Purification and Properties of Deoxyribonuclease A from *Bacillus subtilis*

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An exonuclease, DNase A, was isolated from *Bacillus subtilis* Marburg strain. DNase A had a molecular weight of 35,000 and degraded preferentially double-stranded DNA to mononucleotides. The enzyme could degrade also single-stranded DNA with a reduced rate. The two activities toward double- and single-stranded DNA resided in a single protein. The enzyme required absolutely Mg^{2+} or Mn^{2+} for its activity, and sulfhydryl-protecting reagents stimulated the enzyme activity. The mode of action of this enzyme was predominantly exonucleolytic in a direction from the 5'-end to the 3'-end.

Several deoxyribonucleases (DNases) have been isolated and characterized in *Bacillus subtilis*. Ca^{2+} -dependent extracellular nucleases which degrade native and denatured DNAs, and RNA exonucleolytically to produce 3'-nucleotides in the presence of Ca^{2+} are purified from culture fluids of *B. subtilis* SB19.^{1~4)} A similar nuclease associated with the cell wall-membrane fraction is also reported.⁵⁾ An ATP-dependent DNase which requires ATP and Mg^{2+} for its activity is purified from the extracts of *B. subtilis* Marburg strain.^{6,7)} Two endonucleases, a heat-activated endonuclease⁸⁾ and a Mn^{2+} -stimulated endonuclease,⁹⁾ which introduce a limited number of breaks into DNA, are isolated from *B. subtilis* Marburg strain. Recently DNase activities specific for ultraviolet light irradiated DNA,¹⁰⁾ γ -ray irradiated DNA¹¹⁾ and uracil-containing DNA¹²⁾ were detected in *B. subtilis*.

As compared with a variety of DNases present in *Escherichia coli*, it is expected that another DNase(s) different from those previously reported might exist in *B. subtilis*. In our preliminary report, two DNases (DNase A and DNase B) which could be distinguished from other DNases previously reported in several respects were identified in *B. subtilis*

Marburg strain.¹³⁾ This paper describes the purification and properties of one of the two enzymes (DNase A), which preferentially degrades native DNA to mononucleotides in the presence of Mg^{2+} or Mn^{2+} .

MATERIALS AND METHODS

Bacteria and phage. *Bacillus subtilis* 168TT (thy⁻, trp⁻)¹⁴⁾ was used as an enzyme source. *Escherichia coli* HF4704 (thy⁻)¹⁵⁾ was used for the preparation of ³H-labeled *E. coli* DNA. Coliphage T3 amNG220B, a lysis defective mutant, and a permissive host *E. coli* B3 (thy⁻) kindly supplied by Dr. T. Minagawa were used for the preparation of ³H-labeled T3 DNA.

Reagents. DEAE-cellulose (DE-52) and phosphocellulose (P-11) were the products of Whatman Biochemicals Ltd. Plastic sheets pre-coated with polyethyleneimine (PEI) cellulose was obtained from Macherey-Nagel Co. Sephadex G-100 and Dextran T500 were the products of Pharmacia Fine Chemicals. Phenylmethyl sulfonyl fluoride (PMSF) and polyethylene glycol (6000) was purchased from Sigma Chemicals Co. and Nakarai Chemicals Co., respectively. [6-³H] Thymidine (24 Ci/mmol) was obtained from the Radiochemical Centre Amersham. [γ -³²P]ATP (6·10⁹ cpm/ μ mol) prepared as described previously¹⁶⁾ was kindly given by Dr. M. Takanami.

Enzymes. *E. coli* alkaline phosphatase (nuclease free), micrococcal nuclease, ribonuclease T₁, bovine serum albumin, egg white lysozyme, soybean trypsin inhibitor and horse heart cytochrome C were purchased from Sigma Chemical Co. Ribonuclease A and egg albumin were obtained from P-L Biochemicals, Inc. and ICN Pharmaceuticals, Inc., respectively.

Abbreviation: DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; EDTA-2K-Mg, ethylenediamine tetraacetic acid dipotassium magnesium salt; GSH, reduced glutathione.

Polynucleotide kinase was purchased from Miles Co. Ltd. These were used without further purification.

Preparation of DNA. ^3H -labeled *E. coli* DNA ($2.7 \cdot 10^8$ cpm/ μmol) was isolated from *E. coli* HF4704 grown in amino acid-glucose medium¹⁷⁾ supplemented with [^3H]thymidine. The [^3H]DNA was extracted with phenol and then precipitated with ethanol by the method of Saito and Miura¹⁸⁾ with a slight modification. To remove contaminating RNA, the crude DNA preparation was treated with ribonuclease *A* together with ribonuclease *T*₁ followed by the phenol extraction and ethanol precipitation as described above.

T3 [^3H]DNA ($1.5 \cdot 10^7$ cpm/ μmol) was isolated from the purified T3 bacteriophage particles labeled with [^3H]thymidine by the phenol method. Bacteriophage T3 labeled with [^3H]thymidine was prepared by growing *E. coli* B3 at 37°C in 300 ml of M9 medium²⁰⁾ supplemented with 0.2% casamino acids and [^3H]thymidine (3 $\mu\text{Ci/ml}$). At a cell titer of $1 \cdot 10^8/\text{ml}$, T3 *amNG220B*, a lysis defective mutant was infected at a multiplicity of 9 and incubation was continued for 30 min at 30°C. The culture was quickly chilled in an ice bath and cells were collected by centrifugation. After lysing the cells with lysozyme-EDTA, phages were collected from the lysate by centrifugation at $64,000 \times g$ for 60 min. Phages were purified by repeated differential centrifugation and then by CsCl density gradient centrifugation as described by Richardson.²¹⁾

Preparation of [^3H ,5'- ^{32}P]DNA. *E. coli* [^3H]DNA labeled with ^{32}P at the 5'-end was prepared as described by Kanamori *et al.*⁴⁾ *E. coli* [^3H]DNA was partially digested with micrococcal nuclease. The reaction mixture (3.2 ml) contained 50 mM glycine-KOH (pH 9.2), 9 mM CaCl_2 , 0.2 mg/ml bovine serum albumin, 2.1 mM [^3H]DNA, and 0.1 unit of the enzyme. The amount of polynucleotide is expressed as nucleotide equivalents unless otherwise noted. The mixture was incubated for 15 min at 37°C, the reaction was stopped by addition of EDTA to a final concentration of 20 mM. The partially digested [^3H]DNA was incubated with polynucleotide kinase and [γ - ^{32}P]ATP to label the 5'-end with ^{32}P . The reaction mixture (1 ml) contained 70 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 , 10 mM 2-mercaptoethanol, 50 μmol [γ - ^{32}P]ATP, 1.4 mM [^3H]DNA and 50 units of polynucleotide kinase. After incubation of the mixture for 30 min at 37°C, additional 25 units of polynucleotide kinase were added and the incubation was continued for 30 min. The mixture was deproteinized with phenol and passed through a column of Sephadex G-50 (1.8×17 cm); the void volume fractions were pooled and dialyzed against 20 mM Tris-HCl (pH 7.5)-10 mM NaCl.

T3 [^3H]DNA labeled with ^{32}P at the 5'-end was prepared as described by Richardson²¹⁾ with a slight modification. T3 [^3H]DNA was first treated with

E. coli alkaline phosphatase. The reaction mixture (0.7 ml) contained 50 mM Tris-HCl (pH 7.5), 0.6 mM T3 [^3H]DNA and 0.05 unit of phosphatase. After incubation of the mixture for 1 hr at 37°C, an additional 0.025 unit of phosphatase was added, and the incubation was continued for 30 min. The reaction was stopped by addition of potassium phosphate (pH 7.4) to a final concentration of 5 mM. The mixture was incubated with 10 mM MgCl_2 , 16 mM 2-mercaptoethanol 45 μM [γ - ^{32}P]ATP and 70 units of polynucleotide kinase in a volume of 3.2 ml. After incubation of the mixture for 1 hr at 37°C, additional 50 units of the enzyme were added, and the incubation was continued for 1 hr. The reaction mixture was deproteinized by phenol, and dialyzed extensively against 0.1 M potassium phosphate (pH 7.4)-0.1 M NaCl and then against 10 mM Tris-HCl (pH 7.5)-0.05 M NaCl.

Enzyme assay. The standard reaction mixture (0.25 ml) contained 50 mM glycine-KOH (pH 9.5), 5 mM MgCl_2 , 0.1 mg/ml bovine serum albumin, 10% glycerol, 1 mM 2-mercaptoethanol, 38 μM native *E. coli* [^3H]DNA and enzyme dilutions were made with 50 mM Tris-HCl (pH 8.0)-1 mg/ml bovine serum albumin-20% glycerol-10 mM 2-mercaptoethanol. The mixture was incubated for 30 min at 37°C and the reaction was stopped by addition of 0.25 ml of 1 mg per ml of yeast RNA and 0.25 ml of 15% trichloroacetic acid. After allowing to stand for 30 min at 0°C, the mixture was centrifuged for 10 min at $1,500 \times g$. A portion of the supernatant fluid was removed, diluted with water and the radioactivity was measured in Triton-toluene scintillation fluid. The scintillation fluid consisted of 1 part Triton X-100 to 2 parts toluene, containing 3 g of 2,5-diphenyloxazole and 0.1 g of 2,2'-*p*-phenylene-bis(5-phenyloxazole) per liter. One unit of enzyme activity was defined as the amount catalyzing the production of 10 nmoles of acid-soluble nucleotide in 30 min at 37°C. With T3 [^3H]DNA as substrate, the reaction mixture had the same composition except that the concentration of DNA was 0.33 μM , and the volume of the reaction mixture was reduced to 0.1 ml. The volume of carrier RNA and trichloroacetic acid were also reduced to 0.1 ml. The measurement of radioactivity was the same as above.

Thin layer chromatography of the reaction product. In order to analyze mononucleotides released from oligonucleotide substrate thin layer chromatography of PEI-cellulose was performed by the method of Weimann and Randerath.²²⁾ After the DNase reaction, carrier RNA and trichloroacetic acid were added as in the standard assay, and the mixture was centrifuged for 10 min at $1,500 \times g$. The supernatant fluid was extracted with ether to remove the trichloroacetic acid, and an amount of 0.1 ml of the supernatant was spotted on a PEI-cellulose plate (6.5×20 cm) along with marker

dTMP. The plate was washed with methanol to remove salts, and developed with 0.7 M NaCl for 5 min, 1.0 M NaCl for 15 min and 1.2 M NaCl for 40 min, successively, without intermittent drying. The location of marker dTMP was confirmed by means of an ultra-violet lamp, and then the plate was cut into 1 cm strips. The radioactivity of each strip was measured in toluene scintillation fluid.

Polyacrylamide gel electrophoresis. Analytical disc gel electrophoresis was performed as described by Davis.²⁵⁾ Two polyacrylamide gels (7%) with 2.5% stacking gels were prepared, and 0.2 ml of sample was layered on each stacking gels. DTT (0.5 mM) was added to reservoir to improve recovery of the enzyme. The electrophoresis was carried out at 4°C using a current of 3 mA per gel. One gel was stained with Coomassie brilliant blue, destained by diffusion, and then scanned by a Shimadzu dual-wavelength densitometer type CS-900. The other gel was cut into 3 mm slices. The slices were eluted over night with 0.2 ml of 50 mM Tris-HCl (pH 8.0)-1 mg/ml bovine serum albumin-20% glycerol-1 mM DTT. Aliquots were removed and assayed for DNase activities with T3 DNA as substrate under the conditions described above.

Other methods. Protein was determined by the method of Lowry *et al.*²⁴⁾ Nucleic acid was determined spectrometrically. DNA was denatured by heating for 10 min in a boiling water bath and chilling quickly in an ice bath.

RESULTS

Purification of DNase A

DNase A was purified simultaneously with DNase B, which will be described in the succeeding paper,²⁵⁾ before the phosphocellulose column chromatography step. The result of a typical purification of DNase A are summarized in Table I. All procedures were carried out at 0~4°C unless otherwise noted.

Growth of bacteria. The growth medium consisted of 1% glucose, 1% casamino acids, 50 µg per ml of tryptophan, 100 µg per ml of thymine and subtilis salts.²⁶⁾ *B. subtilis* 168TT grown in 35 liter of the medium with vigorous aeration at 37°C in a fermenter and harvested at the end of logarithmic phase. To avoid autolysis, the culture was chilled rapidly to 4°C by addition of 10 liter of frozen subtilis salts solution and 500 ml of 1 M MgCl₂. The cells were washed with 2 liter of 50 mM Tris-

TABLE I. PURIFICATION OF DNASE A

Fractions	Protein	Total specific	
		activity ^a	activity
	mg/ml	units × 10 ⁻³	units/mg
I. Crude extract	28.2	30.5 ^c	1.57
II. Phase partition	11.9	35.7 ^c	3.85
III. Ammonium sulfate	41.5	26.1	5.42
IV. DEAE-cellulose	9.69	17.0	97.3
V. Phosphocellulose ^b	1.12	8.43	746
VI. Sephadex G-100 ^b	0.099	2.35	2740

^a The enzyme activity was measured with native *E. coli* DNA under the standard assay condition described in "MATERIALS AND METHODS."

^b This step was performed several times on a small scale, and these values are calculated for the large scale procedure.

^c Total activity increase at this step might be due to the removal of factors affecting DNase A activity.

HCl (pH 7.6)-10 mM MgCl₂-5 mM EDTA-2K-Mg-10% glycerol-10 mM 2-mercaptoethanol. The yield of wet packed cells was 270 g.

Preparation of cell extract. The cells were resuspended in 300 ml of 50 mM Tris-HCl (pH 7.6)-10 mM MgCl₂-5 mM EDTA-2K-Mg-5% glycerol-2 mM GSH-1 mM PMSF and disrupted by vigorous agitation with glass beads in a Dyno-Mill (Willy A. Bachofen Maschinen farabrik, Switzerland). The glass beads were removed by centrifugation, the supernatant fluid was pooled, and the glass beads were washed with 400 ml of the same buffer. The combined supernatant fluid was centrifuged for 1 hr at 10,000×g. The resulting supernatant fluid (690 ml) was collected (Fraction I).

Removal of nucleic acids by phase partitioning.²⁷⁾ To Fraction I were added 79.2 ml of 20% (w/w) Dextran T500 and 222 ml of 30% (w/w) polyethylene glycol to give final concentrations (by weight) of 1.6% and 6.4%, respectively. Solid NaCl (232 g) was then added slowly with stirring to a final concentration of about 4 M. The mixture was stirred for 1 hr at 0°C and then centrifuged at 4,000×g for 20 min. The resulting clear, upper polyethylene glycol phase was recovered to give Fraction II (780 ml), and the turbid, lower dextran phase containing the bulk of nucleic acids was

discarded.

Ammonium sulfate precipitation. Fraction II was dialyzed against 0.3 M potassium phosphate (pH 7.4)-2 mM EDTA-10 mM 2-mercaptoethanol to remove NaCl. To the dialyzed solution (1,520 ml) were added 303 g of solid ammonium sulfate slowly with stirring. The mixture was stirred for 1 hr at 0°C, and transferred to a separatory funnel. After allowing to stand for 7 hr at 4°C, the clear lower phase was separated from the turbid upper phase containing polyethylene glycol. To 1,310 ml of the lower phase were added 131 g of ammonium sulfate, and stirred for 1.5 hr at 0°C. The resulting precipitate was collected by centrifugation at $8,000\times g$ for 30 min and dissolved in a minimal volume of 0.3 M potassium phosphate (pH 7.4)-2 mM EDTA-2 mM GSH-10% glycerol. The solution was dialyzed against 0.3 M potassium phosphate (pH 7.4)-2 mM EDTA-10 mM 2-mercaptoethanol-10% glycerol (Fraction III, 116 ml).

DEAE-cellulose chromatography. Fraction III was diluted with 580 ml of 2 mM EDTA-10 mM 2-mercaptoethanol-10% glycerol, and the diluted solution was applied to a column of DEAE-cellulose (4.5×25 cm), which had been equilibrated with 0.05 M potassium phosphate (pH 7.4)-2 mM EDTA-10 mM 2-mercaptoethanol-10% glycerol. The column was washed with 800 ml of 0.075 M potassium phosphate (pH 7.4)-2 mM EDTA-2 mM GSH-10% glycerol at a flow rate of 3.3 ml per min and 18 ml fractions were collected. DNase A was eluted by this wash. The column was then eluted with a linear gradient of 0.075 to 0.50 M potassium phosphate (pH 7.4) containing 2 mM EDTA-2 mM GSH-10% glycerol and DNase B was eluted at the concentration from 0.20 to 0.25 M.¹³⁾ DNase A was precipitated by addition of 0.55 g per ml of ammonium sulfate, and the precipitate was collected by centrifugation at $10,000\times g$ for 30 min. The pellet was dissolved in a minimal volume of 0.05 M potassium phosphate (pH 7.4)-2 mM EDTA-10 mM 2-mercaptoethanol-20% glycerol, and then dialyzed against 0.2 M potassium phosphate

(pH 7.4)-2 mM EDTA-2 mM GSH-20% glycerol (Fraction IV, 18 ml).

Phosphocellulose chromatography. A column of phosphocellulose (2×9 cm) was prepared and equilibrated with 0.05 M potassium phosphate (pH 6.8)-2 mM EDTA-10 mM 2-mercaptoethanol-20% glycerol. Aliquots of Fraction IV (15 ml) was dialyzed against 0.2 M potassium phosphate (pH 6.8)-2 mM EDTA-10 mM 2-mercaptoethanol-20% glycerol, and the dialysate was diluted 4-fold with 2 mM EDTA-10 mM 2-mercaptoethanol-20% glycerol before being applied to the column. The column was washed with 75 ml of 0.05 M potassium phosphate (pH 6.8)-2 mM EDTA-2 mM GSH-20% glycerol, and then a linear gradient elution was performed with 300 ml of the same buffer from 0.05 M to 0.5 M at a flow rate of 0.5 ml per min and 3 ml fractions were collected. Enzyme activity was eluted at 0.3 M of potassium phosphate as a single peak. The peak fractions were pooled, concentrated by dialysis against 30% (w/w) polyethylene glycol containing 0.3 M potassium phosphate (pH 7.4)-2 mM EDTA-2 mM GSH-50% glycerol. The dialysate (Fraction V, 8.4 ml) was stored at -20°C until use.

Sephadex G-100 chromatography. Aliquots of Fraction V (4 ml) was dialyzed against 0.2 M potassium phosphate (pH 7.4)-2 mM EDTA-2 mM GSH-10% glycerol. The dialysate was applied to a column of Sephadex G-100 (2.5×90 cm) equilibrated with the same buffer as used for dialysis. The column was eluted with the same buffer at a flow rate of 10 ml per hr, and 4 ml fractions were collected. The DNase activity was eluted in a single peak at 32% of the bed volume, and the fractions having DNase activity were collected and concentrated by dialysis against 30% (w/w) polyethylene glycol and 50% glycerol as phosphocellulose fraction (Fraction VI, 2 ml).

Polyacrylamide gel electrophoresis. Fraction VI was analyzed by polyacrylamide gel electrophoresis to determine the purity of the enzyme (Fig. 1). Three protein bands were

distinguished in the stained gel. Only one protein band, corresponding to the slowest moving one, had enzyme activity. This protein band comprised roughly 50% of the protein in Fraction VI, as estimated from the areas under the curves traced in densitometry scans of the stained gel.

FIG. 1. Polyacrylamide Gel Electrophoresis of Fraction VI of DNase A.

Details of the experimental procedures are given in the text. The stained protein pattern is shown on the upper side, and the migration is from left (anode) to right (cathode). DNase activities measured with native T3 [^3H]DNA (●) and denatured T3 [^3H]DNA (○) in slices of a parallel gel is given on the bottom side.

Properties of the enzyme

Absolute requirement of divalent cations.

The DNase activity on native DNA was measured as functions of the concentrations of several divalent cations (Fig. 2). The purified enzyme absolutely required divalent cations for its activity, and no activity was detected in their absence. MgCl_2 and MnCl_2 were effective but CaCl_2 was not. The optimal concentration of MgCl_2 was 2 to 5 mM, and MnCl_2 was almost as effective as MgCl_2 at a lower concentration (0.5 to 3 mM) but was inhibitory at a higher concentration. The DNase activity on denatured DNA also required divalent cations (data not shown).

Sulfhydryl requirement. The effect of sulfhydryl-reagents was summarized in Table II. About 40 to 50% stimulation of the activity was observed in the presence of 2-mercapto-

FIG. 2. The Influence of Divalent Cation Concentration on the Activity of DNase A.

The standard assay condition with *E. coli* [^3H]DNA as substrate was used, except that the divalent cation concentration was varied, as shown. To the each assay mixture was added 0.36 unit/ml of Fraction VI enzyme.

TABLE II. EFFECT OF SULFHYDRYL-REAGENTS ON THE ACTIVITY OF DNASE A

Additions ^a	Relative activity (%) ^b
None	100
2-Mercaptoethanol (1 mM)	116
2-Mercaptoethanol (2 mM)	115
2-Mercaptoethanol (5 mM)	143
GSH (0.4 mM)	123
GSH (2.0 mM)	153
DTT (0.2 mM)	136
DTT (0.5 mM)	137
DTT (2.0 mM)	149
<i>p</i> -Chloromercuriphenylsulfonate (0.05 mM)	1
<i>p</i> -Chloromercuriphenylsulfonate (2.0 mM)	0
<i>N</i> -Ethylmaleimide (0.2 mM)	0
<i>N</i> -Ethylmaleimide (0.5 mM)	0

^a The assay conditions were as described in the text, except that 2-mercaptoethanol was omitted from the reaction mixture and the enzyme diluent. Assays were run with 0.36 unit/ml of Fraction VI enzyme and various concentrations of sulfhydryl-reagents. T3 DNA was used as substrate.

^b The value without sulfhydryl-reagent was set at 100%.

ethanol, DTT or GSH. In the absence of 2-mercaptoethanol in the enzyme diluent and in the reaction mixture, addition of 0.05 mM *p*-chloromercuriphenylsulfonate or 0.2 mM *N*-ethylmaleimide completely abolished the enzyme activity.

pH optimum. The pH optimal for the hydrolysis of native or denatured DNA was examined using buffer solutions ranging from pH 6.0 to 10.0 (Fig. 3). Both activities on native and denatured DNA had the same pH optimum at pH 9.5 in glycine-KOH.

FIG. 3. Effect of pH on the Activity of DNase *A*. Activity was measured in the standard assay condition with T3 [³H]DNA as substrate, except that the usual buffer, 50 mM glycine-KOH (□), was replaced by 50 mM Tris-maleate-KOH (○) or Tris-HCl (●). To the each assay mixture was added 0.36 unit/ml of Fraction VI enzyme, and the value in glycine-KOH at pH 9.5 was set at 100%.

Effect of ionic strength. The enzyme activity was inhibited at high ionic strength. The presence of 0.1 M KCl resulted in 50% inhibition and 0.2 M or higher concentration of KCl completely abolished the enzyme activity.

Effect of nucleotides. None of the nucleotides tested had significant effect on the enzyme activity assayed either with native or denatured DNA (Table III).

Stability. At a low enzyme concentration (Fraction IV, 7.1 units/ml) the enzyme was unstable in the enzyme diluent which contained 1 mg/ml bovine serum albumin; the half lives of the enzyme at 37 and 45°C were 30 and 15 min, respectively, and 30% of the activity was lost for 2 hr even at 0°C. The concentrated

TABLE III. EFFECT OF DIFFERENT NUCLEOTIDES ON THE ACTIVITY OF DNase *A*

Additions ^a	Relative activity(%) ^b
None	100
ATP (0.02 mM)	103
ATP (0.05 mM)	117
ATP (0.10 mM)	127
ATP (0.20 mM)	136
dATP (0.25 mM)	130
c-AMP (0.25 mM)	112
c-GMP (0.25 mM)	117

^a Nucleotides were added to the standard assay mixture containing 0.36 unit/ml of Fraction VI enzyme and T3 [³H]DNA as substrate.

^b The value without nucleotide was set at 100%.

Sephadex G-100 fraction (Fraction VI) had been stored without loss of activity for three month at -20°C in 0.3 M potassium phosphate (pH 7.4)-2 mM GSH-50% glycerol. Addition of glycerol (10%) in the assay mixture stabilized the enzyme, and enhanced the enzyme activity by two fold.

Determination of molecular weight by gel filtration. The molecular weight of DNase *A* was determined by Sephadex G-100 column chromatography as described by Andrews.²⁸⁾ The elution of DNase *A* and reference proteins are plotted against the logarithms of their molecular weights (Fig. 4). From this plot, the molecular weight of DNase *A* was estimated to be 35,000.

Substrate specificity and kinetics of hydrolysis. Figure 5 shows the kinetics of degradation of native and denatured T3 DNA by DNase *A*. When 0.36 unit/ml of DNase *A* was added to the reaction mixture, the rate of degradation of native T3 DNA was about 7-fold faster than that of denatured T3 DNA. In the same condition the ratio of the degradation of native *E. coli* DNA to that of denatured *E. coli* DNA was about 4 (data not shown). Degradation of native T3 DNA by DNase *A* reached a plateau at 45 min when 23% of the substrate was rendered acid-soluble, while degradation of denatured T3 DNA continued at a slow rate. At higher concentration of the enzyme (1.4 units/ml) 70% of the native T3 DNA became

FIG. 4. Determination of Molecular Weight for DNase A.

Molecular weight was determined by gel filtration on a 150 ml column of Sephadex G-100. Fraction V was dialysed against 0.2 M potassium phosphate (pH 7.4)-2 mM EDTA-2 mM GSH-10% glycerol and 0.7 ml of the dialysate was applied to the column. The column was equilibrated and developed with the same buffer as used for dialysis. The void volume was 62.5 ml and the elution volume for DNase A was 105 ml. The abbreviation and molecular weights (in parentheses) of the reference proteins are: BSA, bovine serum albumin (67,000); egg albumin (45,000); trypsin inhibitor from soybean (21,500); cytochrome C from horse heart (12,500).

FIG. 5. Time Course of Degradation of T3 [3H]DNA by DNase A.

The reaction mixture had the same composition as the standard reaction mixture, and contained 0.33 μ M of native or denatured T3 [3H]DNA and 0.36 unit/ml of Fraction VI enzyme. Incubation was made at 37°C. Samples (0.1 ml) were removed at indicated times and the radioactivity rendered acid-soluble was measured. (●), native T3 [3H]DNA; (○), denatured T3 [3H]DNA.

acid-soluble, and the degradation almost ceased in 45 min. Addition of more enzyme (1.4 units/ml) at 90 min resulted in 10% of

additional degradation (data not shown).

Mode of degradation. To elucidate the mode of degradation (endonucleolytic or exonucleolytic) of DNase A the reaction products were analyzed by gel filtration on Sephadex G-100.²⁹⁾ When native T3 DNA was degraded by pancreatic deoxyribonuclease and subjected to gel filtration, the reaction products were eluted as a broad peak between the excluded material and dTMP, and retarded on the column with increasing time of reaction (data not shown). In contrast, the products of DNase A at various stages of degradation showed two distinct peaks (Fig. 6). The peak of large molecules at the void volume decreased with reaction time. Concomitantly, the second

FIG. 6. Gel Filtration of Degradation Products of DNA by DNase A.

The reaction mixture contained 50 mM glycine-KOH (pH 9.5), 5 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, 10% glycerol, 5 mM 2-mercaptoethanol, 0.25 mM native *E. coli* [3H]DNA and 6.1 units/ml of Fraction VI enzyme. Incubation was made at 37°C and the reaction was stopped by heating for 10 min at 100°C. Total radioactivity and acid-soluble radioactivity were measured with a portion (50 μ l) each of each sample. Another portion (150 μ l) was diluted to 0.5 ml with 0.1 M NaCl-10 mM EDTA and applied to a column of Sephadex G-100 (0.8 $\times$ 21 cm). The column was developed with the same solution. The radioactivity in 1 ml fractions was measured. The position of dTMP is shown by an arrow. (○), no enzyme, no incubation, 0% acid-soluble radioactivity; (●), 30 min incubation, 30.2% acid-soluble radioactivity; (●), 180 min incubation, 45.2% acid-soluble radioactivity.

peak at the position expected for mononucleotides increased. No materials was detected between the void volume and the position of mononucleotides.

Degradation products. The products of DNase A at various stages of degradation of T3 [^3H]DNA were analyzed by thin layer chromatography on PEI-cellulose. In the solvent system employed in this experiment, mononucleotides are separated from oligonucleotides.²² Most (70%) of the radioactive products always coincided with the mononucleotide marker (dTMP), and little radioactivity was found in oligonucleotides, which were located between the origine and the mononucleotide position (Fig. 7). The distribution

FIG. 7. PEI-Cellulose Thin Layer Chromatography of DNase A Digests.

The reaction mixture had the same composition as the standard assay mixture with T3[^3H]DNA as substrate, except that the concentration of 2-mercaptoethanol was increased to 5 mM, and contained 1.4 units/ml of Fraction VI enzyme, and incubated at 37°C. Samples were removed at the indicated times and then subjected to thin layer chromatography as described under "MATERIALS AND METHODS." (A) 20 min incubation, 31.7% acid-soluble radioactivity; (B) 60 min incubation, 60.0% acid-soluble radioactivity; (C) 180 min incubation, 70.0% acid-soluble radioactivity.

of radioactivity was unaltered from the early stage of the reaction (30% acid-soluble) to the stage when the reaction was almost ceased (70% acid-soluble). These data indicate that DNase A released mononucleotides at the initial stage of degradation.

Action on terminally labeled DNA. To determine the site of exonucleolytic attack, the kinetics of degradation by DNase A of native [^3H]DNA, labeled at 5'-terminus with ^{32}P , was examined (Fig. 8). When *E. coli* [^3H ,5'- ^{32}P]DNA was degraded by the enzyme of two different concentrations, the released ^{32}P was preceded to that of the ^3H in both cases; with the enzyme concentration of 0.12 unit/ml, 48% of the ^{32}P was acid-soluble when 9% of the ^3H was acid-soluble, and of 0.36 unit/ml, 81% of the ^{32}P was acid-soluble when 13% of the ^3H was acid-soluble. A similar results was obtained when native T3 [^3H ,5'- ^{32}P] DNA was used as substrate; when the ^{32}P was 75% acid-soluble, only 0.9% of the ^3H was acid-soluble. These results indicate that the 5' end of DNA was the site of initial attack of DNase A.

FIG. 8. Degradation of [^3H ,5'- ^{32}P]DNA by DNase A.

Fraction VI enzyme in different concentration was incubated with [^3H ,5'- ^{32}P]DNA under the standard assay condition except that the concentration of 2-mercaptoethanol was increased to 5 mM. At intervals samples were removed and the acid-soluble radioactivity of ^3H and ^{32}P were measured. (○) 26 μM *E. coli* [^3H ,5'- ^{32}P]DNA, 0.12 unit/ml enzyme; (●), 31 μM *E. coli* [^3H ,5'- ^{32}P]DNA, 0.36 unit/ml enzyme; (Δ) 40 μM T3[^3H ,5'- ^{32}P]DNA, 0.24 unit/ml enzyme.

DISCUSSION

DNase A was purified about 1700-fold from the crude extract of *B. subtilis* 168TT. The enzyme showed a single peak in Sephadex G-100 chromatography and the molecular weight was estimated to be 35,000. Although the purified fraction (Fraction VI) showed three protein bands in polyacrylamide gel electrophoresis, one of the protein bands (about 50% of the protein in Fraction VI) was coincided with the enzyme activity.

The main difficulties in purification of the enzyme arose from the rapid inactivation on phosphocellulose chromatography and Sephadex G-100 gel filtration. This was due to the low level of enzyme concentration and was prevented by the rapid concentration of the enzyme fraction after each chromatography. Since another inactivation factor(s) as proteases were considered in the crude preparations of the enzyme, the purification method was designed to prevent proteolytic action during the isolation procedure; cells were grown in the medium containing 1% glucose and 1% casamino acids to prevent the production of proteases, and purification was carried out in the presence of protease inhibitor (PMSF, EDTA).

DNase A degrades native DNA preferentially, and denatured DNA with a reduced rate. The two activities resided in a single protein, since the two activities migrate simultaneously in polyacrylamide gel electrophoresis and have the same pH optimum. The enzyme degrades native T3 DNA to a limited extent, and further degradation is observed by addition of more enzyme after the reaction is almost ceased. This phenomena may be interpreted as the inactivation of the enzyme by prolonged incubation. But the high level of the enzyme concentration and prolonged incubation resulted in 80% degradation of the substrate.

DNase A is distinguished from other DNases previously reported in *B. subtilis* on the basis of the size of their products, and their requirements for metal ions and ATP. Ca^{2+} -dependent extracellular nucleases^{1~4)} and similar ac-

tivity associated with cell wall-membrane⁵⁾ fraction require Ca^{2+} for their maximal activities. DNase A is inactive in the presence of Ca^{2+} , DNase A differs from ATP-dependent DNase^{6,7)} in their response to ATP. DNase activities which are specific for ultraviolet light irradiated DNA,¹⁰⁾ γ -ray irradiated DNA¹¹⁾ and uracil-containing DNA¹²⁾ are distinct from the basis of the substrate specificity. Therefore DNase A is a novel DNase purified and characterized from *B. subtilis*.

The mode of action of the enzyme is predominantly exonucleolytic and the major products are mononucleotides. It is shown that the enzyme initiates degradation at the 5'-end of native DNA. Although the possibility that the enzyme initiates degradation at the 3'-end simultaneously at the 5'-end, these results suggest that the enzyme degrades DNA in the direction from 5'-end to 3'-end.

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