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Purification and Properties of the Endonuclease Specific for Apurinic Sites of *Bacillus stearothermophilus**

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An endonuclease specific for apurinic sites when double-stranded DNA is used as substrate has been isolated from the thermophilic bacterium, *Bacillus stearothermophilus;* it is a monomeric protein of about 28,000 daltons, without action on normal DNA strands or on alkylated sites. The enzyme is quite thermoresistant in the presence of other proteins, has an optimal temperature of 60°, needs monovalent cations for optimal activity, is insensitive to EDTA, and is inhibited by divalent cations; it has no associated exonuclease activity. These latter properties are closer to those of *Escherichia coli* thermoresistant endonuclease IV, which is also insensitive to EDTA and has no exonuclease activity, and very different from those of the main endonuclease for apurinic sites of the same bacterium. The *B. stearothermophilus* enzyme is more resistant to urea and detergents than the main *E. coli* endonuclease for apurinic sites and has a higher content of hydrophobic amino acids.

DNA spontaneously loses purines (1) and pyrimidines (2). The rate of base loss is considerably increased by treatment with chemicals like alkylating agents or by exposure to ionizing radiation. Endonucleases specific for apurinic sites in DNA have been found in *Escherichia coli* (3–5), in animals (6, 7), and in plants (8).

E. coli possesses two endonucleases for apurinic sites. The main enzyme, which is responsible for 90% of the cell activity, has been completely purified by Verly and Rassart (9); it is thermolabile, is inhibited by EDTA, needs magnesium ions to be active, and might be the same enzyme as exonuclease III (10, 11). The accessory enzyme, which is responsible for 10% of the cell activity, has been called endonuclease IV (5); it resists heating at 45°, is not inhibited by EDTA, and is devoid of exonuclease activity.

Depurinated DNA has been repaired *in vitro* with three enzymes: the main *E. coli* endonuclease specific for apurinic sites, DNA polymerase I and the four deoxyribonucleosides triphosphates, ligase and its coenzyme (12). Gossard and Verly (11) gave the details of the repair molecular mechanism.

The spontaneous loss of DNA bases must be very high in thermophilic bacteria at the temperature at which they usually live so that we looked for an endonuclease hydrolyzing a phosphoester bond near apurinic sites in *Bacillus stearothermophilus*. The enzyme was found and purified. It is thermoresistant when protected by other proteins; the presence of a high percentage of hydrophobic amino acids, likely grouped in a central core, might explain this property. The *B. stearothermophilus* enzyme behaves more like endonuclease IV than like the main endonuclease for apurinic sites of *E. coli* (endonuclease VI).

MATERIALS AND METHODS

Media and Buffers– Sargeant's medium (13) consists of the following: 20 g of Bacto-tryptone (Difco), 10 g of yeast extract (Difco), 0.32 g of citric acid, 1.3 g of K_2SO_4, 3.2 g of $NaHPO_4 \cdot 2H_2O$, 270 mg of $MgSO_4 \cdot 7H_2O$, 15 mg of $MnCl_2 \cdot 4H_2O$, 7 mg of $FeCl_3 \cdot 6H_2O$, water to make 900 ml. The pH is adjusted to 7.2 with 5 M KOH and the solution is autoclaved at 120° for 30 min; 100 ml of a solution containing 5 g of glucose, sterilized by passing through a 0.45 μm Millipore filter, are finally added. The buffers used are: Buffer I, 0.05 M Tris·HCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, pH 8.0; Buffer II, Buffer I containing 5% glycerol; Buffer III, same as Buffer II, but pH adjusted at 7.5; Hepes buffer, 0.05 M Hepes,[1] 0.125 M KCl, 10 mM EDTA, pH 7.5; SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0.

Culture of Bacillus stearothermophilus and Preparation of Crude Extract– Four Erlenmeyer flasks of 2 liters containing 500 ml of Sargeant's medium are inoculated with *B. stearothermophilus* cells and incubated at 55° with gentle shaking for 18 h. The 2 liters are then poured into a 40-liter fermentor containing the same medium; 250 g of bacteria are harvested by centrifugation at the beginning of the plateau phase and washed in Buffer I containing 0.15 M NaCl. They are frozen at −30°.

After thawing, the bacteria (50 g) are suspended in 250 ml of Buffer I at 0° and crushed in a Manton-Gaulin homogenizer; the apparatus is washed with 125 ml of Buffer I which are added to the homogenate. The suspension is centrifuged at 16,000 × *g* for 30 min and the supernatant collected; this crude extract yields 2,000 enzyme units/g of wet bacteria.

Enzyme Substrates– The preparation of labeled DNA from *Escherichia coli* in the presence of [*methyl*-^{3}H]thymidine, the alkylation of this [^{3}H]DNA with methyl methanesulfonate, and the partial depurination of the alkylated [^{3}H]DNA have been previously described (9, 14). The alkylated [^{3}H]DNA contains 550 methylated sites and the alkylated-depurinated [^{3}H]DNA, 160 apurinic sites, and 390 alkylated sites/10^6 daltons (14); after a treatment with NaOH, 35% of the alkylated-depurinated [^{3}H]DNA becomes acid-

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[1] The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

soluble. The specific radioactivities of these labeled DNAs are adjusted to 60,000 dpm/μg; for the enzymatic reactions, they usually are in Hepes buffer at a concentration of 50 μg/ml.

Labeled T7 phage DNA is prepared according to the method of Crine and Verly (15); it has a specific radioactivity of 150,000 dpm/μg and contains an average of 0.6 break/strand. It is kept at 4° in SSC, 0.01 M $MgCl_2$.

To make alkylated T7 DNA, 0.1 ml of SSC containing 25 μg of T7 [^{3}H]DNA and 0.9 ml of 0.2 M sodium phosphate buffer, pH 7.0, are mixed and methyl methanesulfonate is added to have a 0.02 M final concentration; after 2 h at 37°, the solution is dialyzed three times against 500 ml of SSC, 0.01 M $MgCl_2$. This alkylated T7 [^{3}H]DNA contains approximately 350 methylated sites/strand (16).

To make depurinated T7 DNA, 0.9 ml of 0.01 M acetic acid is added to 0.1 ml of SSC containing 25 μg of T7 [^{3}H]DNA (final pH = 4.0); after an incubation of 30 min at 37°, the solution is neutralized with 0.1 ml of 1 M sodium phosphate, pH 7.0, and dialyzed three times against 500 ml of SSC, 0.01 M $MgCl_2$ (15). This depurinated T7 [^{3}H]DNA contains about 30 apurinic sites/strand.

Enzyme Assay—To 20 μl of alkylated-depurinated [^{3}H]DNA (1 μg; 60,000 dpm) in 0.05 M Hepes buffer, are added 20 μl of enzyme solution in the same buffer. After 10 min at 37°, the tubes are cooled in crushed ice, 100 μl of SSC containing 200 μg of calf thymus DNA and 900 μl of 5.78% perchloric acid are successively added. After shaking, the tubes are kept 15 min at 0°, centrifuged at 12,000 × *g* for 15 min, and the supernatants are assayed for radioactivity. Controls without enzyme, which are always below 1% of the substrate radioactivity, are subtracted from the experimental values. The corrected results are expressed as fractions of the substrate radioactivity (acid-soluble fraction).

As long as it does not exceed 0.1, the acid-soluble fraction is approximately proportional to the amount of enzyme. The enzyme unit is the activity necessary to give an acid-soluble fraction of 0.1 in the assay.

NaOH Treatment of DNA—To 1 volume of DNA solution is added 1 volume of 0.4 M NaOH; after 15 min at 37°, the mixture is neutralized with 1 volume of 0.4 M HCl. This treatment hydrolyzes a phosphoester bond near each apurinic site (4).

Denaturation of T7 DNA by NaOH or Formamide and Estimation of Average Number of Breaks/Strand—After denaturation, the DNA is sedimented on neutral sucrose gradients (12). Alkaline treatment hydrolyzes a phosphoester bond near each apurinic site (17), whereas formamide denaturation at neutral pH leaves them intact (18). Consequently, the sedimentation profile after formamide denaturation yields the average number of breaks/T7 DNA strand; after NaOH denaturation, it yields the sum of breaks and intact apurinic sites. The difference between these values gives the average number of intact apurinic sites (*i.e.* not associated with breaks).

Purification of Enzyme—An 8% streptomycin solution in Buffer I is added dropwise, with constant stirring, to the crude extract from 50 g of bacteria (Preparation I = 440 ml; 44,000 enzyme units; 8 units/mg of protein) at 0° to reach a 0.8% final concentration. After mixing for an additional 15 min, the suspension is centrifuged for 30 min at 16,000 × *g*; the supernatant (Preparation II) is used for further purification.

To Preparation II (505 ml; 43,000 enzyme units; 11 units/mg of protein) at 0°, solid ammonium sulfate is added slowly to reach 55% saturation; after a 30-min centrifugation at 16,000 × *g*, the supernatant is brought to 85% saturation and again centrifuged. The second sediment is dissolved in 100 ml of Buffer II and dialyzed against the same buffer. The dialyzed material (Preparation III) contains 27,000 enzyme units with a specific activity of 26 units/mg of protein.

Preparation III is applied on a DEAE-cellulose (Whatman) column (5 × 15 cm) equilibrated with Buffer II (pH 8.0) at a flow rate of 90 ml/h. The column is washed with 75 ml of Buffer II containing 0.05 M NaCl, then eluted, always at the same flow rate, with 2 liters of a 0.05 to 0.25 M NaCl linear gradient in Buffer II; fractions of 15 ml are collected. Fig. 1*A* shows that two peaks of enzyme activity are eluted between 0.17 and 0.21 M NaCl. Fractions 104 to 116, corresponding to the second peak, are pooled; they contain 23,000 enzyme units with a specific activity of 290 units/mg of protein (Preparation IV).

Preparation IV (195 ml) is dialyzed against Buffer III, then applied, at a rate of 55 ml/h, on a DEAE-Sephadex A-25 column (2.6 × 40 cm) equilibrated with the same buffer (pH 7.5). The column is washed with 100 ml of buffer containing 0.1 M NaCl and the elution is carried out, at the same flow rate, with 1 liter of a 0.1 to 0.2 M NaCl linear gradient in Buffer III; fractions of 10 ml are collected. Fig. 1*B* shows several peaks of activity. Fractions 56 to 75, corresponding to the first peak, are pooled (Preparation V); they contain 11,000 enzyme units with a specific activity of 1,250 units/mg of protein.

To concentrate Preparation V, the 200 ml are dialyzed against Buffer III and poured on a small column (0.9 × 15 cm) of DEAE-cellulose, which is eluted with 0.5 M NaCl in Buffer III; the protein (detected at 280 nm) is found in three 2.5-ml fractions that are pooled. The 7.5 ml are placed on a Sephadex G-75 column (2.6 × 100

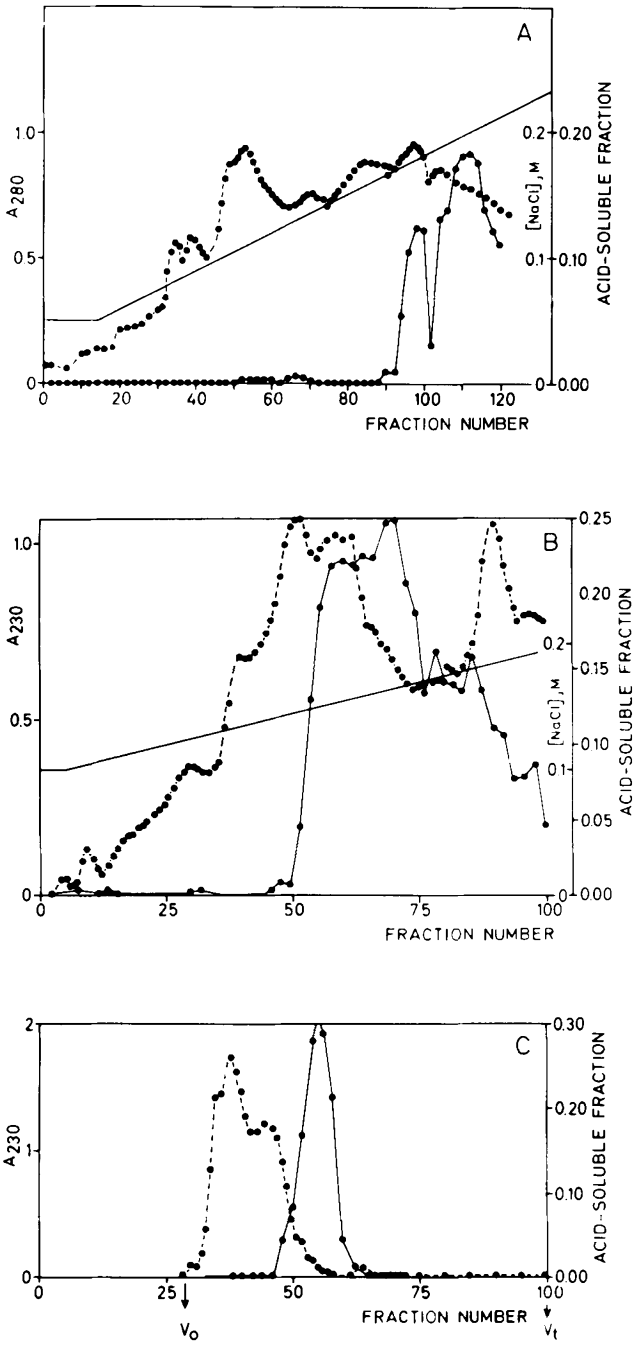

FIG. 1. Chromatographic purification of the *Bacillus stearothermophilus* endonuclease for apurinic sites. Absorbance at 280 or 230 nm (- - -) and enzyme activity (——) on alkylated-depurinated [^{3}H]DNA (acid-soluble fraction) are given for each fraction. *Straight lines* give the slopes of the NaCl gradients. Application of sample and elution are described in the text. *A*, DEAE-cellulose; *B*, DEAE-Sephadex A-25; *C*, Sephadex G-75.

cm) equilibrated with Buffer III containing 0.1 M NaCl; the elution is carried out with the same buffer at a rate of 20 ml/h, and 5-ml fractions are collected. Fig. 1*C* shows that the enzyme is eluted well separated from most of the protein. Fractions 53 to 58 are pooled (Preparation VI); they contain 9000 enzyme units with a specific activity of 5600 units/mg of protein. Electrophoresis on polyacrylamide gel reveals at least four protein bands. At this stage, the enzyme is very labile so that, if the purification is not immediately carried on, the glycerol concentration must be brought to 50% and the solution kept at −30°.

DNA-Sephadex is prepared according to the method of Arndt-Jovin *et al.* (19). Preparation VI (30 ml) is applied, at a rate of 10 ml/h, on a DNA-Sephadex column (0.3 × 5 cm) equilibrated with Buffer III. The column is washed with 10 ml of Buffer III, then eluted, always at the same flow rate, with 0.6 M KCl in Buffer III; fractions of 1 ml are collected. The enzyme appears at the high salt front in only three fractions which give no absorption at 230 nm. The pooled fractions, after dialysis against the Hepes buffer, contain 4,200 enzyme units with a specific activity of 65,000 units/mg of protein (Preparation VII); the glycerol concentration is raised to 50% and the solution kept at −30°.

Table I shows that, from the crude extract, a 8100-fold purification is achieved with an overall yield of about 10%.

Polyacrylamide Gel Electrophoresis in Presence of Sodium Dodecyl Sulfate– The method described by Weber and Osborn (20) is followed using a 2.5% stacking gel (0.5 cm) on top of a 10% separating gel (7 cm). The proteins are denatured, at room temperature or 100°, in 0.1 M Tris·HCl, pH 6.8, containing 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. After addition of bromphenol blue, aliquots (100 μl), containing from 5 to 20 μg of protein, are placed on the stacking gels and the electrophoresis is carried out in 0.05 M Tris, 0.4 M glycine, 0.1% sodium dodecyl sulfate, pH 8.3, buffer at 1.5 mA/tube until the bromphenol blue reaches the end of the gel. The total length (*La*) of the extracted gel and the distance (*Db*) migrated by the bromphenol blue are measured. After fixation and staining with Coomassie blue, the total length of the gel (*Lb*) is again measured and also the distance migrated by the protein (*Dp*). A migration coefficient (*mc*) is then calculated: $mc = (Dp \times La)/(Db \times Lb)$.

Molecular Weight Estimation by Sephadex G-75 Chromatography– A Sephadex G-75 column (1.6 × 70 cm) is equilibrated with Buffer III, 0.1 M NaCl; the void volume (V_0) is measured with blue dextran whereas the total volume (V_t) is determined with glycylglycine. Four different proteins of known molecular weights (ovalbumin, chymotrypsinogen, ribonuclease A, insulin) are used for calibration. The sample (2 mg), dissolved in 2 ml of Buffer III, 0.1 M NaCl, is applied to the column and eluted at a rate of 6 ml/h with the same buffer; 3-ml fractions are collected and their absorbance is read at 280 nm to determine the protein elution volume (V_e). There is a linear relationship between the logarithm of the molecular weight and the elution constant $K_{av} = (V_e - V_0)/(V_t - V_0)$.

Protein Concentration– In the fractions from chromatography, the protein concentration is followed by recording the absorbance at 280 nm or 230 nm; otherwise, it is measured by method of Lowry *et al.* (21) using bovine serum albumin as standard.

RESULTS

Properties of Bacillus stearothermophilus Crude Extract– Samples (200 μl; 2.5 mg of protein) of the crude extract were mixed with 200 μl of [^{3}H]DNA (20 μg/ml), either untreated, alkylated, or alkylated-depurinated, and incubated at 37°; aliquots were taken after 0 to 120 min to measure the acid-soluble radioactivity. Fig. 2 shows that the extract had some action on untreated DNA, more on alkylated DNA, but that its action was far greater when alkylated sites were replaced by apurinic sites (alkylated-depurinated DNA).

Using acetate/barbital buffers (0.14 M sodium acetate, 0.14 M sodium 5,5′-diethylbarbiturate), the optimum pH for the

TABLE I

Purification of Bacillus stearothermophilus endonuclease for apurinic sites

Preparation	Volume	Endonuclease	Specific activity
	ml	*units*	*units/mg protein*
I. Crude extract	440	44,000	8
II. Streptomycin	505	43,000	11
III. Ammonium sulfate	100	27,000	26
IV. DEAE-cellulose	195	23,000	290
V. DEAE-Sephadex	200	11,000	1,250
VI. Sephadex G-75	30	9,000	5,600
VII. DNA-Sephadex	3	4,200	65,000

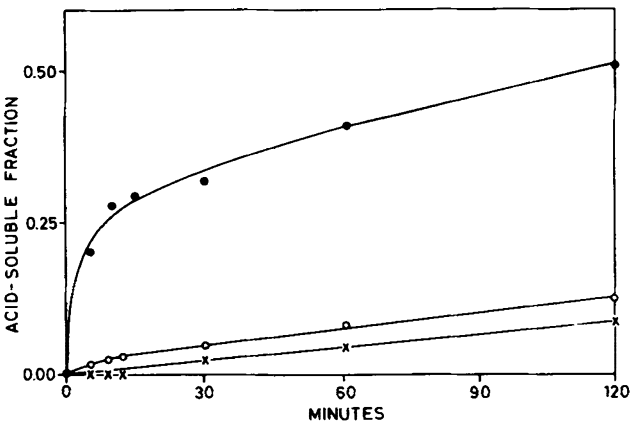

FIG. 2. Action of the crude extract of *Bacillus stearothermophilus* cells on untreated, alkylated, and alkylated-depurinated DNA. Samples (200 μl) of the crude extract in Buffer I were mixed with 200 μl of 0.05 M Hepes, 0.05 M NaCl, 1 mM $MgCl_2$, pH 7.5, buffer containing 4 μg of [^{3}H]DNA, either untreated (×), alkylated (○), or alkylated-purinated (●); the mixtures were incubated at 37° and aliquots taken from 0 to 120 min to measure the acid-soluble radioactivity. The results were corrected for controls with Buffer I instead of the crude extract; they are expressed as fractions of the substrate radioactivity (acid-soluble fraction).

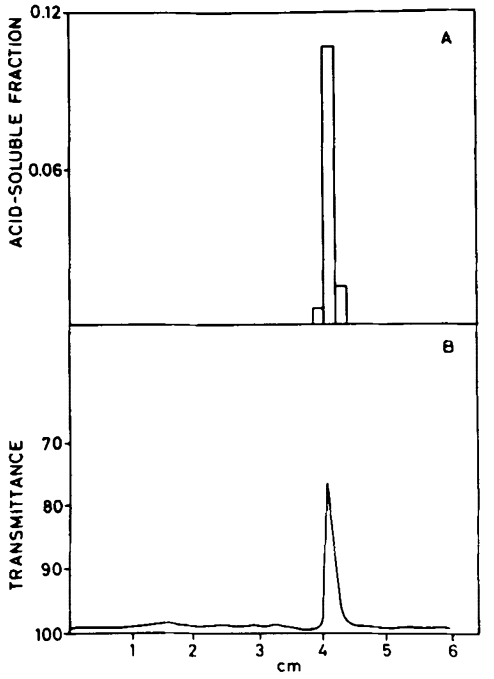

FIG. 3. Polyacrylamide gel electrophoresis of the purified enzyme in the presence of sodium dodecyl sulfate. Two gels were carried out in parallel. One (*A*) was cut in 2-mm slices which were assayed for enzyme activity on alkylated-purinated [^{3}H]DNA (acid-soluble fraction), and the other (*B*) was stained with Coomassie blue, scanned at 550 nm, and the percentage of transmittance recorded.

TABLE II

Amino acid composition of two bacterial endonucleases for apurinic sites

The *Escherichia coli* results are from Verly and Rassart (9). Because no data were available for cysteine, methionine, and tryptophan, the molar percentages do not take account of the possible presence of these amino acids in the proteins. The polarity index is calculated by summing the polar amino acids and half the total of those of the intermediate class (23).

Class and amino acid	*Bacillus stearothermophilus*	*Escherichia coli* main enzyme
	molar %	
Polar		
Arginine	0.0	3.5
Aspartic acid	15.8	11.3
Glutamic acid	4.4	15.1
Lysine	10.0	5.6
Total	30.2	35.5
Intermediate		
Glycine	11.5	16.9
Histidine	3.5	1.8
Serine	6.9	13.0
Threonine	6.0	4.6
Tyrosine	0.0	0.9
Total	27.9	37.2
Nonpolar		
Alanine	11.0	7.6
Isoleucine	5.7	2.8
Leucine	7.8	5.4
Phenylalanine	2.0	3.9
Proline	6.9	3.5
Valine	8.3	4.4
Total	41.7	27.6

TABLE III

Action of Preparation VI on untreated, alkylated, and depurinated T7 DNA

Samples of 100 μl of Preparation VI (16 enzyme units) in Hepes buffer were incubated for 60 min at 37° with 100 μl of the same buffer containing 2 μg of T7 [^{3}H]DNA. Controls without enzyme were carried out in the same way. At the end of the incubation, 600 μl of 0.15 M NaCl, 0.015 M EDTA, pH 7.0, were added and the solution was dialyzed at 4° against the same buffer. Each sample was then split in two parts; one was denatured with NaOH and the other with formamide before sedimentation on neutral sucrose gradients. The average number of breaks/strand was estimated from the sedimentation profile.

T7 [^{3}H]DNA	Enzyme	NaOH	Formamide	Alkali-labile sites
		breaks/strand		
Untreated	−	2.1	2.6	<0
	+	2.3	2.3	0
Alkylated	−	12	7	5
	+	13	13	0
Depurinated	−	33	7	26
	+	30	36	<0

crude extract activity on alkylated-depurinated DNA was found to be at 7.5. Among different buffers at pH 7.5 that were tested, 0.05 M Hepes was found the best for the enzyme activity.

Aliquots of crude extract diluted 100-fold with Buffer I containing 2% bovine serum albumin (20 μl) were incubated with 20 μl of the alkylated-depurinated [^{3}H]DNA solution for 10 min. The optimal temperature for the enzyme was found to be 60°. Below this temperature, the logarithm of the reaction velocity plotted against $1/T$ (T = absolute temperature in K) yields a straight line from which an activation energy of 21,000 cal/mol can be calculated for the enzyme-catalyzed reaction.

TABLE IV

Effect of divalent cations on activity of endonuclease for apurinic sites of Bacillus stearothermophilus

Preparation VI and the alkylated-depurinated [^{3}H]DNA solution were dialyzed three times against 0.05 M Tris·HCl, 0.05 M NaCl, 10 mM EDTA, pH 7.5, then three times against 0.05 M Hepes, 0.05 M NaCl, pH 7.5. To 10 μl of enzyme preparation and 20 μl of substrate solution, were added 30 μl of the same Hepes buffer containing the amount of the chloride salt of the divalent cation needed to reach the indicated concentration. After a 30-min incubation at 37°, the acid-soluble radioactivity was measured; it is expressed in percentage of the value obtained without the divalent cation.

Cation	0.1 mM	0.5 mM	2 mM	6 mM	10 mM
Mg^{2+}	103	89	61	54	43
Ca^{2+}	95	77	57	66	61
Ca^{2+a}	83	84	74	64	74
Mn^{2+}	88	92	77	71	63
Cu^{2+}	79	92	94	83	10
Co^{2+}	109	107	62	12	14
Zn^{2+}	95	96	77	22	14

[a] The incubation medium contained 10 mM $MgCl_2$ plus the indicated concentration of $CaCl_2$.

Physical Properties of Purified Enzyme – Two aliquots of Preparation VII (200 μl), containing 4 μg of protein, were submitted to polyacrylamide gel electrophoresis (see "Materials and Methods"). One of the gels was stained with Coomassie blue; the other was cut in 2-mm slices which were ground in 250 μl of Hepes buffer containing 4% bovine serum albumin and, after centrifugation, the enzyme activity of the supernatant was measured on alkylated-depurinated [^{3}H]DNA. There was a single protein band corresponding to the enzyme activity (Fig. 3).

In another experiment, the solution of Preparation VII, which contained 1% sodium dodecyl sulfate, and three proteins of known molecular weights (ovalbumin, chymotrypsinogen, ribonuclease A) dissolved in the same buffer, were heated at 100° for 2 min and submitted to gel electrophoresis. After staining with Coomassie blue, the migration coefficients were calculated. There was a linear relationship between the logarithm of the molecular weights of the standard proteins and their migration coefficients; Preparation VII gave a single band the migration coefficient of which corresponded to a molecular weight of 28,000.

The molecular weight of the endonuclease was also determined by Sephadex G-75 filtration. Preparation VI was dialyzed against Buffer III and 0.1 M NaCl, and a 2-ml aliquot was filtered through the calibrated Sephadex G-75 column (see "Materials and Methods"). Reference to the calibration curve given by the standard proteins indicated a molecular weight around 27,000.

Preparation VII (180 μg of protein) was dialyzed against water, lyophilized, hydrolyzed in HCl, and analyzed for amino acid content on an automatic JEOL JLC-ASH apparatus. The molar percentages were calculated from the specific absorption determined experimentally with pure amino acids (Table II).

Substrate Specificity of Purified Enzyme – Preparation VI (16 enzyme units) was incubated with 2 μg of T7 [^{3}H]DNA, either untreated, alkylated, or depurinated. Each sample was then split in two parts; one was denatured with NaOH and the other with formamide before sedimentation on sucrose gradients. Table III indicates that the untreated DNA contained no alkali-labile sites (= apurinic sites), the alkylated

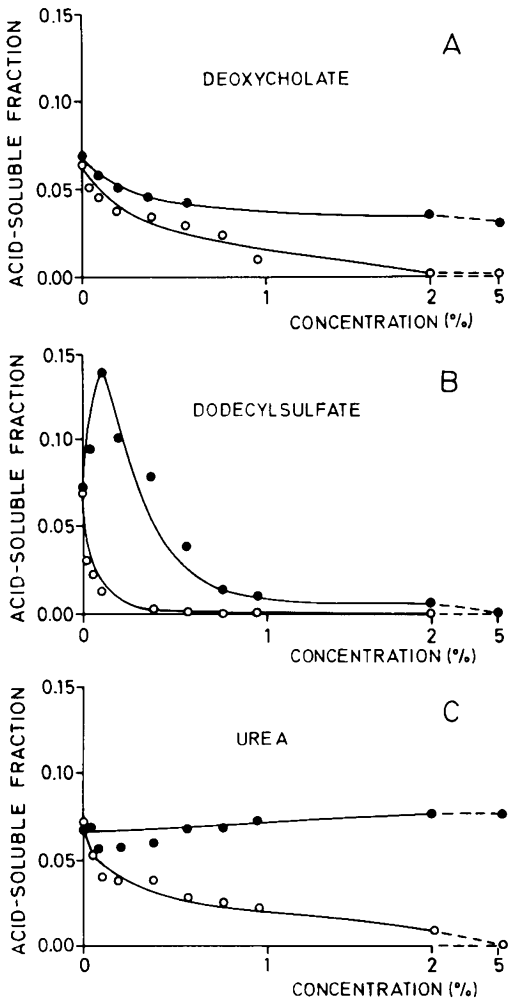

FIG. 4. Action of denaturing agents on *Bacillus stearothermophilus* and *Escherichia coli* endonucleases for apurinic sites. Hepes buffer (80 μl) containing 0.4 μg of alkylated-depurinated [^{3}H]DNA, 0.2 enzyme unit, and the indicated concentrations (weight per cent) of detergent [sodium deoxycholate (*A*); sodium dodecyl sulfate (*B*)] or urea (*C*) was incubated at 37° for 30 min before measuring the acid-soluble radioactivity. After correction for controls without enzyme, the results are expressed as acid-soluble fractions. ●, *B. stearothermophilus* enzyme (Preparation VI); ○, *E. coli* enzyme (Preparation V of Verly and Rassart (9)).

DNA contained, per strand, an average of 5 of them (for 350 alkylated sites; see "Materials and Methods"), and the depurinated DNA an average of 26. Comparison of the formamide results, with and without enzyme, indicates that the alkali-labile sites have been hydrolyzed by the enzyme. On the other hand, the results were the same after a treatment with NaOH whether the enzyme was present or not. This clearly indicates that the enzyme was active only on alkali-labile sites; it thus had no action on normal strands or at alkylated sites (*i.e.* no activity of the *N*-glycosidase or of the endonuclease type).

The action of Preparation VI on heat-denatured DNA was also investigated; there was none on the normal strands, and the enzyme action on depurinated DNA was considerably decreased by the denaturation of the substrate. Preparation VI did not release acid-soluble material from sonicated DNA or DNA activated with pancreatic deoxyribonuclease (EC 3.1.4.5) (Sigma Chemical Co.); the preparation was thus devoid of an activity comparable to that of exonuclease III in *Escherichia coli*.

Factors Affecting Catalytic Properties of Purified Enzyme – Using acetate/barbital buffers, the optimal pH was found to be 7.5 for Preparation VI; the activity curve relative to pH was quite similar to that obtained with the crude extract. The optimal temperature, in the presence of 25% glycerol, was 45° and the enzyme was more labile after purification than in the crude extract. The rate of denaturation of Preparation VI in Hepes buffer, 25% glycerol, was studied at different temperatures; it followed first order kinetics with half-lives of 20 min at 40°, 15 min at 50°, 11 min at 60°, and 8 min at 70°. The addition of 0.4% bovine serum albumin protected the enzyme which could then be heated at 60° for 120 min without loss of activity.

The enzyme activity was influenced by monovalent cations. It passed through a maximum for 0.125 M KCl (440% of the value without KCl) and 0.05 M NaCl (140% of the value without NaCl). Addition of EDTA to Preparation VI up to 10 mM did not inhibit the enzyme activity. On the other hand, Table IV shows that the divalent cations decreased the endonuclease activity.

We compared the action of several denaturation agents on the activity of the *B. stearothermophilus* enzyme (Preparation VI) and of the main *E. coli* endonuclease for apurinic sites (Preparation V of Verly and Rassart (9)). Up to 2% Triton X-100 had no effect on either enzyme, but Fig. 4 shows that the *E. coli* endonuclease for apurinic sites was much more sensitive to sodium deoxycholate, sodium dodecyl sulfate, or urea than the *B. stearothermophilus* enzyme.

DISCUSSION

An endonuclease which hydrolyzes DNA containing apurinic sites has been purified from *Bacillus stearothermophilus* cells. The final product appeared as a single protein band in a polyacrylamide gel electrophoresis performed in the presence of sodium dodecyl sulfate. The molecular weights of the native enzyme measured on Sephadex G-75 and of the sodium dodecyl sulfate-denatured enzyme determined by gel electrophoresis were nearly the same; the enzyme thus appears to be a monomeric protein of about 28,000 daltons.

With double-stranded DNA, the pure *B. stearothermophilus* enzyme is strictly specific for apurinic sites. This was shown using T7 phage DNA labeled with tritium and, after denaturation, the sucrose gradient centrifugation technique. Table III shows that the enzyme introduced no break in untreated DNA, and that the action on alkylated DNA was restricted to the alkali-labile sites. Because NaOH does not produce breaks near alkylated sites (22), the conclusion is that the enzyme has no action on alkylated sites, either of the endonuclease type (see results after denaturation with formamide) or of the *N*-glycosidase type (see results after NaOH denaturation). The pure enzyme did not degrade sonicated DNA or DNA nicked with pancreatic deoxyribonuclease; it is thus without an activity similar to that of *Escherichia coli* exonuclease III. The activity of the endonuclease of *B. stearothermophilus* on apurinic sites is considerably decreased when the DNA is denatured.

Because of its thermoresistance, absence of inhibition by EDTA, and absence of associated exonuclease activity, the endonuclease specific for apurinic sites of *B. stearothermophilus* resembles more closely the endonuclease IV of *E. coli* (5) than the main endonuclease specific for apurinic sites found

in this bacterium by Verly and Paquette (3, 4) and purified by Verly and Rassart (9) (endonuclease VI).

To understand the causes of the heat resistance of the *B. stearothermophilus* enzyme, we compared the enzyme of this thermophilic bacterium to the thermosensitive *E. coli* main endonuclease for apurinic sites. Fig. 4 shows that the *E. coli* enzyme is more readily denatured by urea and detergents. An amino acid analysis revealed a higher percentage of hydrophobic amino acids in the *B. stearothermophilus* endonuclease (Table II); the polarity index, calculated according to Vanderkooi and Capaldi (23), is 54.1% for the *E. coli* enzyme and only 44.2% for that of the thermophilic bacterium. Possibly a more important hydrophobic core might be responsible for the higher resistance to denaturation of the *B. stearothermophilus* endonuclease for apurinic sites.

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