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Purification and Properties of a Neutral Endodeoxyribonuclease from Rat Small Intestinal Mucosa†

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ABSTRACT: An endodeoxyribonuclease has been purified to near homogeneity from rat small intestinal mucosa by a procedure involving Con A–Sepharose affinity chromatography. During the initial steps of purification, the presence of 5 mM $CaCl_2$ was essential for stability of the enzyme activity. The enzyme has a molecular weight of 32 000 and an isoelectric point of 4.7. NaCl, sulfhydryl reagents, and iodoacetate strongly inhibited the reaction, but tRNA did not. The enzyme required divalent cations for activity and had a pH optimum of pH 6.2 with Co^{2+} and pH 7.7 with Mn^{2+}. In both optimum conditions, the enzyme hydrolyzed native DNA more rapidly than denatured DNA, and the average chain lengths of limit digestion products of native and denatured DNA were 8 and 10, respectively, at pH 6.2 and 9 and 11, respectively, at pH 7.7. The enzyme activity to produce acid-soluble fractions from linear DNA substrate was similar in the two optimum conditions, but the activity to nick double-stranded, superhelical circular DNA substrate was significantly higher at pH 6.2 than at pH 7.7. The endonuclease formed single-strand breaks making 5′-phosphoryl and 3′-hydroxyl termini, and deoxythymidine was present at the 5′ termini with a frequency of about 50% in both optimum conditions. Bovine pancreatic DNase I antibody and G-actin inhibited the enzyme activity. Thus this endonuclease is classified as a DNase I.

Pancreatic DNase[1] I, which was first crystallized by Kunitz (1950), is one of the two representative mammalian DNases and has been widely investigated (Laskowski, 1967). Similar activity was found in guinea pig epidermis (Tabachinick, 1964), nuclei of liver cells (Ishida et al., 1974), and other organs (Tanigawa et al., 1974), but the activity does not seem so widely distributed as that of DNase II. The presence of relatively high DNase I like activity in the small intestinal mucosa of rats has been reported (Lee et al., 1972), but the structural identity of the enzyme responsible for this activity with pancreatic DNase I has not been established.

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[1] Abbreviations: DNase, deoxyribonuclease; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

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In studies on the problems of the structural identity and the physiological functions of the DNase I like activity in the small intestinal mucosa of rats, we purified the DNase to near homogeneity from rat small intestinal mucosa by a procedure involving Con A–Sepharose affinity chromatography. We could stabilize the enzyme activity by adding 5 mM $CaCl_2$. The purified enzyme is similar in many characteristics to bovine pancreatic DNase I. In addition, we found two interesting properties of this enzyme: (a) it has two pH optima dependent on Co^{2+} and Mn^{2+}, respectively, and (b) it has marked differences in activity on duplex, circular DNA at the two pH optima.

Experimental Procedures

Materials. ^{32}P-Labeled *Escherichia coli* DNA was prepared as described previously (Anai et al., 1970). pBR322 DNA was a gift from Dr. Y. Sakaki. [γ-^{32}P]ATP was purchased from the Radiochemical Centre, Amersham. Bovine pancreatic DNase I and snake venom and calf spleen phosphodiesterases were products of Sigma. Porcine spleen DNase II and alkaline phosphatase from *E. coli* (BAPF) were from Worthington. Polynucleotide kinase was from Boehringer Mannheim, Yamanouchi, Tokyo. *Eco*RI restriction endonuclease was from Takara Biochemicals, Kyoto. DEAE-cellulose (DE32) and phosphocellulose (P11) were purchased from Whatman. Con A–Sepharose 4B, Sephadex G-100, and DEAE-Sephadex A-25 were obtained from Pharmacia. Bovine serum albumin and egg albumin (2× crystallized) were obtained from Armour and ICN Pharmaceuticals, respectively. Cytochrome *c* and *E. coli* tRNA were products of Sigma. G-Actin was prepared from an acetone powder of rabbit muscle by the method of Spudich & Watt (1971), and the optical density at 280 nm of the solution used was 2.09. Bovine pancreatic DNase I antibody was a gift from Dr. A. Funakoshi. Dextran sulfate (M_r 15 000) was from Nakarai Chemicals, Kyoto, carrier ampholite (pH 4–6) from LKB, and EGTA from Wako Chemicals, Osaka. All other chemicals were the highest grade available commercially and were used without further purification.

Preparation of Crude Extract. Twenty male Wistar strain rats weighing about 300 g were used in each experiment. The animals were killed by a blow on the head, and their small intestine was rapidly removed. To avoid contamination with adherent pancreatic tissue, we discarded the first 10 cm from the gastroduodenal junction. Residual fecal and luminal material was pressed out gently, and the intestine was then promptly placed in ice-cold 0.5% NaCl–0.5% KCl, washed briefly, cut into about 10-cm lengths, and opened longitudinally. These sections were washed 3 times with Krebs–Ringer phosphate buffer, pH 7.4, containing 6% dextran sulfate to remove mucin and food residue. The intestinal mucosa was separated from the muscular layer by scraping the luminal surface with the edge of a microscope slide 3 times, as described by Lieberman et al. (1971).

Intestinal mucosa was suspended in 5 volumes of Krebs–Ringer phosphate buffer, pH 7.4, and centrifuged at 3000*g* for 10 min. The resulting pellet was suspended in 2 volumes of 20 mM Tris-HCl buffer, pH 7.5, homogenized in a Waring blender first at low speed for 2 min and then at high speed for 1 min, and centrifuged at 12000*g* for 10 min. The supernatant was saved, and the pellet was suspended in 2 volumes of the same buffer, homogenized, and centrifuged as above. The two supernatants were combined and passed through four layers of gauze. This supernatant was frozen overnight at −30 °C, then thawed, and centrifuged at 12000*g* for 10 min. The resultant precipitate was discarded, and the supernatant was centrifuged again at 70000*g* for 10 min. The final supernatant fraction so obtained was designated as crude extract (305 mL) and stored at −30 °C until use.

Assay of DNase Activity. Because the enzyme exhibits two pH optima, reactions were carried out under two standard conditions. One reaction mixture (0.3 mL) contained 25 mM cacodylate hydrochloride buffer, pH 6.2, 3 mM $CoCl_2$, 10 μg of bovine serum albumin, 3 nmol of native *E. coli* [^{32}P]DNA, and 0.045 unit of enzyme. The other contained 25 mM Tris-HCl buffer, pH 7.7, 1 mM $MnCl_2$, 10 μg of bovine serum albumin, 3 nmol of native DNA, and 0.045 unit of enzyme. After incubation at 37 °C for 10 min, the reaction was terminated by adding 0.2 mL of 5 mg/mL egg albumin and 0.5 mL of 0.5 N perchloric acid. The radioactivity of the supernatant was counted as described previously (Anai et al., 1979). The production of acid-soluble radioactivity from *E. coli* [^{32}P]DNA up to 40% of input DNA was proportional both to the amount of enzyme added and to the time of incubation. The blank count was less than 1% of the total count. One unit of enzyme was defined as the activity that converted 1 nmol of DNA to an acid-soluble form at 37 °C in 1 min under these conditions. Bovine pancreatic DNase I activity was measured by the production of acid-soluble nucleotides from [^{32}P]DNA in a reaction mixture (0.3 mL) containing 10 mM Tris-HCl buffer, pH 8.0, 1 mM $MgCl_2$, 3 nmol of *E. coli* [^{32}P]DNA, and 0.3 μg of enzyme. For assay of porcine spleen DNase II, the reaction mixture (0.3 mL) contained 100 mM sodium acetate buffer, pH 4.6, 10 mM EDTA, 3 nmol of *E. coli* [^{32}P]DNA, and an enzyme sample. After incubation at 37 °C, acid-soluble radioactivity was determined as described above.

Isoelectric Focusing. The procedures were carried out according to the LKB instruction manual (LKB 8100 Ampholine electrofocusing equipment instruction manual, LKB-Produkter AB, Sweden) with a 110-mL column using pH 4–6 carrier ampholite and a 0–50% sucrose gradient containing 10% ethylene glycol. Electrofocusing was carried out at 450 V for 45 h at 4 °C.

Gel Electrophoresis. Polyacrylamide gel electrophoresis was carried out at 4 °C in 7.5% polyacrylamide gel at 2 mA/gel with 0.38 M glycine–0.05 M Tris-HCl buffer, pH 8.3, for 1.5 h. After electrophoresis, one of the gels was stained and destained as described (Reisfeld et al., 1962). The other was sliced into 1.5-mm sections, which were eluted overnight with 0.1 mL of 20 mM Tris-HCl buffer, pH 7.2, containing 5 mM $CaCl_2$ and 10% ethylene glycol, and the extracts were assayed for endonuclease activity. One percent agarose slab gel electrophoresis was run at 50 V for 18 h at room temperature by using pBR322 DNA as a substrate as described previously (Nakayama et al., 1981).

5'-Terminal Nucleotide Analysis. The analysis was performed by the method of Weiss et al. (1968a), and ascending paper chromatography was carried out as described previously (Nakayama et al., 1981).

Other Methods. The molecular weight of endonuclease was determined with porcine spleen DNase II as a reference protein in a 5–20% sucrose density gradient in 50 mM Tris-HCl buffer, pH 7.5, at 45 000 rpm for 18 h at 4 °C in a Hitachi RPS50 rotor. Fractions collected from the bottom of the gradient were assayed for endonuclease and porcine spleen DNase II activities. The molecular weight was also measured by gel filtration on Sephadex G-100 with marker proteins. The molecular weights of the marker proteins bovine serum albumin, egg albumin, porcine spleen DNase II, and cytochrome *c* were taken as 67 000, 45 000, 38 000, and 12 400, respectively.

Table I: Purification of Neutral DNase from Rat Intestinal Mucosa[a]

fraction	total act.[a] (units × 10^{-3})	total protein (mg)	sp act. (units/mg)	yield (%)
I, crude extract	18.9	445	42.5	100
II, DEAE-cellulose	14.8	120	123	78
III, phosphocellulose	15.2	8.67	1750	80
IV, Sephadex G-100	9.20	1.05	8760	49
V, Con A-Sepharose	1.31	0.092	14200	7.0

[a] Enzyme activity was measured under standard assay conditions with 1 μL of each fraction for 5 min as described under Experimental Procedures.

Products were analyzed on DEAE-Sephadex A-25 in the presence of 7 M urea as described previously (Anai et al., 1970). The protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Results

Enzyme Purification. All operations were conducted at 0–4 °C unless otherwise noted. The purification procedures and results of a typical preparation are summarized in Table I.

Treatment of Crude Extract with Diisopropyl Fluorophosphate. Crude extract (100 mL) obtained as described under Experimental Procedures was stirred with 5 mg of diisopropyl fluorophosphate for 30 min and then dialyzed against 20 volumes of buffer A (20 mM Tris-HCl buffer, pH 8.0, 5 mM $CaCl_2$, and 10% ethylene glycol) for 16 h. The resultant precipitate was removed by centrifugation at 10000*g* for 10 min (78 mL, fraction I).

DEAE-cellulose Chromatography. Fraction I was applied to a DEAE-cellulose column (2.5 × 25 cm) previously equilibrated with buffer A. The column was washed with 400 mL of buffer A, and then DNase activity was eluted with 500 mL of a linear gradient of 0–0.4 M KCl in buffer A. The flow rate was 30 mL/h, and 15-mL fractions were collected. Active fractions eluted with between 0.1 and 0.2 M KCl were pooled (165 mL, fraction II).

Phosphocellulose Chromatography. Fraction II was dialyzed overnight against 10 volumes of buffer B (20 mM acetate buffer, pH 4.7, 5 mM $CaCl_2$, and 10% ethylene glycol) containing 0.075 M KCl and then applied to a phosphocellulose column (2.0 × 20 cm) previously equilibrated with the same buffer, and DNase activity was eluted with 400 mL of a linear gradient of 0.075–0.6 M KCl in buffer B. The flow rate was 20 mL/h, and 10-mL fractions were collected. The peak fractions of DNase activity eluted with between 0.35 and 0.45 M KCl were pooled (100 mL, fraction III).

Sephadex G-100 Filtration. Fraction III was reduced in volume to 2.8 mL by dialysis against poly(ethylene glycol) in buffer C (20 mM Tris-HCl buffer, pH 7.2, 5 mM $CaCl_2$, and 10% ethylene glycol). The sample was applied to a Sephadex G-100 column (2.5 × 90 cm) previously washed with buffer C and eluted at a flow rate of 20 mL/h. Fractions of 5 mL were collected, and those with activity were pooled (65 mL, fraction IV).

Con A–Sepharose Affinity Chromatography. Fraction IV was loaded onto a Con A–Sepharose 4B column (2 mL of bed volume). The column was washed with 150 mL of buffer C, and then the material was eluted with 150 mL of buffer C containing 20% methyl α-D-glucoside. The flow rate for sample application, washing the column, and elution was 4 mL/h, and 5-mL fractions were collected. Fractions with activity emerged at the start of elution were pooled (Figure 1) and dialyzed overnight against 20 volumes of buffer C to remove the glucoside. The preparation was concentrated by dialysis against poly(ethylene glycol) in buffer C and finally dialyzed against 20 mM Tris-HCl buffer, pH 7.5, containing 30% glycerol for 16 h (0.55 mL, fraction V). Unless otherwise noted, this enzyme fraction was used for further experiments.

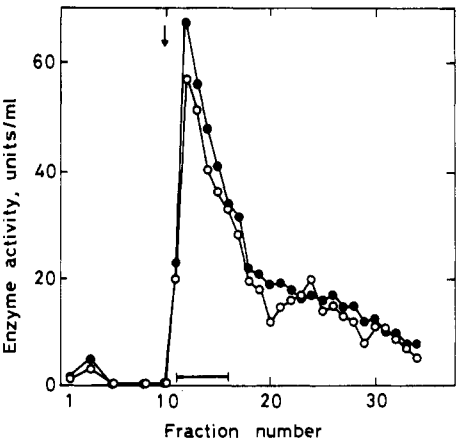

FIGURE 1: Affinity chromatography on Con A–Sepharose. Each fraction (1 μL) was assayed in standard conditions as described under Experimental Procedures. The arrow indicates the start of elution with 20% methyl α-D-glucoside in buffer C. The bar indicates pooled active fractions; (○) enzyme activity at pH 6.2; (●) enzyme activity at pH 7.7.

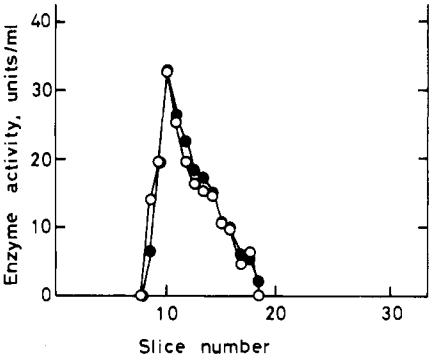

FIGURE 2: Polyacrylamide gel electrophoresis of endonuclease. Fraction V, 30 μg, was layered on a 7.5% gel column (0.6 × 7.5 cm) with 10 μL of 0.1% bromophenol blue. The procedure and conditions were as described under Experimental Procedures. Aliquots (30 μL) from the eluate of each gel slice were assayed for enzyme activity under standard assay conditions; (○) enzyme activity at pH 6.2; (●) enzyme activity at pH 7.7.

Purity of Enzyme. Upon gel electrophoresis of fraction V on a 7.5% polyacrylamide gel (pH 8.3), a clear major band and a faint band were seen (Figure 2). At each pH optimum, the peak of enzyme activity coincided with the major band, but about 60% of the maximum activity was observed at the faint band.

Molecular Weight of Enzyme. The molecular weight of the purified enzyme was estimated to be about 32000 by centrifugation in a 5–20% sucrose density gradient and by Sephadex G-100 gel filtration as described under Experimental Procedures, assuming that the enzyme is a globular protein.

Isoelectric Point, K_m Value, and Optimum Temperature of Enzyme. After electrofocusing as described under Experimental Procedures, 2-mL fractions were collected, and the pH and DNase activity at the two pH optima were determined. The isoelectric point of the purified enzyme was 4.7 ± 0.1 at the respective pH optima (Figure 3). The K_m of the DNase for duplex DNA was 2.4×10^{-9} M at pH 6.2, and the optimum temperature was 35–40 °C, when the reaction was assayed for 10 min.

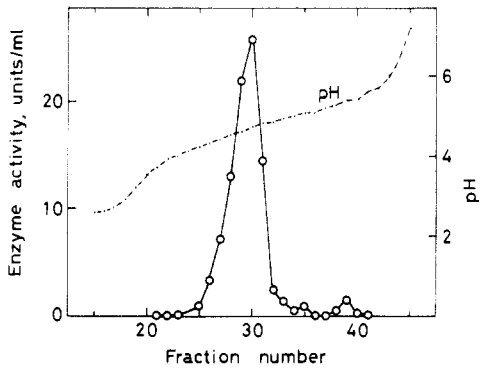

FIGURE 3: Isoelectric focusing of endonuclease. Electrofocusing was performed as described under Experimental Procedures. Fraction V (1300 units), previously dialyzed overnight against 500 mL of 1% glycine containing 10% ethylene glycol, was mixed with the dense gradient solution. After focusing, 2-mL fractions were collected from the bottom of the column. Five microliters of each fraction was assayed for DNase activity as described under Experimental Procedures. The recovery of DNase activity was 13%. Only fractions 15–45 are shown, because no DNase activity was observed in other fractions; (○) DNase activity.

Enzyme Stability. The enzyme activity of the crude extract showed no loss of activity when stored at −30 °C, but overnight storage of the fraction at 0 °C resulted in complete loss of activity. Additions of ethylene glycol and $CaCl_2$ increased the stability of the crude extract. Thus, in the presence of 10% ethylene glycol, the enzyme lost 80% of its original activity during overnight storage at 0 °C and complete activity during storage for 3 days. Additions of 10% ethylene glycol and 5 mM $CaCl_2$ preserved the activity completely for at least 15 days.

The purified enzyme (fraction V) showed no loss of activity for at least 6 months when stored in 20 mM Tris-HCl buffer, pH 7.5, containing 30% glycerol at −20 °C. Incubation of the purified enzyme at 50 °C for 30 min and at 100 °C for 5 min resulted in loss of 30% and 100%, respectively, of its activity.

For removal of Ca^{2+} from the enzyme fraction, the purified enzyme (fraction V) was dialyzed overnight against 20 mM Tris-HCl buffer, pH 7.5, containing 10 mM EGTA and 10% ethylene glycol and then passed through a Sephadex G-25 column previously washed with 20 mM Tris-HCl buffer, pH 7.5, containing 10% ethylene glycol. The enzyme sample, freed from Ca^{2+} and EGTA, lost 35% of its activity on storage at 0 °C for 4 days, but when 5 mM $CaCl_2$ was added, no loss of activity was observed. With and without 5 mM $CaCl_2$, the enzyme lost 25% and 75%, respectively, of its activity when incubated at 50 °C for 30 min. When Co^{2+} (3 mM), Mg^{2+} (5 mM), and Mn^{2+} (1 mM) were added to the enzyme fraction instead of Ca^{2+}, the activity decreased to 90%, 65%, and 60%, respectively, of the initial activity on storage at 0 °C for 4 days and to 45%, 70%, and 50%, respectively, on incubation at 50 °C for 30 min.

Cofactor-Dependent pH Optima. The enzyme required a divalent cation for reaction and showed two pH optima, which were dependent on the divalent cation added. The optimum was pH 6.2 in 25 mM cacodylate hydrochloride buffer in the presence of 3 mM Co^{2+} and pH 7.7 in 25 mM Tris-HCl buffer in the presence of 1 mM Mn^{2+}. The maximum activity obtained at pH 6.2 was about 95% of that at pH 7.7. The enzyme showed similar pH optima in the other buffers tested (Figure 4).

Metal Ion Requirements. Divalent cations were essential for the enzyme activity. Addition of 5 mM EDTA instead of metal ions resulted in complete loss of enzyme activity. In cacodylate hydrochloride buffer, pH 6.2, the maximum activity

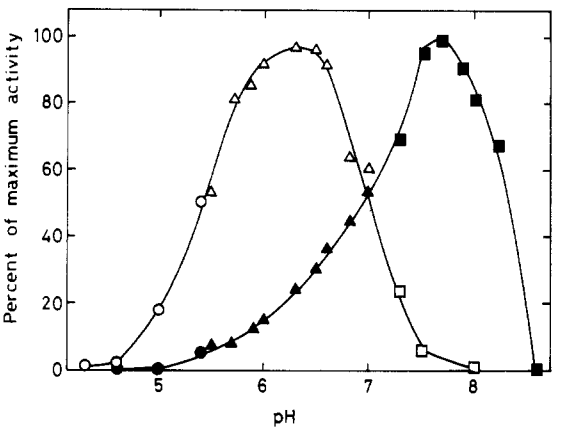

FIGURE 4: Cofactor-dependent pH optima. The reactions were performed for 10 min with 0.045 unit of enzyme in the presence of 3 mM Co^{2+} (open symbols) or 1 mM Mn^{2+} (closed symbols) with the following buffers at 25 mM: pH 4.3–5.4, sodium acetate buffer (○); pH 5.5–7.0, cacodylate hydrochloride buffer (△); pH 7.3–8.9, Tris-HCl buffer (□).

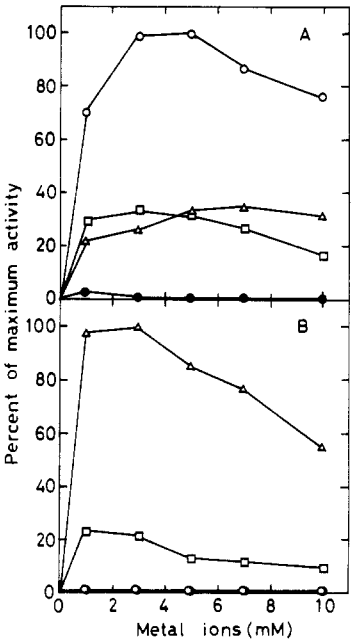

FIGURE 5: Metal ion requirements. Reactions were run in standard assay conditions with the various concentrations of metal ions. (A) pH 6.2; (B) pH 7.7. (○) Co^{2+}; (□) Mg^{2+}; (△) Mn^{2+}; (●) Ca^{2+}.

was obtained with Co^{2+} (3–5 mM). When Co^{2+} was replaced by Mn^{2+} (5–7 mM), Mg^{2+} (3–5 mM), or Ca^{2+} (1 mM), 35%, 33%, or 3%, respectively, of the maximum activity was obtained. In Tris-HCl buffer, pH 7.7, maximum activity was obtained with Mn^{2+} (1–3 mM). With Mg^{2+} (1–3 mM), about 22% of the maximum activity was obtained, but no activity was observed with Co^{2+} or Ca^{2+} (1–10 mM) (Figure 5). Ca^{2+} and Co^{2+} or Mn^{2+} did not have synergistic effects, while the addition of Ca^{2+} (1 mM) with Mg^{2+} (5 mM) at pH 6.2 or Mg^{2+} (3 mM) at pH 7.7 increased the activity to 200% of that obtained in the absence of Ca^{2+}. No activity was detected with Zn^{2+} or Cu^{2+} at both pH optima.

Effects of Reagents. The effects of several reagents on the purified enzyme were tested at the two pH optima under standard assay conditions (Table II). Endonuclease activity was inhibited by salt at both pH optima. Activity was lost completely with excess EDTA over metal ions at both pH values. 2-Mercaptoethanol inhibited the activity, but treatment with 2-mercaptoethanol in the presence of 3 mM $CaCl_2$ did not inactivate the enzyme at either pH optimum in the

Table II: Effects of Reagents[a]

addition	concn (mM)	remaining act. (%) pH 6.2	pH 7.7
none		95	100
NaCl	50	67	70
	100	38	38
KCl	50	73	73
	100	55	60
NH_4Cl	50	80	81
	100	60	58
EDTA	1	80	20
	5	0	0
2-mercaptoethanol	2	95	91
	10	35	12
iodoacetate	8	74	59
	16	25	10

[a] Standard assay conditions were used as described under Experimental Procedures with the additions as indicated.

presence of 5 mM Mg^{2+}. *N*-Ethylmaleimide stimulated the activity about 10–20% at both pH optima. Iodoacetate inhibited the activity at each pH optimum. *E. coli* tRNA had no effect on the DNase activity at the two pH optima. Addition of 10 μg of bovine serum albumin stimulated the activity about 50% at both pH values. Bovine pancreatic DNase I antibody (15 μg) inhibited the activity 45% at pH 6.2 and 40% at pH 7.7. The reaction was also inhibited 50% by 12 μL of G-actin (A_{280} = 2.09) at pH 6.2 and by 5 μL at pH 7.7, whereas bovine pancreatic DNase I was inhibited 90% by 15 μg of antibody and 50% by 4 μL of G-actin (A_{280} = 2.09).

Effect of the Secondary Structure of DNA Substrate on Enzyme Activity. The enzyme activities with native and denatured *E. coli* DNA were determined under each optimum condition, and the average chain length of the products was also estimated. At pH 6.2, native DNA was degraded 1.5 times faster than denatured DNA, and hydrolysis of DNA proceeded until about 80% of both DNA substrates had been converted to an acid-soluble form; the average chain lengths of the products of native and denatured DNA were 8 and 10, respectively. At pH 7.7, the enzyme hydrolyzed native DNA twice as fast as denatured DNA, and the extents of hydrolysis of native and denatured DNA were 70%; the average chain lengths of the products of native and denatured DNA were 9 and 11, respectively.

The products of extensive digestion of native and denatured DNA with the enzyme were analyzed by DEAE-Sephadex A-25 chromatography as described under Experimental Procedures. Very little mono-, di-, or trinucleotide was formed, and more than 90% of the products was larger than pentanucleotides at both pH optima.

Identification of the Terminal Phosphate Groups of Digestion Products. Denatured DNA after limit digestion by the enzyme was incubated with or without alkaline phosphatase and then treated with calf spleen or snake venom phosphodiesterase. The limit digests were sensitive to the venom enzyme but not to the spleen enzyme without prior treatment with alkaline phosphatase, while the products were sensitive to both phosphodiesterases after pretreatment with alkaline phosphatase (data not shown). These results indicate that the endonuclease produces oligonucleotides bearing 5′-phosphoryl and 3′-hydroxyl termini.

Identification of 5′-Terminal Nucleotides of Digestion Products. For determination of whether the endonuclease has any base specificity in the degradation of DNA, native *E. coli* DNA treated with the enzyme was labeled with [γ-^{32}P]ATP at the 5′ termini by polynucleotide kinase after treatment with alkaline phosphatase. Then the products were digested first with pancreatic DNase I and then with snake venom phosphodiesterase, and 5′-mononucleotides were identified by ascending paper chromatography as described under Experimental Procedures. Although all four nucleotides were present in the 5′ termini, about half the 5′ termini was dTMP (Table III).

Table III: Identification of 5′-Terminal Nucleotides of Digestion Products[a]

5′-deoxymononucleotide	^{32}P-labeled radioactivity pH 6.2 cpm × 10^{-2}	%	pH 7.7 cpm × 10^{-2}	%
dAMP	19.0	17.7	40.9	17.5
dGMP	19.7	18.3	41.4	17.8
dCMP	19.7	18.3	40.3	17.3
dTMP	49.2	45.7	110.6	47.4

[a] *E. coli* DNA was partially digested with the endonuclease under standard assay conditions (15% acid soluble), and 5′-terminal nucleotides were identified as described under Experimental Procedures. Controls without the enzyme were run in parallel, and their values were subtracted. Experiments were not done simultaneously under the two optimum conditions.

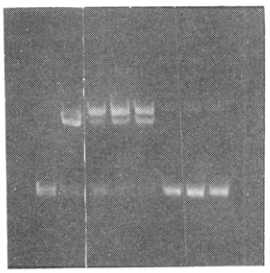

FIGURE 6: Agarose gel electrophoresis of pBR322 DNA treated with endonuclease under the two optimum conditions. pBR322 DNA (form I; 0.8 nmol) was incubated with 1.5 × 10^{-3} unit of endonuclease at pH 6.2 and 4.5 × 10^{-3} unit at pH 7.7 for the indicated times. Each digest was analyzed on 1% agarose slab gel (15 × 25 cm) as described under Experimental Procedures, and pBR322 DNA treated with *Eco*RI was used as a marker. (Lane 1) No enzyme treatment; (lane 2) treatment with *Eco*RI; (lanes 3–5) treatment at pH 6.2; (lanes 6–8) treatment at pH 7.7 [(lanes 3 and 6) 1-min digest; (lanes 4 and 7) 5-min digest; (lanes 5 and 8) 10-min digest].

Mode of Action of Enzyme. pBR322 DNA (form I) was digested with a small amount of the endonuclease for a short time, and the products were subjected to agarose slab gel electrophoresis as described under Experimental Procedures. The initial products generated by the endonuclease were a mixture of an open circular DNA (form II) and a unit length of DNA (form III) at pH 6.2, but even when 3-fold amounts of enzyme at pH 6.2 were added, relatively little of form II and very little of form III were produced at pH 7.7 (Figure 6). These results indicate that the endonuclease makes single-strand breaks in double-stranded DNA at both pH optima and that it acts more rapidly on circular duplex DNA at pH 6.2.

Discussion

We purified a neutral DNase from rat small intestinal mucosa to near homogeneity to clarify its properties and functions. The results of polyacrylamide gel electrophoresis of fraction V revealed that the purified enzyme fraction contained two forms of enzyme activity. Failure to separate the two forms by isoelectric focusing and during all the purification procedures suggests that they have very similar properties.

Multiple forms of bovine and ovine pancreatic DNases have also been reported (Salnikow et al., 1970; Wadano et al., 1979). Our results show that the enzyme is nearly identical with bovine pancreatic DNase I in physical and enzymatic properties but has several interesting characters.

(a) The crude enzyme was stable without addition of $CaCl_2$ when stored at −30 °C, but its activity was rapidly lost in the absence of 5 mM $CaCl_2$ during the early purification steps, although it was stable without adding $CaCl_2$ during later steps. Although the purified enzyme was quite stable without added $CaCl_2$, on treatment with EGTA it showed decreased stability. Addition of $CaCl_2$ to the enzyme that had been treated with EGTA restored its stability, whereas addition of Co^{2+}, Mn^{2+}, or Mg^{2+} had low effect. These results suggest that Ca^{2+} binds to the enzyme tightly and stabilizes its activity.

(b) The pH optima of our enzyme were at 6.2 and 7.7 in the presence of Co^{2+} and Mn^{2+}, respectively. Similar phenomena were observed with bovine DNase I and DNase I of rat pancreatic juice (N. Eshima and M. Anai, unpublished results). Junowicz & Spencer (1973) reported that Mg^{2+}, Mn^{2+}, and Co^{2+} were the most effective activators of bovine DNase I and that with Mg^{2+} or Co^{2+} the enzyme activity decreased above pH 7.0–7.5 whereas with Mn^{2+} the activity increased at about pH 8.0. Thus their observations are similar to ours. Dual pH optima dependent on other cofactors, such as Mg^{2+} and Mg^{2+} plus Ca^{2+}, were reported for bovine DNase I (Price, 1975) and human and pancreatic DNases (Love & Hewitt, 1979). Synergistic effects of Ca^{2+} and Co^{2+} or Mn^{2+} were observed on bovine DNase I (Price, 1975) but not on our enzyme.

(c) Analysis of 5′-end nucleotides of partial digestion products showed that about 50% of the 5′ termini was dT and that the other three nucleotides were present with equal frequency, indicating that the enzyme preferentially introduced breaks on the 5′ side of dT. There are several reports of the preferential cleavage of bovine DNase I on the 5′ side of dT (Scheffler et al., 1968; Ehrlich et al., 1973; Weiss et al., 1968b). Thus our enzyme has a quite similar preference in the cleaving site of DNA substrate to bovine DNase I.

(d) Campbell & Jackson (1980) showed that bovine DNase I generated exclusively form II DNA as the initial product of the reaction in the presence of Mg^{2+}, but a mixture of form II and form III DNA in the presence of Mn^{2+} or Co^{2+}. Our enzyme made single-strand breaks under both optimum conditions; the activity to produce acid-soluble fractions from linear duplex DNA substrate was similar at both pH optima, but the activity to incise double-stranded, superhelical circular DNA substrate was severalfold higher at pH 6.2 than at pH 7.7. This seems to be an interesting property of the enzyme. Detailed analysis of this phenomenon is in progress.

This mode of action and the facts that the average chain length of limit digestion products was larger than 8 and that about 90% of the products were larger than pentanucleotides differ from the results on bovine DNase I. But the molecular weight (M_r 32 000), isoelectric point (4.7), production of 5′-phosphoryl and 3′-hydroxyl termini, and effects of various reagents are identical with the properties of bovine DNase I. Bovine pancreatic DNase I is a glycoprotein (Price et al., 1969), and the specific adsorption of our enzyme to Con A–Sepharose indicates that the enzyme is also a glycoprotein. Bovine DNase I and DNase I of rat pancreatic juice also showed two pH optima dependent on Co^{2+} and Mn^{2+}. In addition, the enzyme was inhibited by G-actin and bovine pancreatic DNase I antibody. Thus the endonuclease from rat small intestine is definitely classified as a DNase I.

Our enzyme produces relatively large limit digests and shows base specificity, suggesting that it functions in DNA replication and metabolism, although we have no data on this at present. It is noteworthy, however, that the villus cells have a rapid turnover time (5 days) and show active DNA replication and metabolism.

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