Purification and Properties of Acid Deoxyribonucleases of Human Gastric Mucosa and Cervix Uteri*

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SUMMARY

The acid deoxyribonucleases in homogenates of human gastric mucosa and cervix uteri were purified extensively by a procedure including DEAE-cellulose and phosphocellulose chromatographies, gel filtration, and isoelectric focusing. The enzymes in the two preparations had similar properties. The active protein was resolved into at least two forms with different isoelectric points, as judged by the formation of acid-soluble fragments. The major component of the activity had an isoelectric point at pH 7.02, and the minor component at pH 6.86. However, other properties of these two forms were similar.

The final preparations of the gastric and uterine acid DNases were free of other types of DNase (DNases I, III, and IV), acid and alkaline phosphatases, acid and basic K\(n\)ases, and nonspecific phosphodiesterase. Both enzymes had a molecular weight of approximately 38,000, as determined by gel filtration on Sephadex G-100, and showed maximum activity at pH 5.2 to 5.5 in 33 mm acetate buffer. They did not require divalent cations for activity, and hydrolyzed native, double-stranded DNA about 10 to 20 times faster than heat-denatured DNA. They did not act on RNA or calcium bis(p-nitrophenyl) phosphate, indicating that they did not contain intrinsic phosphodiesterase activity. The products formed from native DNA were 3'-phosphoryl- and 5'hydroxyl-terminated oligonucleotides. The average chain length of the limit digests with these enzymes was approximately 10 and the predominant species were longer than hexanucleotides.

EXPERIMENTAL PROCEDURE

**Materials**

*Escherichia coli* [\(^{32}\)P]DNA and its thermally denatured form were obtained as described previously (21). Ribosomal RNA containing [5-\(^{3}H\)]uridine was prepared from *E. coli* by extraction with hot phenol, as described by Takeda and Yura (22). Alkaline phosphatase, snake venom phosphodiesterase, and 5'-nucleotidase were partially purified from commercial preparations from Worthington. Polynucleotide kinase was a gift from Drs. K. Shimizu and M. Sekiguchi (Faculty of Science, Kyushu University). It had been prepared from *E. coli* infected by bacteriophage T\(\alpha\), by the method of Richardson (23). Crystalline bovine plasma albumin, Fraction V, was obtained from Armour, soybean trypsin inhibitor and egg albumin (twice crystallized) from Nutritional Biochemicals, and cytochrome c from Boehringer Mannheim.

Calf brain bis(p-nitrophenyl) phosphate and p-nitrophenyl phosphate were products of Daiichi Pure Chemical Co. (Tokyo, Japan). DEAE-cellulose and phosphocellulose were purchased from Brown

**Methods**

**Assay of Acid DNase**—Production of acid-soluble radioactivity from \[^{32}P\]DNA was routinely determined in reaction mixture (0.3 ml) containing 10 nmoles of sodium acetate buffer, pH 5.4, 0.6 nmoles of EDTA, 10 nmoles of E. coli \[^{32}P\]DNA, and 0.5 to 5 units of enzyme. Twenty micrograms of bovine plasma albumin (in freshly prepared aqueous solution) (21) were also added to the medium, when the enzyme preparation had a specific activity of more than 1000. The enzyme preparation was diluted with TE solution. The mixture was incubated for 10 min at 37°C, the acid-soluble fraction was then obtained by adding cold perchloric acid, and the radioactivity of an aliquot was determined as described previously (21). The acid-soluble fraction from control mixture without enzyme contained less than 0.5% of the added radioactivity. One unit of acid DNase activity is defined as the amount of enzyme catalyzing the production of 1 n mole of acid-soluble \[^{32}P\]P in 10 min under the above conditions.

**Assay of Other Enzyme Activities**—DNase I activity was measured in reaction mixture (0.3 ml) containing 30 nmoles of Tris-HCl buffer, pH 7.5, 1.5 nmoles of MgCl₂, 10 nmoles of E. coli \[^{32}P\]DNA, and enzyme samples. Alkaline DNase III and IV were assayed by the procedure of Lindahl (15, 16) with minor modifications as follows. The reaction mixture (0.3 ml) contained 10 nmoles of Tris-HCl buffer, pH 8.5, 1 n mole of MgCl₂, 3 n moles of mercaptoethanol, enzyme sample, and 10 nmoles of denatured E. coli \[^{32}P\]DNA for DNase III, or native E. coli \[^{32}P\]DNA for DNase IV.

**Identification of Acid-soluble Digestion Products**—The susceptibility of the digestion products to alkaline phosphatase and 5' nucleotidase was studied by measuring the formation of \[^{32}P\]-labeled material not adsorbed on Norit, as previously described (21). Hydrolysis of the limit digest of DNA was performed with snake venom and spleen phosphodiesterases using the reaction systems described by Laskowski (27) and Bernardi (28), respectively. Labelling of the 5' terminal of the limit digests with \[^{32}P\]P was carried out by the action of polyadenylate kinase, as reported by Richardson (23). The digestion products of DNA were analyzed by chromatography on a DEAE-Sephadex A-25 column (21).

### RESULTS

**Purification of Enzymes**

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

**Crude Extracts**—Stomachs were obtained by gastrectomy from patients with a duodenal ulcer or cholelithiasis, and uteri by total hysterectomy from patients with prolapse or myoma uteri. Microscopically none of the samples showed malignant changes. Soon after gastrectomy, the stomach was washed with running water and the mucosa was scraped off from the wall with a razor at the level of the muscularis submucosae. Immediately after hysterectomy the cervical portion of the uterus was washed with running tap water and a wedge of tissue was cut, including a large area of surface epithelium with a minimal amount of underlying layers. Acid DNase activity was scarcely detectable in the muscular layers of the two organs. Subsequent procedures were the same for both materials.

The specimens were washed three times in ice-cold 0.5% NaCl-KCl solution, wiped with filter paper, weighed, and cut into small pieces. Tissue was then homogenized for 1 min in a Waring Blender with 10 volumes of cold TE solution. The homogenate was filtered through gauze and the filtrate was centrifuged at 8000 x g for 10 min. The precipitate (cell debris and nuclei) contained little acid DNase activity and was discarded while the supernatant fluid with enzyme activity (Fraction I) was stored in a deep freeze as crude extract.

**DEAE-cellulose Fractionation**—Fraction I (400 ml) was thawed and applied to a column of DEAE-cellulose (3 x 30 cm) previously equilibrated with TE solution. The column was eluted with the same buffer at a flow rate of 100 ml per hour. Fractions of 10 ml of the eluate were collected and assayed for DNase activity. Fractions containing more than 5 units of enzyme activity per ml were pooled (Fraction II).

**Phosphocellulose Chromatography** Fraction II was dialyzed against 10 volumes of 20 mm collidine-HCl buffer, pH 6.5, containing 2 mm EDTA for 12 hours. It was then applied to a

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1 The abbreviation used is: TE solution, 20 mm Tris-HCl buffer, pH 7.5, and 2 mm EDTA.

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**TABLE I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Human gastric DNase II</th>
<th>Human uterine DNase II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Volume</td>
<td>Total protein</td>
</tr>
<tr>
<td>I. Crude extract</td>
<td>400</td>
<td>6,400</td>
</tr>
<tr>
<td>II. DEAE-cellulose</td>
<td>550</td>
<td>1,860</td>
</tr>
<tr>
<td>III. Phosphocellulose</td>
<td>70</td>
<td>17.5</td>
</tr>
<tr>
<td>IV. Sephadex G-100</td>
<td>55</td>
<td>7.5</td>
</tr>
<tr>
<td>V. Electofocusing</td>
<td>4</td>
<td>0.12</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>0.14</td>
</tr>
</tbody>
</table>

**Other Methods**—The molecular weight of the purified enzyme was measured by the method of Andrews (29). Polyacrylamide gel electrophoresis was performed at pH 8.3 using the method of Davies (30). Protein concentration was determined by the method of Lowry et al. (31) with crystalline bovine plasma albumin as a standard. Inorganic phosphorus was determined as described by Lowry and Lopez (32). \[^{32}P\] was counted in an Aloka model TD-307 gas flow counter (Japan Radio Co., Tokyo). \[^{32}P\] was measured in a Beckman model LS-100 liquid scintillation counter.

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**Fractionation of Enzymes**

All operations were conducted at 0–4°C, unless otherwise stated. The purification procedures and results of a typical preparation are summarized in Table I.
column of phosphocellulose (1.5 x 50 cm) equilibrated with 1 liter of the same buffer. The column was washed with 100 ml of the same buffer and then eluted with 800 ml of a linear gradient of 0.2 to 0.6 m NaCl in the same buffer. The flow rate was 50 ml per hour, and fractions of 5 ml were collected. Acid DNase activity was eluted in a first, minor peak, a, and a second, major peak, b, immediately afterward, with approximately 0.35 m and 0.40 m NaCl, respectively. The ratio of activities in the two peaks was fairly constant. Fractions containing more than 5 units of enzyme activity per ml were pooled (Fraction III). Phosphodiesterase and acid phosphatase were not adsorbed on the column. and were eluted with the washing solution. Acid RNase was eluted just after Peak b and overlapped it.

**Gel Filtration on Sephadex G-100**—Fraction III was dialyzed against a solution of 30% polyethylene glycol in TE solution to reduce its volume and then against 0.1 m NaCl-0.02 m sodium acetate buffer, pH 5.5, for 6 hours. The solution obtained was centrifuged for 10 min at 20,000 x g to remove the considerable amount of insoluble material formed during the concentration procedure and dialysis. The supernatant solution (5 ml) was applied to a column of Sephadex G-100 (2 x 110 cm) previously washed with the same buffer. After allowing the enzyme solution to enter the gel, the column was eluted with the same buffer at a flow rate of 7 ml per hour, and 3.5-ml fractions were collected. The peak of acid DNase activity emerged after passage of approximately 200 ml of the eluting solution, and fractions containing enzyme activity of more than 5 units per ml were pooled (Fraction IV). Acid RNase was eluted afterward and was completely separated from acid DNase.

**Isoelectric Focusing**—Fraction IV was dialyzed against a solution of 30% polyethylene glycol in TE solution to reduce its volume to about 10 ml, and then against 2 liters of 1% glycerine solution for 12 hours. The dialyzed fraction was subjected to electrophoresis in a 110-ml LKB electrofocusing column, as described by Vesterberg and Svensson (33). Carrier ampholytes were used to establish the pH gradient. The electrophoresis was run at 700 volts for 60 hours at 4°C. Then fractions of 1 ml were collected and their DNase activity and pH were measured. Two active peaks were observed (Fractions V-A and V-B) (Fig. 1). The fractions in each peak were pooled and dialyzed against TE solution for 24 hours with several changes of the dialyzing solution. Then the preparations were stored at -20°C.

The final yields of human gastric and uterine DNase in the major fraction, Fraction V-B, were about 13 and 14% of the activities present in the crude extracts, representing purifications of 6000- and 4000-fold, respectively.2

**Multiple Forms of Enzymes and Homogeneities of Enzyme Preparations**—During purification of the enzymes from stomach, chromatography on phosphocellulose yielded two active fractions, a and b, which overlapped. Moreover, when these had been combined and subjected to gel filtration isoelectric focusing again showed the presence of two forms of the enzyme (Fig. 1). When the two active peaks, a and b, from the phosphocellulose column were each subjected to isoelectric focusing the electrophoreograms indicated that the protein in the first peak, a, was mainly located in Fraction A, while that in the second, major peak, b, was in Fraction B (Fig. 2). On gel electrophoresis, Fractions V-A and V-B both gave two bands (α and β) in Experiment 2. During purification of the DNases the total activities of the DEAE-cellulose fractions were much greater than the initial activities in crude extracts. Preliminary experiments showed that a fraction inhibiting DNase activity was eluted when the DEAE-cellulose column was washed with 0.5 m NaCl. Accordingly, the increase in activity might be due to removal of some concomitant inhibitor.
hbiting acid DNase activity with about one-third the mobility of that of bromphenol blue, the tracking dye (Fig. 3). The protein of the α-band was found more in Fraction V-A while β-band protein was found in Fraction V-B, although definite evidence for the correspondence of α-band protein to enzyme in Fraction V-A and β-band protein to that in Fraction V-B was not obtained, due to the limited amount of material available.

Fractions V-A and V-B were both about 80% homogeneous, and were contaminated with a different kind of protein possessing no enzyme activity. Similar results were obtained with Fractions V-A and V-B from uterus.

Properties of Enzymes

The properties of the enzymes in Fractions V-A and V-B of the two tissues were studied, and the results obtained with the latter fractions, are mainly described below. Fractions V-A had less activity but except for a difference in their isoelectric point their properties were similar.

Stability—The final preparations of the two enzymes, as well as less purified preparations and crude extracts, were stable at 0° for at least 2 months, and could be stored at −20° for more than 1 year without appreciable loss of activity. The heat sensitivities of the enzymes were studied by preincubating the preparations at various temperatures for 20 min. The enzymes were rather stable up to 55°, but lost 50% of their activity at 60° and were inactivated completely at 70°.

Effect of pH and Temperature on the DNase Reaction—The acid DNases from stomach and uterus exhibited maximum activity at pH 5.2 to 5.5 in reaction mixture containing 33 mm sodium acetate buffer, and at around pH 6.4 in N-tris(hydroxy-methyl)methyl-2-aminoethanesulfonic acid (TES) buffer of the same concentration. The pH curves indicated rather slow decrease of the reaction rate on the acidic side of the optimum range.

The optimal temperature for the two DNase fractions from the two tissues, measured from the initial reaction rates, was about 45°, with a gradient decrease of the reaction rate at lower temperatures.

Inhibition and Activation—The activity of acid DNase is known to vary with the ionic species present in the reaction medium and the ionic strength. Hydrolysis of native DNA by the purified gastric and uterine acid DNases at the optimal pH was inhibited by approximately 50% when Mg2+ (1 mM), Mn2+ (1 mM), Ca2+ (1 mM), or Cu2+ (1 mM) was added to the reaction medium in place of EDTA. Fe3+ (0.1 mM) inhibited the reaction completely, while Na+ (1 mM) did not cause any reduction in activity. 2-Mercaptoethanol, at a concentration of 2%, did not affect the reaction velocity of either enzyme, but bovine plasma albumin, at a level of 0.3 mg per ml, was stimulatory.

Isoelectric Point—The acid DNases from stomach and uterus have the same isoelectric points: Fractions V-A at pH 6.86 and Fractions V-B at pH 7.02 (Fig. 1).

Molecular Weight of Enzyme—Although the DNAse activity from gastric mucosa was found in two fractions on phosphocellulose chromatography, isoelectric focusing, or disc gel electrophoresis, the activities in these fractions were eluted as a single peak from Sephadex G-100, indicating that they have a similar molecular weight. By comparison of the relationship between the elution volumes and the logarithms of the molecular weights of standard proteins (egg albumin, soybean trypsin inhibitor, and cytochrome c) and the elution volumes of the final preparations (Fractions V-A and V-B), it was estimated that the gastric acid DNase is a mixture of molecules with a molecular weight of around 38,000. The same results were obtained with hog spleen DNase II and the two forms of DNase from cervix uteri.

Effect of Enzyme Concentration on DNase Reaction—With purified enzyme preparations the reaction rate was proportional to the amount of protein employed up to a level of enzyme which permitted rapid degradation of approximately 30 to 40% of the DNA substrate.

Substrate Specificity of Enzyme—The reactions of the DNases from the two tissues with native and denatured DNAs were followed (Fig. 4). Both enzymes initially degraded native DNA about 10 to 20 times faster than thermally denatured DNA. Hydrolysis of native DNA proceeded quickly until approximately 30 to 60% of the DNA had been broken down and then more slowly and finally the reaction stopped when 70 to 90% of the DNA had been degraded. E. coli ribosomal RNA was not hydrolyzed by the purified enzyme preparations, as described below.

Identification of Acid-soluble Digestion Products—The reaction products of the enzymes from the two tissues were assayed by measuring the susceptibility of the products to E. coli alkaline phosphatase at different stages during the digestion of native DNA. At the point where hydrolysis of the DNA reached a limit, 10% of the total acid-soluble phosphate was sensitive to the action of alkaline phosphatase. Thus the average chain length of the product was calculated as approximately 9 to 10.

The products formed from native DNA by the enzymes were separated according to their chain length using a column of DEAE-Sephadex A-25 eluted with 7 M urea-0.02 M Tris-HCl buffer, pH 7.5, containing a gradient of increasing NaCl concentration. Table II shows the proportions of small oligonucleotides of from mono- to pentanucleotides after exhaustive digestion of DNA. The two enzymes produced similar amounts of oligonucleotides and no mononucleotides. The amount of dinucleotides was small, and most of the digest consisted of larger oligonucleotides which could not be separated by this technique. These results suggest that the enzymes act endonucleolytically.

The limit digests of DNA by gastric and uterine DNases were converted to mononucleotides and mononucleoside diphosphates by treatment with excess snake venom phosphodiesterase. Of the 32P-labeled products, 80 and 98% were susceptible to the actions of purified 5'-nucleotidase and E. coli alkaline phosphatase, respectively. When the limit digests were treated with spleen phosphodiesterase, approximately 95% of the total 32P became susceptible to alkaline phosphatase. In addition, the limit digests of DNA were labeled with [γ-32P]ATP by mediation of polynucleotide kinase to the same extent, with and without previous incubation with alkaline phosphatase. Therefore, the reaction products were concluded to contain terminal 3'-phosphoryl and 5'-hydroxyl groups.

Absence of Other Enzyme Activities—Less than 1 nmole of p-nitrophenol was liberated when 10 nmoles of p-nitrophenyl phosphate and calcium bis(p-nitrophenyl) phosphate were incubated with 10 units of the final preparations of the two enzymes at pH 5.4 or 7.4 for 2 hours, or at pH 5.4 for 24 hours. In addition, when 100 nmoles of native DNA were incubated...
DNA preferentially. Activities, and were real DNases capable of hydrolyzing native diesterase, acid or alkaline phosphatase or acid or basic RNase natured DNA substrate. Preparations were free from detectable nonspecific phospho-

After incubation for 65 min, 4 units more enzyme were added to each reaction mixture and incubations were continued for totals of 90, 120, and 150 min. O-O, native DNA; O--O, denatured DNA substrate.

**Table II**

<table>
<thead>
<tr>
<th>Products</th>
<th>Total %P eluted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gastric DNase</td>
</tr>
<tr>
<td>Mononucleotides</td>
<td>0</td>
</tr>
<tr>
<td>Dinucleotides</td>
<td>0.8</td>
</tr>
<tr>
<td>Trinucleotides</td>
<td>2.2</td>
</tr>
<tr>
<td>Tetranucleotides</td>
<td>3.4</td>
</tr>
<tr>
<td>Pentanucleotides</td>
<td>3.2</td>
</tr>
<tr>
<td>&gt;Pentanucleotides</td>
<td>90.4</td>
</tr>
</tbody>
</table>

with 20 units of the enzymes for 6 hours at pH 5.3, less than 0.01 nmole of the mononucleotide was released (Table II). When 10 units of the final preparations of the two enzymes were incubated with 100 nmol of E. coli [H]RNA for 2 hours at pH 5.4 or 7.5, less than 1% of the substrate was converted to acid-soluble products. These results indicate that the purified preparations were free from detectable nonspecific phosphodiesterase, acid or alkaline phosphatase or acid or basic RNase activities, and were real DNases capable of hydrolyzing native DNA preferentially. In crude extracts of the two tissues the activities of DNase I and IV were too weak to be detected, but there was a considerable amount of DNase III activity. However, this was removed from the acid DNase fraction by DEAE-cellulose chromatography. When 10 nmole of native or denatured DNA were incubated with 5 units of the final preparations of the two enzymes for 1 hour at pH 7.5 or 8.5, less than 0.01 nmole of acid-soluble nucleotides was formed, indicating that the preparations were not contaminated with these DNases.

**DISCUSSION**

Highly purified preparations of acid DNase were obtained from homogenates of human gastric mucosa and cervix uteri as described in this paper. These preparations appeared about 80% homogeneous by disc electrophoresis, but were free from other nucleolytic enzymes.

All of the acid DNases so far found in mammalian tissues appear to have similar properties (1, 3, 4, 18). The acid DNases reported here are the first enzymes to be purified from human materials of different embryonic origins, and they also had essentially the same properties.

Bernardi and Griffe (34) observed that a highly purified preparation of hog spleen DNase II catalyzes the slow hydrolysis of p-nitrophenol from calcium bis(p-nitrophenyl) phosphate and suggested that the phosphodiesterase activity was an intrinsic property of the acid DNase molecule. Hodes and his co-workers (35) and Slor (36) succeeded in separating these two activities, but Sicard et al. (37) failed to confirm their results. Recently, Dulaney and Touster (18) obtained a preparation of acid DNase from rat liver lysosomes which appeared homogeneous on electrophoresis, and found that it did not show nonspecific phosphodiesterase activity. In this work, during purification of human acid DNases, phosphodiesterase was clearly separated from acid DNase fractions by phosphocellulose chromatography, and the final preparations of DNases were completely free of phosphodiesterase activity. Thus, our results indicate that the two activities belong to different protein molecules.

The human DNases from gastric mucosa and cervix uteri were both resolved into at least two forms, which had different isoelectric points but otherwise similar properties. However, it is possible that formation of active degradation products during the chromatographic procedure may lead to multiplicity of enzyme forms. Multiple forms of acid DNase have been obtained from hog spleen (38), rat liver (18), mouse liver (39), hog liver (40), and beef brain (41). Bernardi et al. (38) prepared two active components of DNase with similar properties from hog spleen by chromatography on CM-Sephadex. They suggested that the acid extraction involved in the purification procedure resulted in the appearance of a minor component, which was formed from the major component by acid hydrolysis and which could be separated from the latter by chromatography.

In the procedure described here for purification of human acid DNases, drastic treatments were avoided and preparations were kept close to neutrality to assure the chemical integrity of the molecules throughout the purification. Each peak of activity from the phosphocellulose column gave a single peak at the identical position when rechromatographed on the same column, and the enzymes in the two final fractions (Fractions V-A, V-B) were recovered in the same positions on repeated electrofocusing. Moreover, varying the time of treatment in a Waring Blender from 30 s to 15 min and repeated freezing and thawing of crude extracts did not affect the pattern of DNase activity at all. Thus it seems unlikely that proteolytic enzymes...
in the materials cause any hydrolysis of the DNase molecule during the purification procedure. However, the properties of these two active forms were very similar. Therefore further investigations are needed on whether the multiple forms of the enzyme in human materials are due to proteolytic modification of the molecules, either at the ends of the chain or by intramolecular cleavage, or whether the enzyme activities reside in different proteins.

REFERENCES

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