Sodium dodecyl sulfate gel electrophoresis showed a single protein band, and it was supposed that the tobacco acid phosphodiesterase consists of subunits of identical molecular weight of 75 000. The elucidation of the quaternary structure of the enzyme requires further study.

It has been reported that plant seedlings and leaf tissues have alkaline and acid phosphodiesterase (Razzell, 1966; Udvardy et al., 1970). It may be interesting to note that no alkaline phosphodiesterase that was stimulated by divalent ions or inhibited by EDTA above pH 8 was found in the homogenate of the cultured tobacco cells.

There has been a considerable interest in the possible significance of adenosine cyclic 3′,5′-monophosphate in metabolic regulation in higher plant as well as in animal or prokaryotic cells. Adenosine cyclic 3′,5′-monophosphate phosphodiesterase activity has been found in plants (Lin and Varner, 1972; Brewin and Northcote, 1973; Vandepeute et al., 1973), but the specificities of these enzymes are still uncertain. Some properties of these enzymes resemble those of the tobacco acid phosphodiesterase. The overall recovery of activity for adenosine cyclic 3′,5′-monophosphate by the purified tobacco acid phosphodiesterase is 20% of that of the crude extract, and this value is comparable to that for recovery of activity for nitrophenyl-pT. It is likely that the plant enzyme previously reported as adenosine cyclic 3′,5′-monophosphate phosphodiesterase is a similar type of enzyme to that reported here.

The physiological significance of the tobacco acid phosphodiesterase is not known at present. However, it seems quite likely that the enzyme hydrolyzes the unique pyrophosphate bonds at 5′-terminal ($m^7G^{5'}$ $ppp^{5'}$ N) of several RNA viruses including the tobacco mosaic virus (Zimmern, 1975), which

was recently elucidated. The action of this enzyme on those bonds of tobacco mosaic virus and possible significance of this enzyme to RNA virus infection are under investigation.

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