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PURIFICATION AND PROPERTIES OF A PHOSPHODIESTERASE FROM AVENA LEAF TISSUES

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SUMMARY

Avena leaf tissues were shown to contain at least two phosphodiesterases which are active on bis-*p*-nitrophenyl phosphate and *p*-nitrophenyl-TMP as substrates; these were found to have pH optima of 5.5 and 9.3. The enzyme with a pH optimum of 9.3 was purified about 130-fold. This phosphodiesterase, which was found in the soluble fraction, resembles phosphodiesterase I from snake venom and animal tissues in a number of general properties including its pH optimum and its sensitivity to metal-chelating (EDTA) and complexing (CN^-) agents. Bivalent ions such as Mg^{2+}, Co^{2+}, Zn^{2+}, and Ca^{2+} increased the enzyme activity; 3 M urea had no effect. The enzyme showed a considerable heat stability. Native DNA and tRNA were highly resistant to the Avena phosphodiesterase, while single-stranded RNA was slowly attacked; however, denatured DNA was rapidly attacked. The enzyme hydrolyzed poly A, poly C and poly U at about the same rates, whereas poly I was cleaved at a lower rate. The action of Avena phosphodiesterase on heat-denatured DNA was found to be exonucleolytic and resulted in the successive liberation of nucleoside 5'-phosphate units. ADP, ATP, FAD, NAD^+ and $NADP^+$ inhibited the enzyme activity. Kinetic experiments with NAD^+ showed that the inhibition was competitive. Cyclic 2',3'-mononucleotides and adenosine 3',5'-cyclic phosphate proved to be noninhibitory. The K_m value for *p*-nitrophenyl-TMP and the K_i for NAD^+ were found to be equivalent (0.4 mM).

INTRODUCTION

It has been shown that the level of "ribonuclease activity" rapidly increases upon leaf excision[1–6], mechanical injury[7,8], parasitic attack[7,9,10], osmotic stress[8,11], and other stress effects[12]. Recently, evidence has been obtained that more than one nuclease is affected in "stressed" tissues[5]. In a previous paper we reported on the purification and properties of a ribonuclease the level of which appears to be primarily affected in excised Avena leaf tissues[13]. Since phosphodiesterase activity, as

assayed in crude extracts, has also been shown to increase in excised Avena leaf tissues[5], the phosphodiesterases of intact and excised Avena leaves were also studied in more detail. In the present paper the purification and properties of a phosphodiesterase isolated from Avena leaf tissues are described and some observations on another phosphodiesterase(s) are reported.

MATERIALS AND METHODS

Determination of enzyme activities

Ribonuclease, phosphodiesterase and phosphatase assays were performed as previously described[5]. When *p*-nitrophenyl-TMP was used as substrate, the phosphodiesterase assay was carried out by the method of HARVEY *et al.*[14]. An increase of 12 *A* units at 400 nm is known to be equivalent to 1 μmole of enzyme-liberated *p*-nitrophenol[15]. One enzyme unit is that amount of enzyme which liberates 1 μmole of *p*-nitrophenol per h under the assay conditions. Specific activity is expressed as enzyme units per mg of protein. Deoxyribonuclease activity was assayed in the same way as ribonuclease except that DNA was used as substrate. Nucleotidase activities were assayed with 3'-AMP and 5'-AMP as substrates at both pH 8.0 and 5.5, as described by WALTERS AND LORING[16]. The release of P_i was determined by the micromethod of CHEN *et al.*[17].

Enzyme purification

Depending on the aim of the experiment, two different procedures were used for partial purification of phosphate ester-splitting enzymes.

Method I. 120 g of 10-day-old *Avena sativa* L. leaf tissue were homogenized in 360 ml of 0.05 M Tris–HCl buffer (pH 7.5) in a prechilled mortar with quartz sand. The homogenate was passed through four layers of cheesecloth and was repeatedly frozen and thawed before the first centrifugation. The homogenate was centrifuged at 40 000 × *g* for 30 min at 4°. The supernatant was brought to pH 4.5 by the addition of 1 M HCl and allowed to stand for 3 h. The resulting precipitate was removed by centrifugation at 8000 × *g* for 15 min. The supernatant was adjusted to pH 5.5 by the addition of 1 M NaOH. Then, solid $(NH_4)_2SO_4$ was added to the supernatant to 45% saturation and the solution was kept overnight at 3°. The precipitate was removed by centrifugation at 8000 × *g* for 30 min. Solid $(NH_4)_2SO_4$ was added to the supernatant to 85% saturation. The solution was allowed to stand overnight in the cold. The sediment obtained upon centrifugation at 8000 × *g* for 30 min was dissolved in 100 ml of 0.1 M sodium citrate buffer (pH 5.5) and the $(NH_4)_2SO_4$ precipitation step, as described above, was repeated. The protein fraction obtained between 45 and 85% $(NH_4)_2SO_4$ saturation was dissolved in 5 ml of 0.01 M Tris–HCl buffer (pH 7.5) and applied, after dialysis against 3 l of distilled water for 12 h, to a 2.4 cm × 100 cm Sephadex G-75 column equilibrated with 0.02 M Tris–HCl buffer (pH 7.5). Elution was carried out with the same buffer. All fractions were assayed for ribonuclease, phosphodiesterase and phosphatase activities (*cf.* Fig. 1).

Method II. Preliminary steps of Method II were identical with those of Method I up to the $(NH_4)_2SO_4$ fractionation step which was carried out only once. The fraction obtained between 45 and 85% $(NH_4)_2SO_4$ saturation was dissolved in 70 ml of 0.1 M sodium acetate buffer (pH 5.5) and the proteins were subjected to acetone precipi-

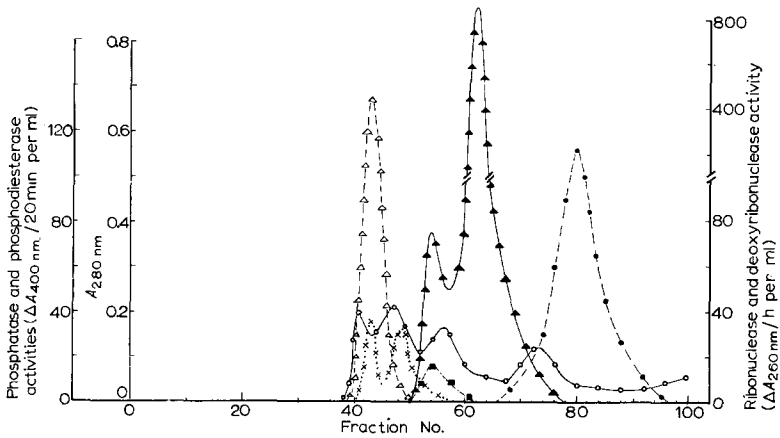


Fig. 1. Chromatography of phosphate ester-splitting enzymes isolated from Avena leaves. Proteins partially purified as described in MATERIALS AND METHODS were applied to a Sephadex G-75 column (2.4 cm × 100 cm) equilibrated with 0.01 M Tris–HCl buffer (pH 7.5). Proteins were eluted with the same buffer and monitored at 280 nm. 3.0-ml fractions were collected. The assay system for RNA-splitting activity contained 1.5 mg yeast RNA, 100 μmoles of acetate buffer (pH 5.5) and 0.4 ml of the effluent in a final volume of 2.0 ml. The incubation mixture was kept at 37° for 30 min. Enzyme activity is expressed as the increase in absorption at 260 nm of acid-soluble (0.3% $La(NO_3)_3$ in 2.5% trichloroacetic acid) digestion products[5]. DNA-splitting activity was assayed in the same way with salmon sperm DNA as substrate. The assay system for phosphodiesterase activity consisted of 1 μmole of *p*-nitrophenyl-TMP, 100 μmoles of acetate buffer (pH 5.5) or Tris–HCl buffer (pH 8.8) and 0.3 ml effluent in a final volume of 2.0 ml. After incubation at 37° for 20 min the reaction was stopped with 1.0 ml of 0.3 M NaOH, and the increase in absorbance at 400 nm was measured. Phosphatase activity was assayed in the same way with *p*-nitrophenyl phosphate as substrate. ○—○, protein; △— — —△, phosphodiesterase (pH 8.8); × . . . ×, phosphodiesterase (pH 5.5); ▲—▲, ribonuclease; ■— · —■, deoxyribonuclease; ●— — —●, phosphatase.

tation at −16°. The precipitate obtained between 30–70% acetone concentration was collected by centrifugation and dissolved in 70 ml of 0.1 M sodium acetate buffer (pH 5.5). Then, the acetone precipitation step, as described above, was carried out once more. The precipitate was centrifuged and the sediment was dissolved in 3 ml of 0.01 M Tris–HCl buffer (pH 7.5). The solution was applied to a 2.3 cm × 100 cm Sephadex G-75 column equilibrated with 0.01 M Tris–HCl buffer (pH 7.5). Proteins were eluted with the same buffer. The fractions rich in phosphodiesterase were collected and lyophilized. The lyophilized product was dissolved in 2 ml of distilled water and dialyzed against distilled water overnight. The solution then was applied to a DEAE-cellulose column (1 cm × 30 cm) and eluted by a 0.0–0.5 M linear NaCl gradient as described by HARVEY *et al.*[14]. A typical elution profile is given in Fig. 2. Fractions 34–40 and 45–51, respectively, were pooled and used for further assays.

Test to establish the mode of cleavage

1.2 mg poly A or poly U were incubated with an enzyme preparation corresponding to 40 μg of protein in the presence of 120 μmoles of Tris–HCl buffer (pH 8.8) and 12 μmoles of $MgCl_2$ in a total volume of 1.2 ml. Aliquots were withdrawn after 1, 2, 3 and 4 h of incubation and kept at 100° for 5 min to inactivate the enzyme. Each sample was then chromatographed on Whatman No. 1 filter paper using the

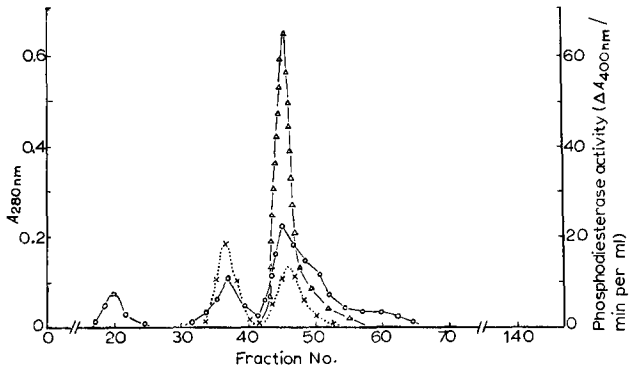


Fig. 2. Chromatography on DEAE-cellulose of two phosphodiesterase-rich fractions obtained from a Sephadex G-75 column. The pooled fractions (*cf.* Fig. 1 Fractions 40–56) were lyophilized, dissolved in 2.0 ml of distilled water, dialyzed against water and applied to a DEAE-cellulose column (1 cm × 30 cm). Elution was carried out with a linear NaCl gradient (0–0.5 M). 2.5-ml fractions were collected. Enzyme activities were assayed as described in the legend to Fig. 1. ○—○, protein; △- - -△, phosphodiesterase (pH 8.8); × · · · ×, phosphodiesterase (pH 5.5).

descending technique in a solvent system of saturated $(NH_4)_2SO_4$–isopropanol–1 M sodium acetate (80:2:18, by vol.)[18]. Authentic samples of 3′- and 5′-mononucleotides were run as reference substances. The spots were marked under ultraviolet light, eluted with 0.1 M HCl for 24 h, and the substances were identified on the basis of their R_F values and spectral properties.

Identification of the products of DNA digestion

The incubation mixture contained 10 mg of heat-denatured DNA, 50 μmoles of Tris–HCl buffer (pH 8.8) and an enzyme preparation corresponding to 70 μg protein in a total volume of 2.0 ml. 0.5-ml aliquots were removed after 2, 4, 6 and 8 h of incubation and, after suitable evaporation, subjected to two-dimensional paper chromatography on Whatman No. 3 filter paper in the following systems: ethanol–1 M ammonium acetate buffer (pH 7.5) (75:30, by vol.) in the first direction and saturated $(NH_4)_2SO_4$–water–isopropanol (80:18:2, by vol.) in the second direction[19]. The ultraviolet-absorbing spots were eluted and identified as described above.

Hydrolysis of purine and pyrimidine nucleoside cyclic phosphates

This was carried out as described earlier[5].

The use of gel filtration to distinguish between endonuclease or exonuclease action

The method of BIRNBOIM[20] was adopted except that Sephadex G-100 was replaced by Sephadex G-50. The composition of the incubation mixtures is described in the legend to Fig. 6.

Estimation of protein content

Protein content in the crude extract was determined by the method of LOWRY *et al.*[21] using bovine serum albumin as standard. In the later stages of purification the protein content was estimated by the ultraviolet absorption method according to KALCKAR[22].

Materials

First leaves of 10–12-day-old *A. sativa* cv. Alaska seedlings grown under ordinary greenhouse conditions were used for enzyme purification.

All the mononucleotides were obtained from the Pabst Laboratories, Milwaukee, Wisc. All the cyclic mononucleotides, *p*-nitrophenyl phosphate disodium salt, bis-*p*-nitrophenyl phosphate sodium salt, ADP, ATP, FAD, NAD^+, $NADP^+$ and the homopolymers were products of Sigma Chemical Co., St. Louis, Mo. Highly polymerized yeast RNA (lot No. 63411), highly polymerized DNA sodium salt from salmon sperm (lot No. 590554), *p*-nitrophenyl-TMP sodium salt (lot No. 840173) and DEAE-cellulose were obtained from Calbiochem, Los Angeles, Calif. tRNA from brewers yeast (Cat. No. 1110-50) was purchased from Schwarz Bioresearch, Orangeburg, N.J. Sephadex G-50 and G-75 were products of Pharmacia Fine Chemicals, Uppsala. The other chemicals used were of analytical grade.

RESULTS

Enzyme purification

We applied purification Method I whenever the aim of the experiments was to obtain a relatively full array of phosphate ester-hydrolyzing enzymes present in the Avena leaf. Results of a representative experiment are shown in Fig. 1. It may be seen that the chromatography on Sephadex G-75 of relatively crude preparations obtained by Method I yielded one alkaline and two acid phosphodiesterase peaks, as well as one phosphatase peak, *plus* two peaks with RNA-splitting activity one of which also contained DNA-splitting activity.

Method I was used when the amount of various phosphate ester-splitting enzymes in intact and excised leaves was compared (for preliminary results see DISCUSSION).

The characterization of phosphodiesterases of the Avena leaf was carried out on preparations obtained by purification Method II. This method resulted in phosphodiesterase preparations completely free of ribonuclease, deoxyribonuclease, phosphatase and 3'- and 5'-nucleotidase activities. DEAE-cellulose chromatography (the last step of Method II) resolved the phosphodiesterase activity reproducibly into two peaks (Fig. 2). Fractions corresponding to the first phosphodiesterase peak exhibited enzyme activity at acidic pH only. The second phosphodiesterase peak contained mainly alkaline phosphodiesterase activity, but it appeared to have some phosphodiesterase activity at pH 5.5 as well. (The problem as to whether this phosphodiesterase activity should be regarded as a contamination of the alkaline phosphodiesterase with an acid phosphodiesterase will be discussed later.)

By the use of Method II a 130-fold purification was obtained with respect to the alkaline phosphodiesterase activity (Fig. 2, Fractions 45–51). The yield was close to 20% (Table I).

The degree of purification of the acid phosphodiesterase (Fig. 2, Fractions 34–40) was less (about 30-fold). Therefore, the properties of this enzyme were not studied in detail. Still, for comparative purposes, some preliminary data on the acid phosphodiesterase will also be included in the present paper to show that the Avena leaf contains (at least) two different phosphodiesterases.

TABLE I

PURIFICATION OF ALKALINE PHOSPHODIESTERASE FROM AVENA LEAF TISSUE

1 enzyme unit is defined as that amount of enzyme which liberates 1 μmole of *p*-nitrophenol per h under the assay conditions.

Fraction	*Vol. (ml)*	*Phosphodiesterase activity (units)*	*Total protein (mg)*	*Specific activity (units/mg protein)*	*Degree of purification*	*Yield (%)*
Crude extract*	440	217.3	2520	0.086	1	100
$(NH_4)_2SO_4$-fraction	70	138.4	380	0.364	4.2	63.7
Sephadex G-75, Fractions 40–56	69	62.5	9.6	6.51	75.7	28.8
DEAE-cellulose, Fractions 45–51	17.5	36.9	3.37	11.0	128	17.0

* Prepared from 120 g of Avena leaf tissue.

pH optimum

As shown in Fig. 3 the pH optimum of the alkaline phosphodiesterase was found to be close to 9.3 with both *p*-nitrophenyl-TMP and bis-*p*-nitrophenyl phosphate as substrates. The pH optimum of the acid phosphodiesterase preparation was 6.0.

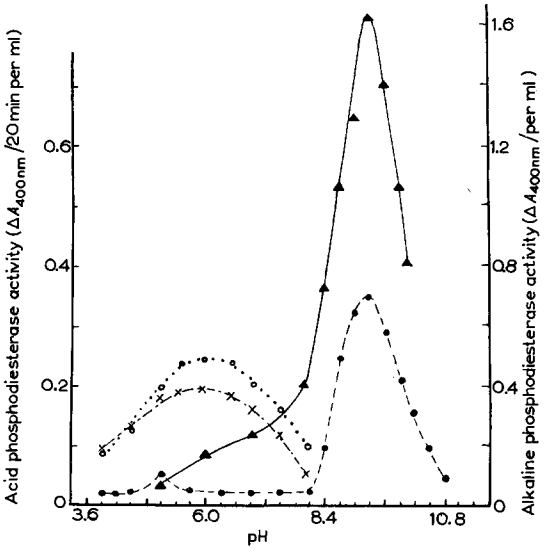


Fig. 3. Effect of pH on the activity of Avena phosphodiesterases. Standard assay conditions. Buffers used: pH 3.6–5.6 sodium acetate–acetic acid buffer; pH 5.0–8.0 Tris–acetate buffer; pH 7.2–9.0 Tris–HCl buffer; pH 8.5–10.8 glycine–NaOH buffer. ▲—▲, alkaline phosphodiesterase assayed with *p*-nitrophenyl-TMP as substrate; ●— — —●, alkaline phosphodiesterase assayed with bis-*p*-nitrophenyl phosphate as substrate; ○ · · · ○, acid phosphodiesterase preparation assayed with *p*-nitrophenyl-TMP as substrate; × — · — ×, acid phosphodiesterase preparation assayed with bis-*p*-nitrophenyl phosphate as substrate.

Stability

The alkaline phosphodiesterase preparation showed a considerable heat stability. Only about 30% of the original activity was lost when the enzyme was incubated in 0.1 M Tris–acetate buffer (pH 6.8) at 80° for 5 min (Table II). After a 20-min heat treatment the inactivation became almost complete. Repeated freezing and thawing also tended to inactivate the enzyme. Much enzyme activity was lost upon dialysis of the preparation against distilled water.

Since the alkaline phosphodiesterase preparation exhibited some activity at acidic pH as well, it seemed worthwhile to investigate whether or not the activity measured at pH 5.5 can be eliminated by heat treatment. As shown in Table II the alkaline phosphodiesterase preparation, after exposure to 80° for 5 min in 0.1 M

TABLE II

EFFECT OF HIGH TEMPERATURE ON THE STABILITY OF PHOSPHODIESTERASES ISOLATED FROM AVENA LEAF TISSUES

0.1 M Tris–acetate buffer (pH 7.5) solutions were heated to 80°. Alkaline and acid phosphodiesterase preparations, respectively, were added to the preheated solutions and the heating was continued for the time indicated. The enzyme assays were carried out on aliquots under standard conditions at the pH values indicated below.

Time of exposure to 80° (min)	*Activity recovered after heat treatment (%)*			
	Alkaline phosphodiesterase assayed at*			*Acid phosphodiesterase** assayed at*
	*pH 8.9****	*pH 8.9†*	*pH 5.5****	*pH 5.5****
0	100	100	100	100
5	72.4	60.0	2.8	0
10	34.0	37.1	0	0
15	20.0	11.0	0	0
20	4.0	2.0	0	0

* *cf.* Fig. 2, Fractions 45–51.
** *cf.* Fig. 2, Fractions 34–40.
*** Assayed with *p*-nitrophenyl-TMP as substrate.
† Assayed with bis-*p*-nitrophenyl phosphate as substrate.

Tris–acetate buffer (pH 7.5), almost completely lost its activity at pH 5.5. This observation would suggest that the alkaline phosphodiesterase preparation, as obtained from the DEAE-cellulose column, contained some contamination with a heat-labile acid phosphodiesterase which can, however, be eliminated by heat treatment. The acid phosphodiesterase preparation (Fractions 34–40, Fig. 2) also proved to be much more heat labile than the alkaline phosphodiesterase (Table II).

Activators and inhibitors

As shown in Table III the alkaline phosphodiesterase was activated by Mg^{2+}, Co^{2+}, Zn^{2+} and Ca^{2+} (2 mM salt solutions) by 97, 74, 45 and 35%, respectively. Slight inhibitions, ranging from 5 to 24%, were noted in the presence of Na_3AsO_4, urea, P_i, Cu^{2+} and NaF. Chelating (EDTA) and complexing (CN^-) agents inhibited the enzyme significantly indicating a requirement for bivalent metal ion(s).

TABLE III

EFFECT OF ACTIVATORS AND INHIBITORS ON PHOSPHODIESTERASES ISOLATED FROM AVENA LEAF TISSUES

Standard assay conditions. Bis-*p*-nitrophenyl phosphate was used as substrate.

Sunstance added	*Concn. (mM)*	*Alkaline phosphodiesterase**		*Acid phosphodiesterase***	
		*Activity****	*Percent of control*	*Activity****	*Percent of control*
None	0	0.62	100	0.43	100
$MgCl_2$	2	1.23	197.0	0.29	67.8
$CuSO_4$	2	0.47	76.0	—	—
$ZnCl_2$	2	0.91	145.2	0.27	62.9
$HgCl_2$	2	0.10	15.4	—	—
$CaCl_2$	2	0.84	134.6	0.33	76.2
KH_2PO_4	2	0.52	82.7	—	—
EDTA	2	0.09	13.9	0.42	97.9
NaF	2	0.48	76.4	—	—
Na_3AsO_4	2	0.59	95.7	—	—
NaCN	2	0.14	21.6	—	—
Urea	3000	0.58	92.8	—	—

* *cf.* Fig. 2, Fractions 45–51.
** *cf.* Fig. 2, Fractions 34–40.
*** Activity is expressed as ΔA_{400} /20 min.

It may be seen in Table III that the various ions affected the activities of the acid and alkaline phosphodiesterase differently.

Hydrolysis rate with various nucleic acids and synthetic polyribonucleotides

Degradation of various nucleic acids in the presence of identical amounts of alkaline phosphodiesterase preparations was followed over a period of 3 h. Highly polymerized RNA was hydrolyzed at a medium speed; tRNA and native DNA proved

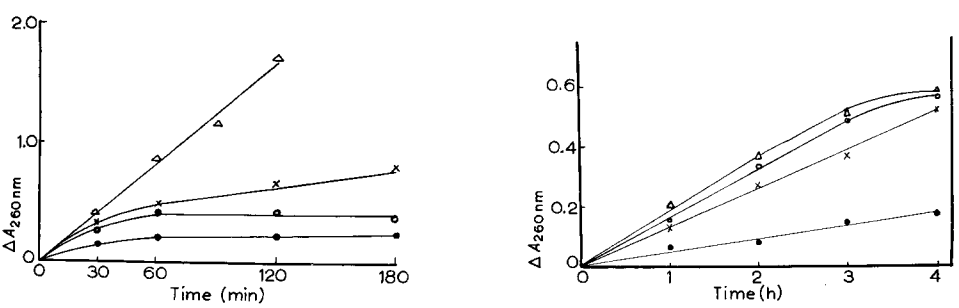


Fig. 4. Hydrolysis of various nucleic acids by alkaline phosphodiesterase from Avena leaves. The incubation mixture contained 1.2 mg substrate, 120 μmoles of Tris–HCl buffer (pH 8.8), 12 μmoles of $MgCl_2$ and an enzyme preparation corresponding to 40 μg protein in a total volume of 1.2 ml. Aliquots of 0.3 ml were withdrawn at the times indicated and blown into 0.3 ml of 0.3% uranyl acetate in 2.5% $HClO_4$ kept in an icebath. Subsequent steps were the same as those of the standard assay. ○—○, tRNA; ×—×, highly polymerized RNA; ●—●, highly polymerized DNA; △—△, heat-denatured DNA.

Fig. 5. Hydrolysis of synthetic polynucleotides by alkaline phosphodiesterase from Avena leaves. The details of the experiment were the same as those described in the legend to Fig. 4. ●—●, poly I; ×—×, poly C; ○—○, poly A; △—△, poly U.

to be fairly resistant to the enzyme; denatured DNA was split rapidly (Fig. 4). Poly A, poly C and poly U were hydrolyzed at the same or at a somewhat lower rate than highly polymerized RNA (Fig. 5). The lowest rate was obtained with poly I.

Hydrolysis of nucleoside cyclic phosphates

As shown by direct spectrophotometric assay (cytidine and uridine 2',3'-cyclic phosphates) and paper-chromatographic identification of the 3- and 6-h digestion products of all four nucleoside cyclic phosphates, the alkaline phosphodiesterase failed to cleave the purine and pyrimidine nucleoside 2',3'-cyclic phosphodiester bonds.

Mode of action

Results of a representative gel-filtration experiment designed to test the endo- or exonuclease nature of the alkaline phosphodiesterase are shown in Fig. 6. At zero-time incubation a single peak representing undigested heat-denatured DNA was eluted (Fig. 6A). For comparison, the position of 5'-AMP eluted under identical conditions is also shown in the Figure. The solution pattern of preparations obtained after various incubation times is shown in Fig. 6B. The pattern obtained points to a clearly exonucleolytic mode of action. The reliability of the method of distinguishing between endo- and exonuclease action was ascertained by using another enzyme fraction (Fractions 54–56 in Fig. 1) instead of the purified phosphodiesterase. This preparation, which was shown to split both RNA and DNA, yielded medium-size intermediate hydrolysis products which, in the system used, clearly separated both from the undigested DNA molecules and the mononucleotide fraction. This pattern represents an endonucleolytic or mixed type of attack.

To establish whether the enzyme produces 3'- or 5'-mononucleotides from polyribonucleotides, aliquots of poly A and poly U digests were chromatographed as described in MATERIALS AND METHODS. Only spots of 5'-AMP, 5'-UMP and small amounts of the corresponding nucleosides were detected after 4 h of digestion.

As expected, similar results were obtained by two-dimensional chromatography of enzymatic digests of heat-denatured DNA. Besides 5'-mononucleotides, nucleosides

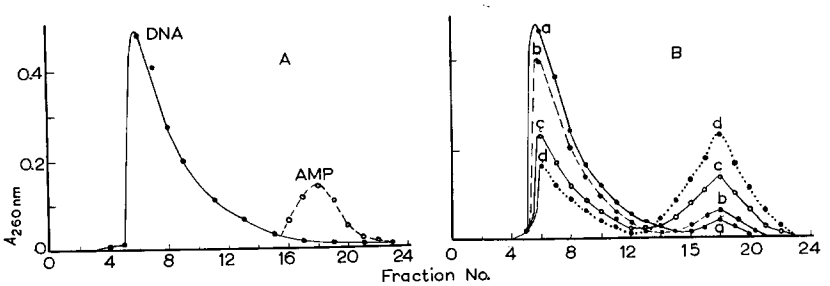
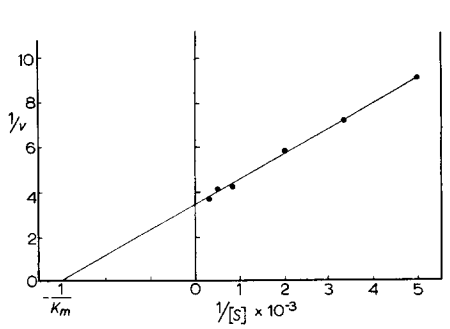


Fig. 6. Gel filtration of breakdown products obtained upon hydrolysis of heat-denatured DNA with alkaline phosphodiesterase from Avena leaves. A. Chromatography of a mixture of 200 μg heat-denatured DNA and 50 μg 5'-AMP on a Sephadex G-50 column (2.3 cm × 15 cm). B. Gel filtration of the breakdown products of heat-denatured DNA at various stages of digestion by alkaline phosphodiesterase. 1 ml of the incubation mixture contained 200 μg of heat-denatured DNA, 50 μmoles of Tris–HCl buffer (pH 8.8), 5 μmoles of $MgCl_2$ and an enzyme preparation corresponding to 30 μg protein. Elution patterns of 1-ml aliquots of the incubation mixture kept at 37° for various incubation times: a, 45 min; b, 90 min; c, 3 h; d, 6 h.



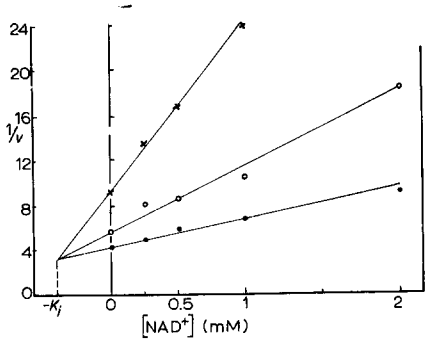


Fig. 7. Lineweaver–Burk plot of the rate of hydrolysis of *p*-nitrophenyl-TMP by alkaline phosphodiesterase from Avena leaf tissues. v = μmoles of *p*-nitrophenol liberated in 20 min by phosphodiesterase preparations corresponding to 5 μg of protein. Substrate concentration in M.

Fig. 8. Competitive inhibition by NAD^+ of the hydrolysis of *p*-nitrophenyl-TMP by alkaline phosphodiesterase from Avena leaves. DIXON plot.[23] v = μmoles of *p*-nitrophenol liberated in 20 min by phosphodiesterase preparations corresponding to 5 μg of protein. Concentration of *p*-nitrophenyl-TMP: ×—×, 0.2 mM; ○—○, 0.5 mM; ●—●, 1.25 mM.

and 3′,5′-nucleoside diphosphosphates were observed after incubation of DNA with the enzyme for 2, 4, 6 and 8 h.

Kinetic studies

Results of a typical kinetic experiment are shown in Fig. 7. The K_m value was found to be 0.4 mM for *p*-nitrophenyl-TMP as substrate.

Table IV summarizes data on the inhibition by pyridine nucleotide coenzymes, flavine adenine dinucleotide and nucleoside di- and triphosphates of the enzymatic hydrolysis of *p*-nitrophenyl-TMP. All the compounds listed inhibited the enzyme considerably. The type of inhibition by NAD^+ was established by varying the NAD^+ concentration at three levels of *p*-nitrophenyl-TMP and plotting the data according to DIXON AND WEBB[23] (Fig. 8). A fully competitive type of inhibition was clearly indicated. The K_i for NAD^+ with *p*-nitrophenyl-TMP as substrate was found to be equivalent to the K_m value (0.4 mM). Cyclic 2′,3′-mononucleotides and 3′,5′-AMP had no inhibitory effect.

TABLE IV

INHIBITION BY NUCLEOTIDES OF THE ENZYMATIC HYDROLYSIS OF *p*-NITROPHENYL-TMP BY ALKALINE PHOSPHODIESTERASE FROM AVENA LEAF TISSUES

Enzyme activity is expressed as ΔA_{400}/20 min.

Substance added	*Enzyme activity at two concentrations of inhibitor*			
	1 mM		*0.5 mM*	
	Activity	*%*	*Activity*	*%*
None	1.47	100.0	1.47	100.0
ADP	0.53	36.4	0.75	51.3
ATP	0.76	51.9	0.90	61.5
FAD	0.76	51.9	0.83	56.8
NAD^+	0.51	35.2	0.96	65.2
$NADP^+$	1.12	76.4	1.29	88.0

DISCUSSION

The nature of increase in the level of nucleolytic enzymes in excised ageing and/or injured leaf tissues is of a considerable interest. It has been suggested that the increase in ribonuclease level in detached (ageing) tobacco leaf tissues can be explained by an increase in the number of ribonuclease-containing lysosomes which disintegrate in the later stages of senescence[2]. Another author claimed that histon-bound ribonuclease is the enzyme the activity of which increases dramatically in excised barley leaf tissues[4]. Recent work carried out in our laboratory has indicated that several nucleolytic enzymes are affected upon leaf excision and/or injury[5,13], an observation which is in line with early studies of DIENER[7] on the enzyme content of crude extracts from mechanically injured tobacco leaves. It is clear from all these observations that the nature of nucleases participating in the overall "nuclease" response of the ageing and/or injured cells must be ascertained before premature conclusions as to the mechanism and significance of the phenomenon are drawn. In the framework of such a research program the phosphodiesterases of the Avena leaf were studied.

Although phosphodiesterases have been detected in a number of higher plant species[14,24–26], even in multiple forms[26,27], thorough investigations on plant phosphodiesterases are scarce[14,25]. The present results constitute the first detailed study of the properties of an alkaline phosphodiesterase purified from green leaf tissues.

Avena leaf tissues were shown to contain at least two distinct phosphodiesterases the properties of which differed in many respects (pH optimum, heat sensitivity and ion requirement).

The alkaline phosphodiesterase of the Avena leaf proved to be similar to animal phosphodiesterase I with respect to almost all characteristics tested[29]. Its exonucleolytic type of attack has been shown unequivocally. Experiments with synthetic polyribonucleotides have demonstrated that the enzyme has no significant base specificity. Characterization of the hydrolysis products have indicated that the enzymatic cleavage occurs stepwise (most probably from the 3′-hydroxyl end of the chain) and produces 5′-nucleoside phosphates. Inhibition of the enzyme activity by pyridine nucleotide coenzymes, flavine adenine dinucleotide and nucleoside di- and triphosphates has been demonstrated. It has been found in kinetic experiments that the K_i value for NAD^+, in the presence of *p*-nitrophenyl-TMP as substrate, is equivalent to the K_m value for *p*-nitrophenyl-TMP (0.4 mM). All the above characteristics of the plant enzyme are shared with the alkaline phosphodiesterases from animal tissues or venom. Both animal and plant alkaline phosphodiesterases exhibit the common property of a broad substrate specificity[30]. However, the plant phosphodiesterases, including the Avena enzyme, appear to be more heat stable than phosphodiesterase I. The Avena enzyme apparently does not hydrolyze 2′,3′-cyclic nucleotides at all. A low rate of hydrolysis of 2′,3′-UMP has been reported for snake venom phosphodiesterase[33].

Phosphodiesterases have been isolated so far from two higher plants, barley[25] and carrot[14]. These enzyme preparations show a number of features which are similar to or identical with those observed for the enzyme from Avena leaf tissues. Some differences, however, are observed. Thus, the phosphodiesterase from malt has been reported to have a pH optimum of 7.0; it has been suggested that it is activated by $(NH_4)_2SO_4$ and Na_2SO_4; no Mg^{2+} requirement has been shown[25].

The acid phosphodiesterase of the Avena leaf (pH optimum 5.5) may be similar to that detected in germinating garlic[24] and characterized in greater detail in preparations from calf spleen[31] and hog[32].

The nature of phosphodiesterase activity at pH 5.5 in the region of the alkaline phosphodiesterase peak eluted from the DEAE-cellulose column (Fig. 2) requires further investigation. One possibility is that the alkaline phosphodiesterase has some activity at pH 5.5. The pH optimum curves (Fig. 3) do not entirely exclude this explanation. The results of heat sensitivity tests summarized in Table II are compatible with another alternative as well: the alkaline phosphodiesterase preparation might have contained some contamination with an acid phosphodiesterase. This phosphodiesterase, however, did not interfere with the characterization of the alkaline phosphodiesterase, since it could be eliminated by heat treatment and, even if present in traces, it had no activity at pH 8.9, the optimum of the alkaline phosphodiesterase.

Recent observations indicate that in contrast to a soluble relative purine-specific ribonuclease, the level of which dramatically increases in excised Avena leaf tissues[5,13], the amount of the alkaline phosphodiesterase described in the present paper is but slightly altered upon leaf excision. The amount of acid phosphodiesterase(s), however, increases in excised Avena leaves. A more detailed characterization of these enzymes and a study of their behavior upon leaf excision is in progress.

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