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Purification and Properties of a Neutral Endodeoxyribonuclease from Rat Pancreatic Juice

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DNase I was purified from rat pancreatic juice by a procedure including DEAE-cellulose and phosphocellulose chromatographies, Sephadex G-75 gel filtration and concanavalin A-Sepharose affinity chromatography. The purified enzyme contained no detectable activities of acid DNase, acid or alkaline RNase, acid or alkaline phosphatase or nonspecific phosphodiesterase. The molecular weight of the enzyme was estimated to be 37,500 by Sephadex G-75 gel filtration. Its isoelectric point is 4.7 ± 0.1. The enzyme required divalent cations and shows dual pH optima that are dependent on divalent cations : in the presence of Co^{2+}, the optimum pH is 6.0 in 17 mM cacodylate-HCl buffer and in the presence of Mn^{2+}, the optimum pH is 8.0 in 17 mM Tris- HCl buffer. The enzyme hydrolyzes native DNA about 2 times faster than denatured DNA, producing 5′-phosphoryl and 3′-hydroxyl terminated oligonucleotides with an average chain length of about eight nucleotides. The enzyme converts double-helical pBR322 DNA (form I) to unit length DNA (form Ⅲ) via open circular DNA (form Ⅱ). Thus the mode of action of the enzyme is endonucleolytic with single-strand break. The enzyme was inhibited with G-actin of rabbit muscle and antiserum against bovine pancreatic DNase I. The existence of no species-specificity in the inhibition of DNase I enzymes by rabbit muscle actin was considered.

Pancreatic DNase I is one of the two representative mammalian DNases and has been widely investigated.[9] Similar activity was found in rat small intestinal mucosa,[12] guinea pig epidermis,[18] nuclei of liver cells[7] and other organs.[19] To establish the identity of these enzyme activities, we have previously purified and characterized the some properties of neutral endodeoxyribonucleases from rat small intestinal mucosa[17] and guinea pig epidermis.[4] The properties of DNase of rat small intestinal mucosa are similar to those of bovine pancreatic DNase I in many respects. Because no reports on the detailed properties of rat pancreatic DNase are available at present, we have purified and characterized DNase I of rat pancreatic juice to compare its properties with those of DNase I of rat small intestinal mucosa.

On the other hand, G-actin of rabbit muscle has been shown to be a specific protein inhibitor of bovine pancreatic DNase

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I.[11] The inhibition has been supposed not to have species-specificity in the reaction of complex formation.[10] But recently Lacks[8] showed that actin of rabbit muscle did not inhibit any of the DNase I enzymes of the rat. Another purpose of this work is, therefore, to examine the inhibitory activity of rabbit muscle G-actin on the purified pancreatic DNase of the rat.

In this paper we describe the purification and some properties of DNase I of rat pancreatic juice and the inhibitory effect of G-actin of rabbit muscle on the enzyme.

EXPERIMENTAL PROCEDURES

Materials - Pancreatic juice was obtained from male Wister rats weighing 250-300 g which were anaesthetized with amobarbital intraperitoneally. The pancreatic duct was cannulated and the pancreatic juice and bile were collected continuously over a period of 10 h into a test tube immersed in ice water. The collected pancreatic juice and bile were stored at -40℃ until use. ^{32}P-labeled *Escherichia coli* DNA was prepared as described previously.[1] ^{32}P-labeled *E. coli* ribosomal RNA was prepared by the method of Littauer and Esisenberg.[13] pBR322 DNA was prepared as described previously.[21] Concentrations of DNA and RNA were expressed as nucleotide residues. *E. coli* alkaline phosphatase was purified by the method of Garen and Levinthal[5] with some modification. Snake venom and calf spleen phosphodiesterases and bovine pancreatic DNase I was products of Sigma. DNase I of rat small intestinal mucosa was purified as described previously.[17] DEAE-cellulose (DE32) and phosphocellulose were purchased from Whatman. Sephadex G-75, DEAE-Sephadex A-25 and Con A-Sepharose 4B were obtained from Pharmacia. G-actin of rabbit muscle and rabbit antiserum against bovine pancreatic DNase I were generous gifts from Dr. K. Fujii and Dr. A. Funakoshi, respectively, of Kyushu University. Bovine serum albumin and egg albumin were obtained from ICN Biochemicals. Cytochrome c and carrier ampholite (pH 3.5-10) were products of Sigma and LKB, respectively. Methyl α-D-glucoside was obtained from Nakarai Pure Chemicals. All other chemicals were of standard reagent grade.

Methods - Assay of rat pancreatic juice DNase : The assay measures the conversion of ^{32}P-labeled *E. coli* DNA to acid-soluble products. Because the enzyme shows two pH optima, reactions were carried out under two standard conditions. One reaction mixture (0.3 ml) contained 17 mM cacodylate-HCl buffer, pH 6.0, 10 mM $CoCl_2$, 3 nmol of native *E. coli* [^{32}P]DNA, and enzyme preparation. The other contained 17 mM Tris-HCl buffer, pH 8.0, 5 mM $MnCl_2$, 3 nmol of native *E. coli* [^{32}P]DNA, and enzyme preparation. After incubation for 30 min at 37℃, the acid-soluble radioactivity was measured as described previously.[3] One unit of the enzyme activity was defined as the amount catalyzing the production of 1 nmol of acid-soluble nucleotides under the conditions described above.

Assay of other enzymes : *E. coli* alkaline phosphatase was assayed as described previously[1]. Bovine pancreatic DNase I was assayed as described previously.[4] The activities of acid DNase, acid and alkaline RNase, acid and alkaline phosphatase and nonspecific phosphodiesterase were assayed as described previously.[4]

Other methods : Isoelectric focusing and polyacrylamide gel electrophoresis were

performed as described previously.[4] Protein concentration was determined by the method of Lowry *et al.*[14] or Mcknight[16] with bovine serum albumin as a standard or determined spectrophotometrically at 280 nm using an absorption coefficient of 1.0 (mg/ml)$^{-1}$cm^{-1}.

RESULTS

Enzyme Purification - A summary of the purification procedure is given in Table I. The entire purification procedure was performed at 0-4℃, unless otherwise noted.

Treatment of pancreatic juice with diisopropyl fluorophosphate : Pooled rat pancreatic juice (35 ml) was stirred with 1.75 mg of diisopropyl fluorophosphate for 30 min and then dialyzed two changes of 500 ml of buffer A (20 mM Tris-HCl buffer, pH 7.5 , 5 mM $CaCl_2$ and 10 % ethylene glycol) overnight . The dialyzed sample was centrifuged at 10,000 × *g* for 10 min (Fraction I, 32 ml).

DEAE - cellulose chromatography : The supernatant was applied to a DEAE-cellulose column (1.8 × 19 cm) previously equilibrated with 500 ml of buffer A, and then enzyme was eluted with a linear gradient of 0 to 0.4 M NaCl in buffer A, the total volume of the gradient being 100 ml. The flow rate was 14 ml/h, and 6-ml fractions were collected. The fractions eluted between 0.08 M and 0.16 M NaCl were pooled (Fraction II , 79 ml).

Phosphocellulose chromatography : Fraction II was dialyzed against 1 liter of buffer B (20 mM sodium acetate buffer, pH 4.7 , 5 mM $CaCl_2$ and 10 % ethylene glycol) overnight. The dialysate, 77 ml, was applied to a phosphocellulose column (1.3 × 15 cm) previously equilibrated with 500 ml of buffer B and then enzyme was eluted with a linear gradient of 0 to 0.4 M NaCl in buffer B, the total elution volume was 200 ml. The flow rate was 15 ml/h and 4- ml fractions were collected. The fractions eluted between 0.13 M and 0.22 M NaCl were pooled (Fraction III , 49 ml) and concentrated by dialysis against poly (ethylene glycol) solution.

Sephadex G-75 gel filtration:The concentrated fraction was applied to a Sephadex G-75 column (2.0 × 95 cm) previously washed with buffer A and eluted with the same buffer. The flow rate was 15 ml/h, and 5-ml fractions were collected. The fractions containing more than 93 units enzyme per ml were pooled (Fraction IV, 20 ml).

Con A - Sepharose affinity chromatography : Fraction IV was applied to a Con A-Sepharose column (2 ml of bed volume), washed with buffer A containing 20 %

TABLE 1. Purification of rat pancreatic juice DNase. Enzyme activity was measured under the standard assay conditions at pH 6.0 as described under "EXPERIMENTAL PROCEDURES."

Fraction	Activity (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)
I. Crude extract	4,950	73.4	67.4	100
II. DEAE-cellulose	6,830	44.8	152	138
III. Phosphocellulose	3,520	3.7	951	71
IV. Sephadex G-75	2,470	0.66	3,740	50
V. Con A-Sepharose	594	0.02	29,700	12

methyl α-D-glucoside. The flow rate was 6 ml/h and 5-ml fractions were collected. The peak fractions were pooled and concentrated by dialysis against buffer A. Unless otherwise indicated, the following studies were performed with Fraction V.

Properties of the Enzyme-Stability: When stored at -40℃, the activity was retained unchanged for at least 6 months.

Purity: Polyacrylamide gel electrophoresis of the purified enzyme gave two major and two minor bands, and the DNase activity coincided with one of the minor bands. But the enzyme fraction contained no detectable activities of DNase II, acid and alkaline DNase, acid and alkaline phosphatase or nonspecific phosphodiesterase.

Isoelectric point, molecular weight and optimum temperature: The isoelectric point of the enzyme was 4.7±0.1 (Fig. 1). The molecular weight of the enzyme was estimated to be

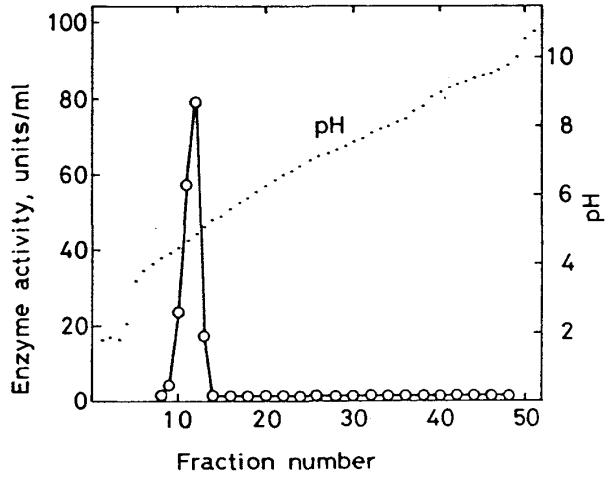

Fig. 1. Isoelectric focusing of endonuclease. The Sephadex G-75 fraction (20 ml, 21,000 units) was dialyzed against 1 liter of 1% glycine solution overnight. The dialyzed fraction (19 ml) was subjected to isoelectric focusing at 300 volts for 72 h in an LKB electrofocusing column (110 ml) using a pH 3.5-10 carrier ampholite. After electrofocusing, fractions of 2 ml were collected and assayed for enzyme activity and pH. ○, DNase activity.

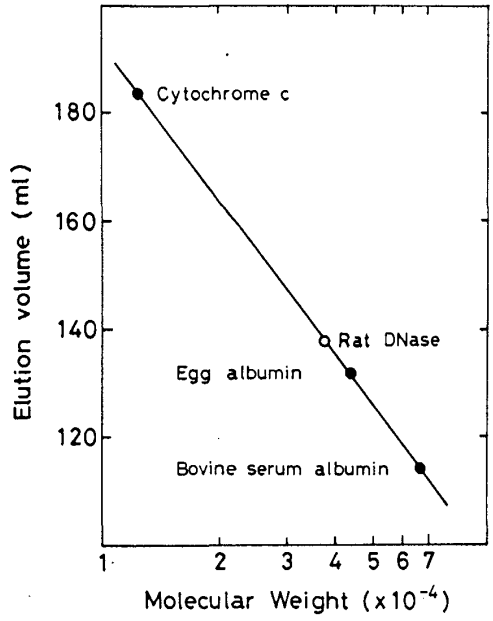

Fig. 2. Estimation of molecular weight of rat pancreatic DNase I by Sephadex G-75 gel filtration. Rat endonuclease (20 units) was subjected to a Sephadex G-75 column (2×95 cm) previously washed with 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 M KCl with bovine serum albumin (Mr 67,000), 5 mg egg albumin (Mr 45,000), 5 mg and cytochrome c (Mr 12,300), 1 mg as molecular weight markers. The proteins were eluted from the column with the same buffer.

37,500 by Sephadex G-75 gel filtration with several molecular weight markers (Fig. 2). The optimum temperature was 45℃ when assayed under standard reaction conditions for 30 min.

Effect of divalent cations on the pH optimum: The purified enzyme required divalent cations. The enzyme showed dual pH optima that were dependent on the divalent cations present, particulary Mn^{2+} and Co^{2+} (Fig. 3). In the presence of Mn^{2+}, the optimum pH was 8.0 in 25 mM Tris-HCl buffer. In the presence of Co^{2+}, the pH optimum was 6.0 in 25 mM cacodylate-HCl buffer. The maximum activity obtained at pH 6.0 was slightly higher than that obtained at pH 8.0.

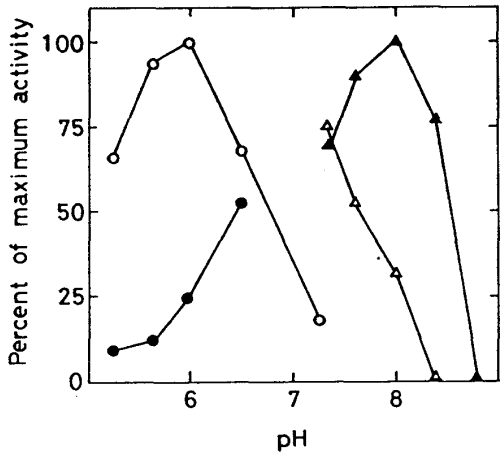

Fig. 3. Two pH optima dependent on divalent cations. The reactions were performed for 10 min with 0.045 unit of enzyme in the presense of 10 mM Co^{2+} (open symbols) or 5 mM Mn^{2+} (closed symbols) with the following buffers at 17 mM pH 5.5 – 7.0, cacodylate-HCl buffer (○) ; pH7.3-8.9, Tris-HCl buffer (△).

Metal ion requirement : The effects of various divalent cations on the enzyme activity at the respective pH values were examined. In cacodylate-HCl buffer, pH 6.0, the maximum activity was obtained with Co^{2+}. But Mg^{2+} or Mn^{2+} showed about 50% of the maximum activity. In Tris-HCl buffer, pH 8.0, Mn^{2+} showed the maximum activity. With Mg^{2+}, about 25% of the maximum activity was obtained, but no activity was observed with Co^{2+}.

Effect of the secondary structure of DNA substrate on enzyme activity : The enzyme activities with native and denatured *E. coli* DNA's were determined under each optimum codition. At pH 6.0, native DNA was degraded about 2 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 DNA was about 8, but after the extensive digestion, the average chain length decreased to about 5. At pH 8.0 the similar results were obtained.

Digestion products of native DNA : The composition of the products of hydrolysis of native DNA was analyzed on a DEAE-Sephadex A-25 column in the presence of 7 M urea[2]. The amount of mono-, di-, tri-, tetra-, and pentanucleotides were 4.3, 6.4, 7.6, 9.6 and 10.4%, respectively. The products longer than pentanucleotides were about 60%. No essential differences were observed between the degradation products at both optimal pH's.

Structure of the termini of the products : For determination of the termini produced by rat pancreatic juice DNase, the digestion products were incubated separately with two phospodiesterases , snake venom phosphodiesterase, which requires a free 3´-OH and produces 5´-mononucleotide, and calf spleen phosphodiesterase , which requires free 5´-OH and produces 3´-mononucleotide . Mononucleotides were produced only in the presence of venom phosphodiesterase, indicating that the rat pancreatic juice DNase cleaved DNA producing 5´-phosphate and 3´-OH termini.

Mode of action of the enzyme : To determine the mode of action of the 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. The initial products generated by the endonuclease at both pH's were a mixture of an open circular DNA (form II) and a unit length of DNA (form III) (Fig. 4). These results indicate that the endonuclease makes single-strand breaks in double-stranded DNA at both pH optima with similar hydrolyzing activity.

Fig. 4. Agarose gel electrophoresis of pBR322 DNA treated with the enzyme under the two pH optimal conditions. pBR322 DNA (form I ; 0.8 nmol) was incubated with 1.5×10^{-3} unit of endonuclease at each pH for the indicated times. Each digest was analyzed on 1% agarose slab gel (1.5 × 25 cm) as described under "EXPERIMENTAL PROCEDURES." (Lane1) No enzyme treatment; (lane 2-4) treatment at pH 6.0; (lane 5-7) treatment at pH 8.0; (lane 2 and 5) 1-min digest; (lane 3 and 6) 5-min digest; (lane 4 and 7) 10-min digest.

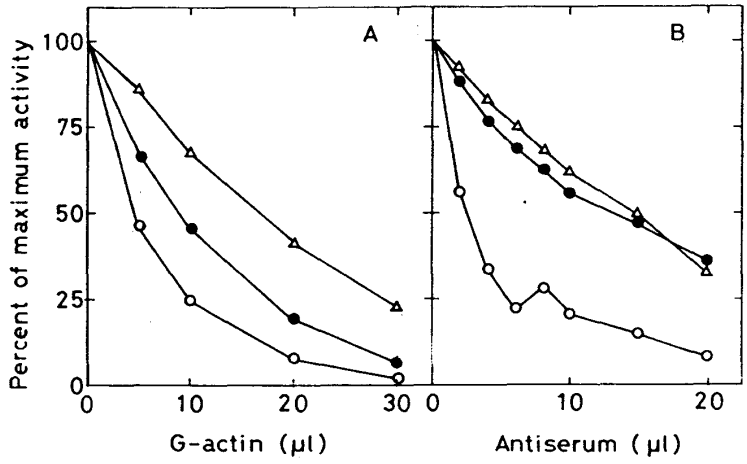

Fig. 5. Inhibition of rat pancreatic DNase, rat intestinal DNase and bovine pancreatic DNase I with rabbit muscle G-actin and antiserum against bovine pancreatic DNase I. (A) Each enzyme and the indicated amount of G-actin were mixed in 25 μl of solution and then the complete reaction mixtures were set up in the solution and activity was assayed as described under "EXPERIMENTAL PROCEDURES." The optical density at 280 nm of the solution of G-actin was 2.09. (B) Each enzyme and various amount of antiserum were mixed in 70 μl of solution and incubated for 20 min at 20℃. Aliquots (20 μl) were removed from the solution and assayed under standard assay condition at pH 6.0 as described under "EXPERIMENTAL PROCEDURES." ○, Bovine pancreatic DNase I ; ●, rat pancreatic DNase ; △, rat intestinal DNase.

Inhibition of DNase by G-actin and antiserum against bovine pancreatic DNase I : Rabbit muscle G-actin, a specific inhibitor for bovine pancreatic DNase I, inhibits rat pancreatic juice DNase as well as rat intestinal DNase (Fig. 5A). Antibody against bovine pancreatic DNase inhibited rat pancreatic DNase and rat intestinal DNase at the same degree (Fig. 5B)

DISUCUSSION

To compare the properties of neutral endodeoxyribonuclease from rat small intestinal mucosa with those of rat pancreatic DNase I, we firstly tried to purify DNase I from the rat pancreas. But no activity of DNase I was found in pancreatic extracts. Similar observations were also reported[8,9]. Therefore we purified the enzyme from the pancreatic juice of the rat. The purified enzyme was not homogeneous as judged by polyacrylamide gel electrophoresis, but the purified enzyme preparation contained no detectable enzyme activities that interfered with studies on its enzyme properties.

The DNase of rat pancreatic juice was adsorbed to concanavalin A-Sepharose. The similar adsorption to concanavalin A-Sepharose was observed with bovine and ovine pancreatic DNase I[20], guinea pig epidermal DNase[4] and rat small intestinal DNase I.[17] Concanavalin A is known to adsorb carbohydrates specifically and many examples of the use of this binding property of concanavalin A-agarose in purification of

glycoproteins have been reported.[20] These indicate that rat pancreatic juice DNase is a glycoprotein.

The enzyme shows dual pH optima which depend on the divalent cations present. Thus, in the presence of Co^{2+} or Mn^{2+}, the optimum activity was at pH 6.0 or 8.0, respectively. The similar dual pH optima have also been found for neutral endo-deoxyribonuclease of guinea pig epidermis[4] and DNase I of small intestinal mucosa.[17] Rat pancreatic juice DNase shows many similarities to DNase of rat small intestinal mucosa. Thus, both enzymes have dual pH optima which depend on the divalent cations and similar isoelectric points. Both enzymes hydrolyzed native DNA about 2 times faster than denatured DNA producing oligonucleotides with 3´- OH and 5´-phosphoryl termini of about eight nucleotide length. These enzymes were shown to be immunologically similar to bovine pancreatic DNase I because both enzyme activities were inhibited by antiserum against bovine pancreatic DNase I. Although small differences were observed in the molecular weights and the composition of the limit digests, the two enzymes were essentially identical in many respects. But more detailed studies are required to establish the identity of the two enzymes.

The specific interaction of bovine pancreatic DNase I and skeletal muscle or cytoplasmic actin has been well shown in recent years.[11, 15, 6] The two proteins form stable 1 : 1 complex of high affinity. The widespread occurance of actin in cells and its high affinity for DNase I suggested that the interaction of these two proteins may be of general significance for either the function of actin in cell motility or the physiological function of DNase I.[8] Furthermore it appears that no species-specificity exsists in the reaction of complex formation. On the contrary, Lacks[8] examined inhibition of various DNase I enzymes of the rat by rabbit muscle actin in gels and in solution and showed that actin did not inhibit any of the DNase I enzymes of the rat.

Our results in this paper, however, clearly showed that G-action of rabbit muscle inhibited rat pancreatic juice DNase I, and our previous papers showed that G-actin of rabbit muscle inhibited highly purified DNase I enzymes of rat small intestinal mucosa[17] and guinea pig epidermis.[4] Thus our results support the notion that muscle G-actin inhibits DNase I enzymes without species-specificity. The discrepancy between his results and ours remained unresolved, and further studies on inhibition of purified DNase I enzymes of various organs of the rat by rabbit muscle actin were required.

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