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Purification and Properties of a Plant Endonuclease Specific for Apurinic Sites*

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An endonuclease which hydrolyzes depurinated DNA has been isolated from *Phaseolus multiflorus* embryos; it has a molecular weight around 40,000. The enzyme is specific for apurinic sites; it has no action on normal DNA strands or on alkylated sites, and is without exonuclease activity. The rate of phosphoester bond hydrolysis near apurinic sites is far greater in native than in denatured DNA. The endonuclease is not inactivated by 10 mM EDTA, but is activity is however stimulated by Mg^{2+} or Mn^{2+}. Its optimum pH is 7.5 to 8.0, and its optimum temperature 40° although, at this temperature, it is rapidly denatured; even low NaCl concentrations inhibit the enzyme activity. The endonuclease for apurinic sites of *P. multiflorus* is a non-histone protein of chromatin; the properties (like thermosensitivity or susceptibility to ionic strength) of the enzyme *in situ*, working on chromatin DNA, might be different from those described for the isolated endonuclease in homogenous aqueous solution.

An endonuclease specific for apurinic sites was first found in *Escherichia coli* by Verly and Paquette (1); this enzyme was completely purified by Verly and Rassart (2) and was used to repair depurinated DNA *in vitro* (3). The endonuclease specific for apurinic sites of *E. coli* is also exonuclease III (4–6). Gossard and Verly (6) have shown that the endonuclease hydrolyzes a phosphoester bond on the 5′ side of the apurinic site leaving a 3′-OH and a 5′-P; the exonuclease III activity of the enzyme then removes a few nucleotides in the 3′-5′ direction leaving the apurinic site on the DNA molecule. The exonuclease III activity of the endonuclease for apurinic sites might have the role of an anti-ligase preventing the closure, by the polynucleotide ligase, of the bond just opened by the endonuclease activity. This exonuclease activity does not however appear as absolutely necessary because a fair repair of apurinic sites seems to occur in *xthA* mutants devoid of exonuclease III; these mutants have a minor endonuclease for apurinic sites (endonuclease IV) which is devoid of exonuclease activity (7).

Endonucleases specific for apurinic sites have also been purified from rat liver (8) and calf thymus (9); the calf thymus enzyme has no exonuclease activity (10).

We had previously shown the presence of an endonuclease for apurinic sites in the roots, leaves, and embryos of a higher plant (11, 12). This paper deals with the purification and properties of the enzyme from *Phaseolus multiflorus* embryos.

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MATERIALS AND METHODS

Description of Buffers – Buffer A, 0.05 M Tris/HCl, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol, pH 8.0; Buffer B, 0.02 M Tris/HCl, 1 mM EDTA, 0.2 mM dithiothreitol, 10% glycerol, pH 8.1; Buffer C, 0.05 M Tris/HCl, 0.05 M NaCl, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol, pH 8.0; Honda's buffer, 0.025 M Tris/HCl, 4 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 0.25 M sucrose, 2.5% Ficoll, 5% dextran, 0.1% bovine serum albumin, pH 8.5; Michaelis' buffer, 0.028 M sodium acetate, 0.028 M sodium 5,5-diethylbarbiturate, 0.012 M NaCl, and HCl to reach the chosen pH; NaCl/citrate, 0.15 M NaCl, 0.015 M sodium citrate, pH 8.0.

Substrates – The preparation of labeled DNA from *Escherichia coli* grown in the presence of [*methyl*-^{3}H]thymidine, the alkylation of this normal [^{3}H]DNA with methyl methanesulfonate, and the partial depurination of the alkylated [^{3}H]DNA, have been previously described (2, 13). The alkylated [^{3}H]DNA contained 550 methylated sites and the alkylated-depurinated [^{3}H]DNA, 160 apurinic sites and 390 methylated sites per 10^6 daltons (13); after a treatment with NaOH, the acid-soluble fraction was usually around 0.35 for the alkylated-depurinated [^{3}H]DNA. The specific radioactivity of these labeled DNAs was about 50,000 dpm/μg; for the enzymic reactions, they usually were in NaCl/citrate at a concentration of 20 μg/ml.

To prepared nicked [^{3}H]DNA, 2 units of pancreatic deoxyribonuclease (3.1.4.5; Sigma Chemical Co.) and 20 μg of normal [^{3}H]DNA in 1 ml of 17 mM Tris/HCl, 10 mM sodium citrate, 0.1 M NaCl, 0.17 mM $MgCl_2$, pH 7.5, were incubated for 15 min at 37°. The mixture was heated at 77° to inactivate the enzyme without denaturing the DNA, then cooled in ice.

Enzyme Assay – To 20 μl of alkylated-depurinated [^{3}H]DNA (0.4 μg; 20,000 dpm) in NaCl/citrate, were added 20 μl of enzyme solution in Buffer A; the $MgCl_2$ final concentration was 10 mM. After a 15-min incubation at 37°, the tubes were cooled in crushed ice, then 100 μl of NaCl/citrate containing 200 μg of calf thymus DNA and 900 μl of 5.78% perchloric acid were successively added. After 15 min at 0°, the tubes were centrifuged at 12,000 × *g* for 15 min, and the supernatants were assayed for radioactivity. Controls without enzyme, which were always below 1% of the substrate radioactivity, were subtracted from the experimental values. The corrected results are expressed as fractions of the substrate radioactivity (acid-soluble fraction).

The acid-soluble fraction is approximately proportional to the amount of enzyme as long as it remains below 0.12. The enzyme unit is the activity necessary to give an acid-soluble fraction of 0.1 in the assay.

NaOH Treatment – To 1 volume of DNA solution was added 1 volume of 0.4 M NaOH; after 15 min at 37°, the mixture was neutralized with 1 volume of 0.4 M HCl. The acid-soluble radioactivity in 5% perchloric acid was then determined as above.

Formamide Denaturation – The DNA solution (100 μg in 2 ml of NaCl/citrate) was dialyzed at 4° against 250 ml of 95% formamide (formamide freshly redistilled under reduced pressure, 0.015 M

EDTA (95:5; v/v), adjusted to pH 7) with two changes. The temperature was then raised at 37° for 2 h. The formamide was finally eliminated by dialysis against a suitable buffer.

Radioactivity Determinations – The solution to be analyzed was diluted with water to a volume of 1 ml and prepared for counting by addition of 10 ml of a scintillation mixture made of 1 part of Triton X-100 and 2 parts of toluene containing 0.4% (w/v) 2,5-diphenyloxazole (PPO) and 0.01% (w/v) of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene (dimethyl-POPOP). An internal ^{3}H-labeled hexadecane standard was used; radioactivity was measured in a Packard Tri-Carb scintillation spectrometer and expressed in disintegrations per min.

Phaseolus multiflorus Embryos – The seeds were washed in a 1% sodium hypochlorite solution, rinsed with water, and placed between two layers of cotton wool soaked with water. After a 4-day germination in the dark at 25° in an atmosphere containing 90% humidity, the embryos were separated from the cotyledons and frozen in liquid nitrogen. This biological material was kept at −35° until use.

DNA-cellulose – The DNA-cellulose was prepared according to the method of Bautz and Dunn (14) using calf thymus DNA (Sigma Chemical Co). The wet material contained 200 μg of DNA/ml.

Assay for Proteins – Protein concentration was measured by Lowry's method (15) using bovine serum albumin as standard, or by absorption at 280 nm.

RESULTS

Preparation and Properties of Crude Extract – Embryos of *Phaseolus multiflorus* were ground with a Polytron in Honda's buffer. The homogenate was filtered through several layers of cheesecloth, then centrifuged at 100,000 × *g* for 90 min. Samples (200 μl) of this crude extract were mixed with 200 μl of NaCl/citrate containing 4 μg of [^{3}H]DNA, either normal, alkylated, or alkylated-depurinated; the final $MgCl_2$ concentration was 5 mM. These mixtures were incubated at 37° and aliquots were taken after 0 to 120 min to measure the acid-soluble radioactivity. The results were corrected for controls with Honda's buffer instead of the crude extract. Fig. 1*A* shows that the extract had little action on normal DNA; its action was slightly greater on alkylated DNA, but became important only when alkylated sites were replaced by apurinic sites (alkylated-depurinated DNA). Two aliquots were taken at each time from the incubation mixture containing the alkylated-depurinated [^{3}H]DNA, and one of them was treated with NaOH before measuring the acid-soluble radioactivity; it can be seen in Fig. 1*A* that incubation with the extract led to a quick disappearance of the alkali-labile sites. Taken together, these results indicate that the crude embryo extract contained an endonuclease which hydrolyzed a phosphoester bond near apurinic sites; the optimum pH for its activity was found to be 7.5 to 8.0.

To see whether the crude extract might contain a DNA *N*-glycosidase removing alkylated purines, 200 μl of NaCl/citrate containing 4 μg of [^{3}H]DNA, either normal or alkylated, were incubated at 37° with 200 μl of extract or 200 μl of Honda's buffer; the aliquots, taken after 120 min, were treated with NaOH before measuring the acid-soluble radioactivity. Table I shows that the crude extract had no more action, leading to alkali-labile sites or directly to strand breaks, on alkylated DNA than on normal DNA. There is thus no glycosidase activity, removing alkylated bases from DNA, demonstrable with this technique.

A comparison was made between two buffers for the preparation of the 100,000 × *g* supernatant from *P. multiflorus* embryos; per mg of protein, the extract in Honda's buffer contained 7 units of endonuclease for apurinic sites whereas the extract in Buffer A contained 25 units of the enzyme.

Purification of Enzyme – To the 100,000 × *g* supernatant from 620 g of embryos ground in Buffer A (1500 ml; 214,000 enzyme units) at 4°, an 8% streptomycin sulfate solution in Buffer A was added dropwise with constant stirring to a final concentration of 0.8%. Stirring was continued for 15 more min before centrifuging the suspension for 15 min at 12,000 × *g*; the supernatant (Preparation I) was kept for further purification.

The ammonium sulfate concentration range within which the enzyme precipitates was determined in a pilot experiment. Then, to Preparation I (1670 ml) cooled at 0°, solid ammonium sulfate (431 g) was added to reach 45% saturation; after a 15-min centrifugation at 12,000 × *g*, the supernatant was brought to 85% saturation (503 g of ammonium sulfate) and submitted to another centrifugation. The sediment from the second centrifugation was dissolved in 100 ml of Buffer A and dialyzed against 4 × 1 liter of the same buffer. The dialyzed material (Preparation II; 125 ml) contained 133,000 enzyme units with a specific activity of about 60 units/mg of protein.

A column (2.6 × 50 cm) of DEAE-cellulose (Sigma Chemical Co.) was equilibrated with Buffer A at 4°. Preparation II was applied at a rate of 25 ml/h and the column was washed with 150 ml of the same buffer. The elution was carried out, at the

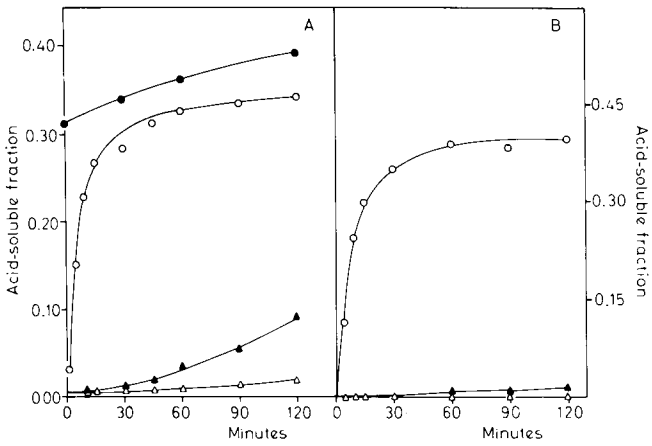

FIG. 1. Action of the crude extract from *Phaseolus multiflorus* embryos and of the purified enzyme on normal, alkylated, and alkylated-depurinated DNA. NaCl/citrate (200 μl) containing 4 μg of [^{3}H]DNA, either normal (△), alkylated (▲), or alkylated-depurinated (○), was incubated at 37° with 200 μl of 100,000 × *g* supernatant prepared in Honda's buffer (*A*) or with 200 μl of Preparation IV (*B*), and aliquots were taken from 0 to 120 min to measure the acid-soluble radioactivity. The results were corrected for controls where extract or Preparation IV was replaced by the corresponding buffer; they are expressed as fractions of the substrate radioactivity (acid-soluble fraction). In the case where the alkylated-depurinated [^{3}H]DNA was incubated with the crude extract, two aliquots were taken at each time, one of which was treated with NaOH before the determination of the acid-soluble radioactivity (●).

TABLE I

Failure to demonstrate N-glycosidase removing alkylated purines from DNA in crude extract from Phaseolus multiflorus embryos

Two hundred microliters of NaCl/citrate containing 4 μg of [^{3}H]DNA, normal or alkylated, were incubated with 200 μl of the crude extract or of the corresponding buffer. After 120 min at 37°, 20-μl aliquots were treated with NaOH and the acid-soluble radioactivity was determined; the results are given in fractions of the substrate radioactivity.

Substrate DNA	Acid-soluble fraction		
	Extract	Buffer	Δ
Alkylated	0.057	0.046	0.011
Normal	0.008	0	0.008

same flow rate, with 1 liter of a 0 to 0.15 M NaCl linear gradient in Buffer A; fractions of 10 ml were collected. Fig. 2*A* shows that the endonuclease for apurinic sites was eluted between 0.03 and 0.05 M NaCl. Fractions 20 to 33 were pooled (Preparation III); they contained 85,000 enzyme units with a specific activity of around 300 units/mg of protein.

Preparation III (140 ml) was mixed with an equal volume of Buffer B and applied, at a rate of 10 ml/h, onto a column (1.6 × 40 cm) of DNA-cellulose previously equilibrated with Buffer B. The column was washed with Buffer B until the absorbance at 280 nm dropped below 0.02; the enzyme was then eluted with 0.62 M KCl in Buffer B, always at the same flow rate, and 5-ml fractions were collected (Fig. 2*B*). Fractions 106 to 108 were pooled (Preparation IV); they contained 25,000 enzyme units with a specific activity of 28,000 units/mg of protein.

Chemical Properties of Purified Enzyme—Two Sephadex G-75 columns were prepared; one (2.6 × 95 cm) was equilibrated with Buffer B and the other (1.6 × 60 cm) with Buffer C. The void volume (V_0) was measured with blue dextran (Pharmacia), whereas the total volume (V_t) was determined with riboflavin. Four different proteins of known molecular weights were used for calibration. The sample (5 mg), dissolved in 3 ml of the appropriate buffer, was applied to the column and eluted at a rate of 6 ml/h; 3-ml fractions were collected and their absorbance was read at 280 nm to determine the protein elution volume (V_e). For the two columns, there was a linear relationship between the logarithm of the molecular weight and the elution constant $K_{av} = (V_e - V_0)/(V_t - V_0)$. Preparation IV (3 ml) was filtered through the same columns and the enzyme activity assayed on the collected fractions. In each case, the elution constant of the enzyme was very near that of ovalbumin (Table II) which indicates that the endonuclease for apurinic sites of *P. multiflorus* has a molecular weight around 40,000.

Preparation IV was placed in a dialysis bag and the solution was concentrated by burying the bag in Sephadex G-25 powder. Aliquots of the concentrated solution containing about 4 μg of protein were submitted to gel electrophoresis either in the native form at pH 8.3, or after denaturation with sodium dodecyl sulfate at pH 8.0. Staining with Coomassie blue revealed one major protein band in each case (Fig. 3).

To measure the enzyme's thermosensitivity, 200-μl aliquots of the purified enzyme in Buffer A, 10 mM $MgCl_2$, were heated during 30 min at various temperatures between 30 and 70°, then cooled in ice before measuring the residual activity (Table III). Some denaturation already occurred at 30°. The kinetics of the denaturation was followed at 40°; the logarithm of the residual activity was a linear function of time with a half-life of 15 min.

Catalytic Properties of Purified Enzyme—Two-milliliter volumes of Preparation IV and of the solution of alkylated-depurinated [^{3}H]DNA (20 μg/ml) were dialyzed against 5 × 500 ml of Buffer A containing 10 mM EDTA, then against 5 × 1 liter of Buffer A. Aliquots of 20 μl of these dialyzed solutions were mixed together with 10 μl of $MgCl_2$ solution to reach a 0 to 500 mM final concentration, and incubated for 15 min at 37° before measuring the acid-soluble radioactivity. Table IV shows that the enzyme was active in absence of divalent cations; its activity was however increased 4-fold when the Mg^{2+} concentration was raised to 10 to 15 mM, but higher concentrations progressively inactivated the enzyme. The experiment was repeated with the $MgCl_2$ solution replaced by an equal volume of $CaCl_2$, $MnCl_2$, $ZnSO_4$, or $CuSO_4$ solution to have a 10 mM final concentration in each case: only Mn^{2+} could substitute for Mg^{2+}; with Ca^{2+}, Zn^{2+}, and Cu^{2+}, the activity was the same as in absence of these divalent cations (Table IV).

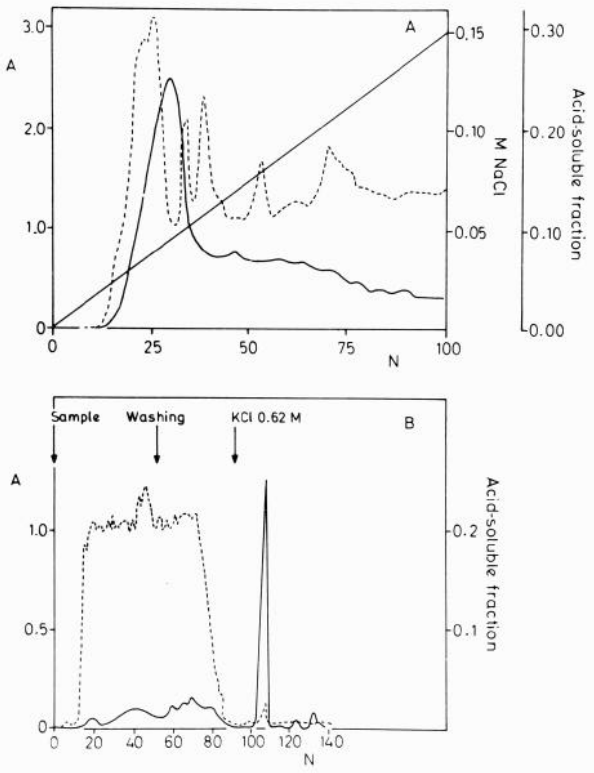

FIG. 2. Chromatographic purification of the endonuclease for apurinic sites. Absorbance at 280 nm (*A*, ····) and enzyme activity on alkylated-depurinated [^{3}H]DNA (acid-soluble radioactivity relative to the substrate total radioactivity; *continuous line*) are given for each fraction (*N* = fraction number). *A*, DEAE-cellulose, the proteins precipitated between 45 and 85% saturation in ammonium sulfate, dissolved in Buffer A, were chromatographed on a column (2.6 × 50 cm) of DEAE-cellulose. After washing with Buffer A, the elution was carried out with 1 liter of a 0 to 0.15 M NaCl linear gradient (*straight line*) in Buffer A; 10-ml fractions were collected. *B*, DNA-cellulose, Fractions 20 to 33 of the preceding chromatography were pooled, mixed with an equal volume of Buffer B, and applied onto a column (1.6 × 40 cm) of DNA-cellulose. After washing with Buffer B, the enzyme was eluted with 0.62 M KCl in Buffer B; 5-ml fractions were collected.

TABLE II

Determination of enzyme molecular weight using Sephadex G-75

Two columns of Sephadex G-75 were used with different solvents: a column (2.6 × 95) with Buffer B; a column (1.6 × 60 cm) with Buffer C. The protein (5 mg), dissolved in 3 ml of the appropriate buffer, or 3 ml of Preparation IV, was put on the column; the elution was carried out at a rate of 6 ml/h and 3-ml fractions were collected. The proteins used as standards were detected by their absorbance at 280 nm, and the enzyme by its activity on alkylated-depurinated [^{3}H]DNA. In each case, the K_{av} value was calculated.

Sample	M_r	K_{av}	
		Buffer B	Buffer C
Ovalbumin	43,000	0.16	0.11
Chymotrypsinogen	25,700	0.33	0.23
Ribonuclease A	13,700	0.71	0.41
Insulin	5,700	0.99	0.60
Preparation IV		0.17	0.11

Preparation IV and the solution of alkylated-depurinated [^{3}H]DNA were dialyzed against Buffer A containing 10 mM

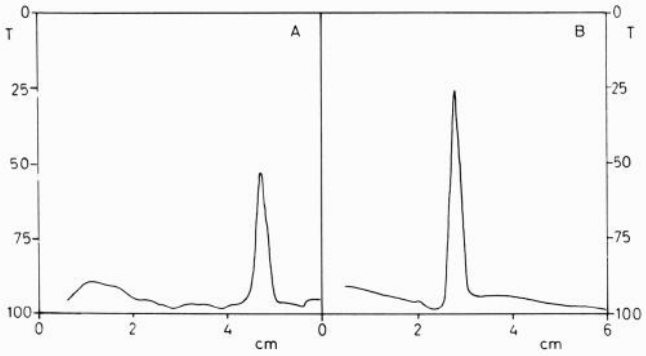

FIG. 3. Gel electrophoresis of the purified enzyme. In each case, the 3-mA current was stopped when the bromphenol blue reached the end of the gel. The gels, after staining with Coomassie blue, were scanned at 550 nm and the per cent transmittance (*T*) recorded *versus* the migrated distance (*cm*) toward the anode. *A*, native enzyme, the gels were prepared according to the method of Davis (16). The sample (100 μl) containing about 4 μg of protein, mixed with some bromphenol blue, in 40% sucrose, was applied onto the stacking gel, and the electrophoresis was carried out in 25 mM Tris, 0.2 M glycine, 5 mM EDTA, pH 8.3, buffer. *B*, denatured enzyme, the gels were prepared according to the method of Shapiro *et al.* (17) and Weber and Osborn (18). The concentrated Preparation IV was incubated for 30 min at 37° with an equal volume of 10 mM Tris/acetate, 5 mM EDTA, 1% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10% glycerol, pH 8.0, buffer. After addition of some bromphenol blue, an aliquot (100 μl) containing about 4 μg of protein was used for the electrophoresis which was carried out in 0.05 M Tris/acetate, 5 mM EDTA, 0.1% sodium dodecyl sulfate, pH 8.0, buffer.

TABLE III

Thermolability of purified enzyme

Samples (200 μl) of the purified enzyme, in Buffer A with 10 mM $MgCl_2$, were heated for 30 min at the indicated temperature, then cooled in ice. Aliquots (20 μl) were incubated with 20 μl of alkylated-depurinated [^{3}H]DNA in NaCl/citrate for 5 min at 37°C before measuring the acid-soluble radioactivity. The results, corrected for a control without enzyme, are expressed as fractions of the substrate radioactivity. Without heat treatment, the enzyme gave an acid-soluble fraction of 0.145.

Temperature	Acid-soluble fraction
20°	0.123
30	0.091
35	0.080
40	0.035
45	0.030
50	0.026
60	0.007
70	0.002

$MgCl_2$. Aliquots of 20 μl of these dialyzed solutions were mixed together with 10 μl of NaCl solution to reach a 0 to 1 M final concentration, and incubated for 15 min at 37° before measuring the acid-soluble radioactivity. Fig. 4 indicates that NaCl inhibits the enzyme: at 0.15 M, the endonuclease had already lost two-thirds of its activity.

The enzyme's optimum pH is 7.5 to 8.0 (Fig. 5*A*) and its optimum temperature is 40° (Fig. 5*B*). An Arrhenius plot of the logarithm of the reaction velocity against the inverse of the absolute temperature for the data below 303 K, gives a straight line from which an activation energy of 13,000 cal/mol can be calculated for the enzyme-catalyzed reaction.

Samples (200 μl) of Preparation IV were mixed with 200 μl of NaCl/citrate containing 4 μg of [^{3}H]DNA either normal, alkylated, or alkylated-depurinated; the $MgCl_2$ concentration was 10 mM and the pH 8.0. These mixtures were incubated at

TABLE IV

Effect of divalent cations on activity of purified enzyme

Preparation IV and the solution of alkylated-depurinated [^{3}H]DNA were dialyzed against 10 mM EDTA in Buffer A, then against Buffer A alone. Aliquots of 20 μl of these dialyzed solutions were mixed together with 10 μl of $MgCl_2$, $CaCl_2$, $MnCl_2$, $ZnSO_4$, or $CuSO_4$ solution to reach the final concentration given in the table; after 15 min at 37°, the acid-soluble radioactivity was measured. The results, corrected for a control without enzyme, are expressed as fractions of the substrate radioactivity.

Cation	Final concentration	Acid-soluble fraction
	mM	
None	0	0.051
Mg^{2+}	0.5	0.058
	1	0.058
	5	0.143
	10	0.215
	15	0.216
	20	0.177
	100	0.038
	250	0.002
	500	0.002
Ca^{2+}	10	0.062
Mn^{2+}	10	0.240
Zn^{2+}	10	0.050
Cu^{2+}	10	0.070

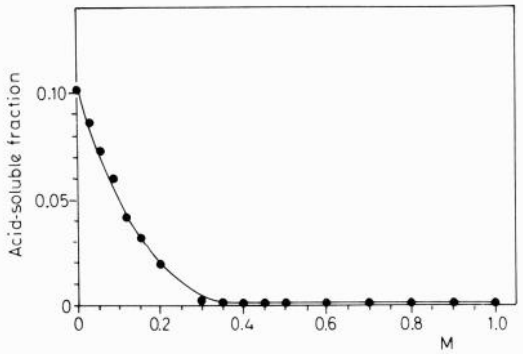

FIG. 4. Enzyme inhibition by NaCl. To mixtures of equal volumes of the purified enzyme and the alkylated-depurinated [^{3}H]DNA in Buffer A containing 10 mM $MgCl_2$, NaCl was added to reach various final molarities (M). The acid-soluble radioactivity was measured after an incubation of 15 min at 37°; the results are expressed as fractions of the substrate total radioactivity.

37° and aliquots taken after 0 to 120 min to measure the acid-soluble radioactivity. The results were corrected for controls without enzyme. Fig. 1*B* shows that the purified enzyme had no action on normal DNA. This was also true when the DNA was denatured. There was a slight action on the alkylated DNA; but the alkylated DNA became a very good substrate only when alkylated sites were replaced by apurinic sites (alkylated-depurinated DNA): after 60 min, the acid-soluble radioactivity had reached a maximum equal to the value given after a NaOH treatment.

To know whether the slight action observed on alkylated DNA was only on the apurinic sites always present in this substrate, 200 μl of alkylated [^{3}H]DNA solution were incubated with 200 μl of Preparation IV or 200 μl of Buffer B; aliquots, taken after 0 to 120 min, were treated with NaOH before measuring the acid-soluble radioactivity. At any time, the results were the same whether the enzyme was present or not during the incubation preceding the NaOH treatment.

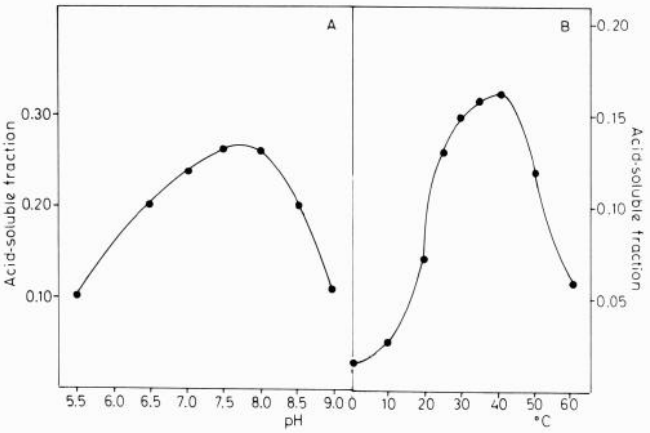

FIG. 5. Action of pH and temperature on purified enzyme. In both experiments, the results were corrected for suitable controls without enzyme; they are expressed as fractions of the substrate total radioactivity. *A*, aliquots of Preparation IV and of the alkylated-depurinated [^{3}H]DNA solution were dialyzed against Michaelis' buffers of various pH values, containing 10 mM $MgCl_2$. Mixtures of 20 μl of the enzyme solution and 20 μl of the substrate solution at the same pH were then incubated for 15 min at 37° before measuring the acid-soluble radioactivity. *B*, mixtures of 100 μl of Preparation IV and 100 μl of alkylated-depurinated [^{3}H]DNA in NaCl/citrate, containing 10 mM $MgCl_2$, were incubated 15 min at temperatures varying from 0–60° before measuring the acid-soluble radioactivity.

Alkylated-depurinated [^{3}H]DNA (100 μg in 2 ml of NaCl/citrate) was denatured with formamide. After exhaustive dialysis against 0.005 M potassium phosphate buffer, pH 6.8, the sample was incubated 6 h at 60°, then chromatographed on hydroxyapatite to isolate the single-stranded DNA according to the method of Bernardi (19). Aliquots of 20 μl of Preparation IV were then incubated with 20 μl of NaCl/citrate containing 0.4 μg of alkylated-depurinated [^{3}H]DNA, single-stranded or double-stranded. Table V shows that the enzyme had little if any action on apurinic sites in denatured DNA.

The action of the purified enzyme on alkylated-depurinated [^{3}H]DNA was not decreased by the addition of 10 times as much normal DNA. The purified endonuclease for apurinic sites had no action on RNA; pancreatic ribonuclease (3.1.4.22) did not hydrolyze native, but was active on denatured alkylated-depurinated [^{3}H]DNA.

Preparation IV (200 μl) was incubated at 37° with 200 μl of NaCl/citrate containing 4 μg of [^{3}H]DNA nicked with pancreatic deoxyribonuclease (3.1.4.5); no increase of acid-soluble radioactivity could be detected in aliquots taken after 0 to 120 min. The solution remaining after 120 min was chromatographed on DEAE-Sephadex in 0.005 M Tris/HCl, pH 7.6, buffer, containing 7 M urea, according to the method of Junowicz and Spencer (20); the linear LiCl gradient did not elute any radioactivity up to 0.4 M, indicating the absence of mononucleotides.

DISCUSSION

The crude extract of *P. multiflorus* embryos has an endonuclease activity which hydrolyzes DNA containing apurinic sites (Fig. 1*A*). No effect directed toward alkylated sites could be demonstrated, either of the endonuclease type leading directly to strand breaks, or of the DNA *N*-glycosidase type leading to alkali-labile sites (Table I).

A much higher content of endonuclease for apurinic sites was found in the crude extract when the embryos were ground in Buffer A, a hypotonic solution which destroyed most of the nuclei, rather than in Honda's buffer which left most of them intact. The endonuclease for apurinic sites thus appears to be mainly localized in the nucleus of the cells; it was found in the non-histone proteins of chromatin (12).

TABLE V

Action of purified enzyme on apurinic sites in single strand DNA

Alkylated-depurinated [^{3}H]DNA was denatured in formamide, incubated at 60° for 6 h, and the single-stranded [^{3}H]DNA was isolated on hydroxyapatite. Aliquots (20 μl) of the native or denatured [^{3}H]DNA in NaCl/citrate, were incubated for 15 min at 37° with 20 μl of Preparation IV or of 0.4 M NaOH, before measuring the acid-soluble radioactivity; the results, corrected for controls without Preparation IV or NaOH, are expressed as fractions of the substrate radioactivity. It is noteworthy that the single strand DNA contained less alkali-labile sites than the double strand DNA from which it was prepared; the difference might be due to the loss of small DNA pieces during the purification on hydroxyapatite.

DNA sample	Acid-soluble fraction	
	NaOH	Enzyme
dpm		
Single-stranded, 14,200	0.19	0.03
Double-stranded, 12,900	0.36	0.14

From the crude extract prepared in Buffer A, the endonuclease for apurinic sites was purified approximately 1100-fold with a yield of 12%. The final product appeared fairly pure: gel electrophoresis of the native enzyme at pH 8.3, or of the sodium dodecyl sulfate-denatured enzyme at pH 8.0, showed one major protein band in each case (Fig. 3). Inactivation at 40° followed first order kinetics with a single half-life of 15 min showing that the purified preparation contained only one enzyme acting on alkylated-depurinated DNA. Using Sephadex G-75, the enzyme was found to have a molecular weight around 40,000 (Table II).

The purified enzyme was active on depurinated DNA (Fig. 1*B*): it liberated acid-soluble fragments up to a maximum corresponding to the acid solubility given by an alkaline treatment consecutive to an incubation, without enzyme, of the same duration. It is known that an alkaline treatment hydrolyzes a phosphoester bond near each apurinic site (21, 22). The action of the enzyme on denatured depurinated DNA was very weak (Table V); it is possible that the activity was restricted to regions which had reconstituted double helices. The enzyme had no action on normal strands, either in native (Fig. 1*B*) or denatured DNA. Its slight action on alkylated DNA (Fig. 1*B*) was restricted to the apurinic sites always present in this substrate: the amount of acid-soluble radioactivity liberated from alkylated [^{3}H]DNA was the same whether it was incubated with the enzyme and then treated with NaOH, or incubated for the same time without enzyme and also treated with NaOH. The purified enzyme and NaOH thus acted on the same sites. If NaOH is known to introduce breaks near apurinic sites, it is without effect or rather stabilizes the glycosidic bond of the alkylated bases (23). The conclusion is that the enzyme acted only on the apurinic sites present in the alkylated DNA and not at all on the far more numerous alkylated sites.

The plant endonuclease for apurinic sites had no action on RNA. On the other hand, pancreatic ribonuclease did not hydrolyze double-stranded depurinated DNA although, according to Durand and Thomas (24), it breaks down apurinic acid; but this acid, lacking purines, necessarily is single-stranded. We indeed found that pancreatic ribonuclease was active on depurinated DNA after denaturation. The opposite DNA strand prevents the ribonuclease action on alkali-labile apurinic sites, as the enzyme action on RNA is suppressed

when the RNA is hybridized to a complementary DNA strand or when it is double-stranded.

The main endonuclease for apurinic sites of *Escherichia coli* is the same as exonuclease III (4–6); the minor endonuclease for apurinic sites of this bacterium (endonuclease IV) is however devoid of such an activity (7). We were also unable to detect an exonuclease associated with our purified enzyme.

Although the plant enzyme had a high affinity for DNA-cellulose so that it was readily purified by chromatography on this material, the addition of normal DNA did not decrease the activity of the purified enzyme on apurinic sites; this was true even when the amount of normal DNA was 10 times greater than the amount of depurinated DNA.

Dialysis against 10 mM EDTA, followed by removal of the chelating agent, did not inactivate the enzyme (Table IV); however, addition of Mg^{2+} or Mn^{2+} to a final 10 mM concentration in the incubation medium increased the enzyme activity 4- to 5-fold, whereas Ca^{2+}, Zn^{2+}, or Cu^{2+} had no effect. It might be that a divalent cation, Mg^{2+} or Mn^{2+}, is a natural cofactor of the enzyme, but that the concentration of EDTA was not high enough to remove it completely. Concentrations of Mg^{2+} greater than 15 mM progressively inhibited the enzyme. The endonuclease was also progressively inhibited by increasing NaCl concentrations (Fig. 4).

The optimum pH was between 7.5 and 8.0 (Fig. 5*A*), the optimum temperature 40° (Fig. 5*B*), but, at this temperature, the purified enzyme had a half-life of only 15 min (Table III). The temperature chosen for most experiments, *i.e.* 37°, was a compromise to have a reaction rate high enough while avoiding two quick a denaturation of the enzyme.

The thermolability and also the high sensitivity to ionic strength of the purified enzyme in homogeneous solutions have probably little to do with the *in vivo* situation where the physiologically active enzyme is integrated into the chromatin structure.

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