DNase Specific for Uracil-Containing Bacteriophage DNA

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A DNase from *Bacillus subtilis* which specifically hydrolyzes native DNA of phage PBS 1 has been purified and characterized. The mode of action of the enzyme is endonucleolytic, yielding deoxyuridine and oligonucleotides of various sizes. The primary site of enzymatic attack is deoxyuridylic acid in the DNA. A mild nitrous acid treatment of thymine-containing thymus DNA, which deaminates 30% of the cytosine residues, renders the DNA susceptible to the DNase. Nicked DNA from coli phage T5 and hydroxymethyluracil-containing DNA from phage PBS 15 are not sensitive to this DNase.

Our previous investigation on DNase activities in phage PBS 1-infected cells revealed the presence in *Bacillus subtilis* of a DNase specific for uracil-containing PBS 1 DNA (9). It was also reported that during the development of PBS 1 this DNase was inhibited by a protein induced by the phage (9). In the present study attempts have been made to purify the enzyme and to identify the primary site of hydrolysis.

MATERIALS AND METHODS

Culture methods, preparation of crude extracts, preparation of DNA, and analytical methods were as described previously (9).

Phage PBS 15 (hydroxyuracil replaces thymine in the DNA) was propagated with *B. subtilis* SB19E as host. Phages T4 and T5 for *Escherichia coli* were propagated on strain B in a modified M-9 medium (4).

The reaction mixtures for DNase assay contained 0.05 ml of DNA (330 μ g/ml), 0.015 ml of 0.1 M β -mercaptoethanol, 0.025 ml of 5×10^{-4} M CaCl₂, 0.1 ml of 1 M Tris-chloride (pH 8.5), aliquots of enzyme, and distilled water in a total of 0.27 ml.

The products of the enzyme reaction were examined by chromatography on DEAE-Sephadex A-25. A large amount of digestion products was obtained by hydrolyzing unlabeled PBS 1 DNA. In the reaction mixture 0.45 M Tris-chloride buffer was replaced by 0.12 M glycylglycine-KOH buffer (pH 8.3) to reduce the amount of total solute. The extent of hydrolysis in the glycylglycine buffer was the same as in 0.45 M Tris-chloride buffer. After an incubation at 37 C for 10 h, urea was added to the reaction mixture to a concentration of 7 M. After the addition of urea, the total volume became three times the original volume and the concentration of all the solutes other than urea was approximately 0.04 M. The hydrolysate was applied on DEAE-Sephadex A-25 (1 by 15 cm) previously equilibrated with 0.02 M Tris-chloride (pH

7.6) containing 7 M urea (3). After washing the column with 0.02 M Tris-chloride (pH 7.6)/7 M urea, elution was accomplished by a linear gradient of 0.14 M to 0.28 M NaCl in 0.02 M Tris-chloride/7 M urea, and finally with 1 M NaCl in the same buffer. Fractions were concentrated by adsorbing on a column of DEAE-cellulose previously equilibrated with 0.01 M NH₄HCO₃ and eluting with 1 M NH₄HCO₃. The ammonium bicarbonate was removed by evaporating in vacuo. The concentrated fractions were treated with snake venom phosphodiesterase (100 μ g/ml) at 37 C for 12 h to examine mononucleotides present in the fractions. Nucleotides in the hydrolysates were analyzed by paper chromatography with a mixture of isopropanol:hydrochloride:water (170:41:39 by volume) as solvent.

RESULTS

Purification of DNase active on native PBS 1 DNA. Frozen cells of strain SB19E were resuspended in 0.001 M potassium phosphate buffer (pH 6.8) and cell free extracts were prepared as described previously (9). The extracts were dialyzed against the potassium phosphate buffer overnight and applied on a column of hydroxylapatite-C which was previously equilibrated with the potassium phosphate buffer. The charged column was eluted successively with 0.001, 0.05, and 0.16 M potassium phosphate buffer. The DNase active on native PBS 1 DNA was found only in the fraction eluted with 0.16 M potassium phosphate buffer. Protein in this fraction was precipitated with ammonium sulfate at 60% saturation. Ammonium sulfate was removed by dialysis in 0.001 M potassium phosphate buffer (pH 6.8). The dialysate was applied on a column of DEAE-cellulose (100 ml in a column 2 cm in diameter) previously equilibrated with 0.001 M potassium phosphate buffer. The column was washed first with 120 ml of 0.001 M potassium

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phosphate buffer, then eluted with a linear gradient of NaCl (0 to 0.3 M NaCl in 0.001 M potassium phosphate buffer). The total volume of the gradient was 400 ml. Five-milliliter fractions were collected. The DNase activity on native PBS 1 DNA appeared as a broad peak, which included two separate protein peaks (Fig. 1). The active fractions were pooled and concentrated with ammonium sulfate in 60% saturation. Ammonium sulfate was removed by dialysis in 0.001 M potassium phosphate buffer.

The specific activity and recovery of the DNase during the purification steps are shown in Table 1. The final product had a specific activity 50 times higher than that of crude extracts and contained almost no nucleic acids as judged from its UV absorption spectrum.

Attempts to increase the specific activity with CM-cellulose, ECTEOLA-cellulose, Sephadex G-200, or DEAE-Sephadex A-25 were unsuccessful.

Properties of the purified DNase active on native PBS 1 DNA. All preparations except the final product from DEAE-cellulose chromatography could be stored at -5 C without any loss of activity. The purified DNase after chromatography on DEAE-cellulose was stable at 4 C, but was inactivated in the frozen state. The inactivation took place even in liquid nitrogen.

With the purified DNase the highest activity was obtained at pH 8.5. The rate of hydrolysis

was affected by the concentration of buffer used. The optimal concentration of Tris-chloride buffer for the enzyme activity was 0.5 M. As in the case of partially purified preparation in the previous report (9), the DNase required the presence of Ca^{2+} for its optimal activity (Table 2). The requirement for Ca^{2+} was specific and no DNase activity was observed with Mg^{2+} or Mn^{2+} .

The enzyme activity was strongly inhibited by potassium phosphate. The inhibition of DNase by phosphate is probably due to the removal of Ca^{2+} from the mixture by the

TABLE 1. *Purification of the DNase active on native PBS 1 DNA^a*

Purification steps	DNA degraded (mg/h per mg of protein)	Protein (mg)	Recovery (%)
Crude extract	0.67	40,000	100
Hydroxylapatite C	0.67	16,000	60
$(\text{NH}_4)_2\text{SO}_4$ (0.4 to 0.6 saturation)	6.7	1,650	41
DEAE-cellulose	19.5	30	2.2
$(\text{NH}_4)_2\text{SO}_4$ (0.6 saturation)	33.3	10	1.3

^a The DNase activity was assayed by the radioisotope method.

FIG. 1. *Elution profile of the DNase on DEAE-cellulose. The DNase was assayed by the radioisotope method. The amount of protein applied on the column (2 by 34 cm) was 600 mg.*

TABLE 2. Effect of calcium on the purified DNase^a

Concn of calcium (mM)	DNA degraded (mg/h per mg of protein)	Relative act
0	3.4	10
0.5	25.8	76
1.0	29.5	88
5.0	34.0	100
10.0	19.8	58
100.0	3.5	10

^aThe DNase was assayed by the optical method.TABLE 3. Degradation of various DNAs by the purified DNase active on native PBS 1 DNA^a

Source of DNA	DNA degraded (mg/h per mg of protein)	Relative act
<i>B. subtilis</i> (SB19E)	0	0
Calf thymus	0	0
Phage T4	0.3	1
Phage PBS 15	1.7	5
Phage PBS 1	34.0	100

^aThe DNase activity was assayed by the optical method. In this experiment 0.05 ml of DNA (470 μ g/ml) was used in a total volume of 0.27 ml of reaction mixtures.

formation of insoluble complexes. The addition of yeast RNA at concentrations up to 30 μ g/ml to the reaction mixture did not inhibit the DNase activity.

Native *B. subtilis* DNA, calf thymus DNA, T4 DNA, and PBS 15 DNA were not hydrolyzed by the purified enzyme (Table 3). Furthermore, the addition of thymus DNA to the reaction mixture had no effect on the extent of hydrolysis of native PBS 1 DNA even at a concentration three times that of the phage DNA (Table 4). Therefore, it seems that calf thymus DNA does not interact with active site(s) of this enzyme.

No RNase or nucleotidase activity was detected in the purified preparation. The purified preparation, however, showed a DNase activity on heat-denatured DNA. Heat-denatured DNA from T4, PBS 15, and *B. subtilis* were degraded by this preparation (Fig. 2). The initial rate of degradation of PBS 1 DNA was greatest among the DNAs tested. It was not clear whether the DNase activity on heat-denatured DNA was due to the presence of another enzyme. The sensitivity of the two enzyme activities (on native and denatured PBS 1 DNA) to heat was found to be different. The DNase activity on native PBS 1 DNA was completely inactivated after 30 min of incubation at 55 C (Fig. 3). On the other hand, 12% of the original activity on

denatured PBS 1 DNA was found after the same heat treatment. In addition to the difference in heat sensitivity, the DNase activity on native PBS 1 DNA was inhibited by an inhibitor induced by the phage, whereas the DNase activity on denatured DNA was not affected at all by the inhibitor (9). These observations suggest that the purified preparation obtained in the present study might still contain two enzymes: one active on native PBS 1 DNA and the other active on heat-denatured DNA.

Mode of action of the DNase active on native PBS 1 DNA. It has been shown that the PBS 1 DNA molecule contains four single-strand breaks (12), whereas T4 DNA and *B. subtilis* DNA, which are resistant to the enzyme, contain no single-strand breaks. To determine whether the specificity of this enzyme was due to the presence of single-strand breaks in the DNA, the sensitivity of *B. subtilis* DNA in which single strand breaks were artificially introduced was examined.

Single-strand breaks were introduced by

TABLE 4. Effect of thymus DNA on hydrolysis of PBS 1 DNA by the purified DNase^a

Calf thymus DNA added (μ g/ml)	DNA degraded (mg/h per mg of protein)	Relative act
0	34.0	100
30	33.6	99
60	34.6	102
170	34.4	101

^aThe DNase activity was assayed by the optical method.

FIG. 2. Degradation of denatured DNAs by the purified enzyme.

FIG. 3. Heat inactivation of the DNase activities. Heating was performed with the complete reaction mixture without substrate. The reaction was initiated by transferring the mixture to 37 C and adding the substrate to the mixture. Symbols: ●, native PBS 1 DNA; ○, denatured host DNA.

treatment with a low concentration of DNase (10). The sedimentation profiles in alkaline sucrose gradients of DNA treated with various amounts of pancreatic DNase are shown in Fig. 4. The DNA treated with pancreatic DNase sedimented in the gradients at a much slower rate than did the untreated control DNA. Since the control DNA and the DNA treated with 0.13 U of pancreatic DNase per ml sedimented at the same rate in neutral sucrose gradient (the result is not shown), the slower rate of sedimentation of the treated DNA in alkaline sucrose gradients would indicate the presence of single-strand breaks.

Single-strand breaks were also introduced by ascorbate according to Bode (2). Sedimentation in sucrose gradients was done in the same way as in the case of DNA treated with pancreatic DNase. *B. subtilis* DNA treated with ascorbate sedimented at a greatly reduced rate in alkaline sucrose gradients, indicating again the presence of single-strand breaks in the treated DNA.

B. subtilis DNA treated with 0.13 U of pancreatic DNase per ml or with 10^{-2} M ascorbate, as well as T5 DNA which contained single-strand breaks (1), were resistant to the action of the DNase (Table 5). *B. subtilis* DNA

treated with higher concentrations of pancreatic DNase or ascorbate was degraded by the DNase. Presumably higher concentrations of these agents produce not only single-strand breaks but also single-stranded regions which are susceptible to the DNase active on denatured DNA.

Another unique property of PBS 1 DNA which is absent in DNA of other sources is the replacement of thymine by uracil (7). To determine whether the strict specificity of the DNase toward native PBS 1 DNA was due to the presence of uracil, cytosine was converted to uracil in thymus DNA by nitrous acid according

FIG. 4. Sedimentation analysis of *B. subtilis* DNA treated with pancreatic DNase. The incubation mixture (1.52 ml) contained DNA from SB19E (1.5 ml of optical density of 8.6 at 260 nm in 0.01 M Tris-chloride (pH 8.0)/0.02 M NaCl, 0.01 ml of 1 M MgCl₂ and 0.01 ml of pancreatic DNase. The mixture was incubated for 30 min at room temperature. The reaction was terminated by adding 0.01 ml of 0.1 M EDTA and the mixture was dialyzed against 0.1 M NaCl. The volume of DNA layered on the top of the gradient was 0.5 ml. Sedimentation was performed in 0.02 M K₂PO₄ at pH 12.1 in a gradient of sucrose (5 to 20%). Samples were centrifuged in a Spinco SW25.1 rotor at 15 C for 24 h at 24,000 rpm.

TABLE 5. *Degradation of DNAs carrying single-strand breaks^a*

Source of DNA	Treatment	DNA degraded (mg/h per mg of protein)	Relative act
PBS 1	None	34.2	100
SB19E	Pancreatic DNase		
	0.13 U/ml	0	0
	0.66 U/ml	11.0	32
	Ascorbate		
	10 ⁻³ M	0	0
	10 ⁻¹ M	8.9	26
T5	None	1.7	5

^aThe DNase activity was determined by the optical method. The reaction mixture (1 ml) for ascorbate treatment contained 0.5 ml of 0.01 M Tris-chloride buffer (pH 7.50)-EDTA (10⁻³ M), 0.5 ml of DNA (optical density of 6.2 at 260 nm), and various amounts of ascorbic acid. The mixture was incubated for 7 h at 37 C and the reaction was terminated by dialyzing it against 0.1 M NaCl.

to the method of Shapiro and Chargaff (6). Since low pH causes depurination and backbone breakage of DNA (8), DNA treated with low pH without nitrous acid was included in the experiment as control.

Thymus DNA treated at pH 3.3 with or without nitrous acid was hydrolyzed extensively by the DNase (Table 6). Under these conditions the DNA was fragmented and could not be recovered as fibers by the addition of ethanol. On the other hand, under a milder condition (pH 4.0 for 30 h), control DNA was not degraded. Thymus DNA deaminated under this mild condition was degraded extensively (Table 6).

As nitrous acid deaminates also guanine and adenine (5), it is not clear whether the sensitivity of the nitrous acid-treated thymus DNA to the DNase is due to only the presence of uracil in the molecule. Therefore, to obtain further information on the mode of action of the DNase, the products of hydrolysis of several DNAs, untreated by nitrous acid, were examined by DEAE-Sephadex A-25 chromatography and paper chromatography. The results of the analysis are summarized in Table 7. The only nucleoside obtained was dUrd. In the mononucleotide fraction and oligonucleotide fractions up to tetranucleotide, only dGMP and dAMP were present. In the fraction of oligonucleotides larger than hexanucleotides, dAMP,

dGMP, and dCMP were present but dUMP was absent.

Calf thymus DNA treated with nitrous acid at pH 4.0 as described above was digested with the DNase. Digestion products consisted of nucleosides, mononucleotides, and oligonucleotides (Table 8). In the nucleoside fraction dUrd was the only compound detected. The mononucleotide fraction consisted of dAMP and dGMP. The combined fractions of tri- and tetranucleotides consisted of dAMP, dGMP, and dIMP. The pentanucleotide fraction and oligonucleotides larger than hexanucleotides consisted of dAMP, dGMP, dTMP, and dCMP. As in the case of digestion products of native PBS 1 DNA, the only nucleoside obtained was dUrd and no nucleotides containing uracil were found. These results suggest that dUMP in the DNA is probably the primary site of hydrolysis by this enzyme. However, it is not clear from the present data whether dUrd is formed by the action of contaminating phosphatases or other nucleases. Preliminary results showed that our

TABLE 6. *Degradation of nitrous acid-treated calf thymus DNA^a*

Treatment	Cytosine converted (%)	Total content of uracil (mol%)	DNA degraded (mg/h per mg of protein)	Relative act
72 h at pH 3.3 with nitrous acid	75	15.4	26.2	77
Without nitrous acid	0	0	26.8	79
30 h at pH 4.0 with nitrous acid	30	6.2	27.2	80
Without nitrous acid	0	0	0	0
Control PBS 1 DNA		36.0	34.0	100

^aThe treatment of DNA with nitrous acid was as follows: 135 mg of NaNO₂ was added to a solution of 5 mg of calf thymus DNA in 2.5 ml of distilled water. The pH of the mixture was adjusted to an appropriate value by glacial acetic acid. The mixture was incubated at 37 C for 30 or 72 h in a securely closed test tube. The treated DNA was dialyzed against 1 × SSC (0.15 M NaCl and 0.015 M sodium citrate) and finally against 0.1 M NaCl. The DNase activity was assayed by the optical method. The amount of cytosine converted was determined by paper chromatography of the treated DNA after complete hydrolysis by the mixture of pancreatic DNase (100 µg/ml) and snake venom phosphodiesterase (100 µg/ml).

TABLE 7. Analysis of digestion products

Substrate	Fraction	Content of various bases (mol %) ^a				
		A	G	C	T	U
Native PBS 1 DNA	Nucleosides	0	0	0		100
	Mononucleotides	89	11	0		0
	Dinucleotides	70	30	0		0
	Trinucleotides	72	28	0		0
	Tetranucleotides	81	19	0		0
	Larger than hexanucleotides	53	19	28		0
Denatured PBS 1 DNA	Nucleosides	0	0	0		100
	Mononucleotides	89	11	0		0
	Dinucleotides	74	26	0		0
	Trinucleotides	73	27	0		0
	Tetranucleotides	80	20	0		0
	Larger than hexanucleotides	44	25	31		0
Denatured host DNA	Nucleosides	0	0	0	0	
	Mononucleotides	91	9	0	0	
	Dinucleotides	53	47	0	0	
	Trinucleotides	47	53	0	0	
	Tetranucleotides	61	49	0	0	
	Larger than hexanucleotides	26	18	26	30	

^a A, dAMP; G, dGMP; C, dCMP; T, dTMP; U, dUrd.

TABLE 8. Digestion products from nitrous acid-treated thymus DNA

Fraction	Content of various bases (mol%) ^a					
	A	G	C	T	I	U
Nucleosides	0	0	0	0	0	100
Mononucleotides	65	35	0	0	0	0
Dinucleotides	19	81	0	0	0	0
Trinucleotides	20	18	0	0	62	0
Tetranucleotides	20	18	0	0	62	0
Pentanucleotides	9	38	8	45	0	0
Larger than hexanucleotides	27	42	7	24	0	0

^a A, dAMP; G, dGMP; C, dCMP; T, dTMP; I, dIMP; U, dUrd.

DNase preparation was free from phosphatase activities.

As described in the previous section, the purified DNase still contained a DNase active on heat-denatured DNA. To investigate the mode of action of the DNase active on heat-denatured DNA, digestion products of heat-denatured PBS 1 and host DNA were examined by the same technique. The digestion products of heat-denatured PBS 1 DNA were very similar

to those of native PBS 1 DNA (Table 7).

In the hydrolysate of denatured *B. subtilis* DNA, mononucleotides and oligonucleotides smaller than hexanucleotides were found to contain only dAMP and dGMP. Nucleosides were not detectable. Oligonucleotides larger than hexanucleotides contained dAMP, dGMP, dTMP, and dCMP (Table 7).

DISCUSSION

It has been known that certain DNases show affinity toward specific substrates such as alkylated DNA, UV-irradiated DNA, etc. The present data show that the single-strand interruptions present in PBS 1 DNA are not the initial sites of hydrolysis of the DNase specific for PBS 1 DNA. The observation that deaminated thymus DNA in which 30% of cytosine residues are converted to uracil is fully susceptible to the DNase suggests that the primary site of hydrolysis may be dUMP in the DNA. Analysis of the products of hydrolysis presented in this paper also supports the above conclusion.

According to Wovcha and Warner (11), a DNase activity associated with DNA polymerase I of *E. coli* hydrolyzes dUMP-containing portions of repaired DNA faster than that

containing dTMP. Our enzyme isolated from *B. subtilis* seems to be distinct from the DNase associated with DNA polymerase of *E. coli*, since the *B. subtilis* enzyme does not hydrolize dTMP-containing DNA at all and has an optimal pH and a divalent cation requirement which are different from those of the *E. coli* enzyme. Nevertheless the function of the *B. subtilis* enzyme may be similar to that of the *E. coli* enzyme, as both the DNases may eliminate segments of DNA which contain dUMP instead of dTMP in these cells. In PBS 1-infected cells, on the other hand, an inhibitor induced by the phage interacts with the DNase (9) so that the synthesis of uracil-containing phage DNA may proceed.

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