Protein Inhibitor of Acid Deoxyribonucleases
IMPROVED PURIFICATION PROCEDURE AND PROPERTIES*

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A method is described for the extensive purification of acid deoxyribonuclease (acid DNase) and its specific inhibitor from beef liver, the existence of which had been only supported by indirect evidence. By the use of insolubilized acid deoxyribonuclease, eight other proteins interacting with the enzyme have been detected. One of them (molecular weight, 59,000) was identified as responsible for phosphodiesterase activity which is often a contaminant of DNase preparations. Acid DNase (free of phosphodiesterase) and its inhibitor have been obtained as homogeneous proteins, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular weight of acid DNase and its inhibitor are, respectively, 26,500 and 21,500; those of other proteins range from 17,000 to 112,000. The properties of beef liver acid DNase are similar to those described for the enzymes extracted from other sources. The same alteration of DNase kinetics by this inhibitor, as that previously demonstrated with an impure protein has been confirmed; the sigmoidal shape observed at pH 5 for the plot of initial rate versus substrate concentration progressively disappears with increasing pH. We have also demonstrated that RNA, which inhibits the acid DNase through a competitive binding to the catalytic site, is able, like the substrate, to reverse the binding of inhibitor to the enzyme.

The function of acid DNase is unknown, although it may be involved in such important steps as DNA replication, recombination, integration, excision after irradiation, or viral induction. Several studies have shown the presence of acid DNase in nuclear fraction of rat liver (1), mouse liver (2), and HeLa cells (3). However most of acid DNase activity in mammalian cells was found to be associated with lysosomes (4). Therefore it seems likely that acid DNases are involved in the degradation of DNA. In any case, the activity of acid DNases may be regulated by different effectors within the cells.

We have reported (5) the presence, in mouse liver, of a protein inhibitor of this enzyme. This natural inhibitor is also active on beef spleen and Helix aspersa acid DNases (6) but inactive on pancreatic DNase and Escherichia coli endonuclease (5). The present paper reports on the purification of both acid DNase and its inhibitor to homogeneity.

For this purpose, we have developed a technique of affinity chromatography (7) which takes advantage of the interaction between the enzyme and its inhibitor. This method has permitted the isolation of nine proteins interacting with acid DNase. One of these proteins is the inhibitor, another carries the phosphodiesterase activity towards bis(p-nitrophenyl) phosphate which is usually difficult to separate from the acid DNase.

EXPERIMENTAL PROCEDURE

Materials

Beef liver was removed within 1 hour after slaughter, immediately frozen in Dry Ice, and then stored at -20°C.

8-H-labeled DNA was prepared from T4 phage according to Richardson et al. (8), as modified by Laval and Paoletti (9). Its specific activity was $14.2 \times 10^5$ cpm per nmol of nucleotide. Radioactivity was determined in a toluene-2,5-diphenyloxazole (PPO) (0.4%) 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene (dimethyl POPOP) (0.01%) scintillation fluid. DNA was denatured before use by heating at 100°C for 10 min followed by quenching in ice. Fish sperm DNA was purified according to Aubin et al. (10). Total yeast RNA was obtained from Chaoy Laboratory.

Helix aspersa acid DNase (fraction VIII) (6) was purified in our laboratory by Dr. J. Laval. Spleen phosphodiesterase and snake venom phosphodiesterase were purchased from Worthington.

Other materials were obtained from commercial sources.

Preparation of Immobilized Acid DNase

Coupling techniques were those described by Kato and Anfinsen (11) and Cucrstees and Scept contain (12).

Sepharose 4B (40 ml) was suspended in the same volume of distilled water after extensive washing. After raising the pH to between 11 and 11.5 with 2 N NaOH, 40 ml of freshly prepared cyanogen bromide (9 g) solution were added and the pH was maintained between 11.0 and 11.5 with 2 N NaOH for 4 to 5 min at room temperature. The activated Sepharose was then washed with 2 liters of distilled water and 1 liter of 0.1 M NaHCO3 solution and finally resuspended in 40 ml of distilled
water. An equal volume containing 30 nmol (11.0 g) of octanethiolamine was added and the pH was adjusted to 10 with 0.1 N HCl. After a 16-hour reaction at 4°C, the beads were washed with the following solvent: distilled water, 0.1 M NaHCO₃ solution, distilled water again, and resuspended in 40 ml of distilled water. An equal volume containing 40 nmol (4 g) of succinic anhydride was added and the pH was adjusted to 5.0 and maintained at 5°C with 10 N NaOH. The mixture was incubated 5 hours at 4°C and the gel was washed as previously described. The succinylaminooctyl-Sepharose was mixed with at least 4 x 10⁸ units (21.5 mg) of pure acid DNase obtained by a modification of the method described below (two cycles of hydroxylapatite chromatography) and 1 g of N-ethyl-N-(3-dimethylaminopropyl)carbodiimide, giving a final volume of 160 ml. The pH was adjusted to 4.8 with acetic acid and the mixture was incubated 20 hours at 20°C. The binding of the enzyme to the beads was monitored by measuring DNAase activity of the insoluble derivative. The insoluble acid DNase (acid DNase-succinylaminooctyl-Sepharose; DSOS) was stored in 0.3 M potassium phosphate, pH 6.8, at 4°C and retained its full biological properties for over 1 year.

**Assay of Acid DNase Activity**

This assay measures the conversion of 3H-labeled T₄ phage DNA into acid-soluble fragments. The incubation mixture (0.125 ml), pH 5.0, contained 1.25 μmol of sodium acetate, 12.5 μg of bovine serum albumin, 4 nmol of T₄ phage DNA unless otherwise stated, and enzyme. After a 30-min incubation at 37°C, 0.01 ml of bovine albumin (50 mg/ml) and 0.2 ml of 1 N perchloric acid were added. After 5 min at 0°C, the mixture was centrifuged for 10 min at 7,700 x g. The radioactivity was determined on 0.2 ml of the supernatant. One unit of enzyme activity was defined as that which converts 0.1 nmol of DNA into acid-soluble form in 1 min at 37°C.

**Assay of Inhibitory Activity**

The inhibitory activity was measured by determining the amount of protein given a 50% decrease in the rate of DNA degradation by acid DNase. In the course of inhibitor purification the assay was strongly complicated by the presence of acid DNase in the fractions. Therefore, the following procedures were employed.

**Step a. Determination of Acid DNase Units in Each Fraction**—The total number of units of acid DNase for each purification step, must be determined, under conditions where the inhibitor is not active (high concentration of substrate to reverse the enzyme-inhibitor interaction). The assay of acid DNase activity previously described was used.

**Step b. Standard Curve of Acid DNase Activity in Inhibition Condition**—A standard curve of reaction velocity was established, with different concentrations of pure enzyme, under conditions where the inhibitor would be active (low concentration of substrate): the reaction mixture and the experimental process were the same as in the standard assay of acid DNase activity except 3H-labeled T₄ DNA (1 nmol), acid DNase (from 0.03 to 3 units), and incubation time (10 min).

**Step c. Determination of Inhibitory Activity**—To determine the inhibitory activity of each fraction toward the acid DNase contained in the same fraction, a comparison was done (for some dilutions of the fraction) between the true activity measured under conditions where the inhibitor is not active (see Step a) and the apparent activity measured under conditions where the inhibitor is active (see Step b). The number of apparent units was obtained according to the standard curve. The ratio between apparent units and true units x 100 gives the percentage of inhibition of the acid DNase, which can then be plotted against protein concentration. The unit of inhibitory activity was defined as the amount of protein required for 50% inhibition of the acid DNase. In the last stages of inhibitor purification, the latter was thoroughly devoid of acid DNase and the assay of inhibitory activity was easier. To obtain the inhibition kinetics of the enzyme, the procedure described above (Step b) was used with 0.25 unit of acid DNase (Fraction VII) and inhibitor. An assay was carried out without inhibitor and from the inhibition curve obtained the concentration of inhibitory which causes a 50% decrease of the acid DNase activity was determined.

**Other Enzymatic Assays**

**RNase Assays**—Enzyme (0.05 ml) and inhibitor (0.025 ml) were incubated with 1 mg of total yeast RNA in a mixture (1 ml) containing 100 μmol of sodium acetate, pH 5.0, (acid RNase) or 50 μmol of potassium phosphate, 100 μmol of sodium chloride and 10 μmol of magnesium chloride, pH 7.3 (alkaline RNase). After 30 min at 37°C, 1 ml of 1 N HCl in ethanol, and 0.1 ml of bovine albumin (4 mg/ml) were added. After 5 min at 0°C the mixture was centrifuged at 7,700 x g for 1 hour and the absorbance of the supernatant at 260 nm was determined. One unit is the amount of protein catalyzing the conversion of 1 μmol of RNA into acid-soluble form in 1 min.

**Phosphatase Assays**—Purified preparations (0.02 ml) were assayed by measuring the liberation of p-nitrophenol from 3 ml of 1 mm sodium p-nitrophenylphosphatase according to Chater et al. (13) either in 150 mm sodium acetate, pH 5.0, or in 150 mm Tris-acetate, 10 mm MgCl₂, pH 7.8. One unit is the amount of enzyme catalyzing the liberation of 1 μmol of p-nitrophenol per min.

**Phosphodiesterase Assays**—Purified preparations (0.02 ml unless otherwise stated) were assayed on bis (p-nitrophenyl)phosphate in 150 mm sodium acetate, pH 5.0, according to Privat de Garilhe and Laskowski (14).

**Exonuclease Assays**—Portions (0.02 ml) of each purified preparation were tested according to the method of Bernardi and Bernardi (15) with fish sperm DNA “core” as substrate.

**Polyacrylamide Gel Electrophoresis**

Acid DNase, protein inhibitor, and other proteins at different stages of purification were examined by electrophoresis (16) on 7.5% polyacrylamide gel. The procedure described by Shapiro et al. (17) and modified by Dunker et al. (18) was used to determine molecular weights with bovine serum albumin, ovalbumin, chymotrypsinogen A, and cytochrome c as molecular weight markers. In this case, 0.1 mg of protein (0.1 ml) was mixed with an equal volume of 2% 2-mercaptoethanol, 8 M urea, and 2% sodium dodecyl sulfate, then incubated 60 min at 45°C. The samples (0.1 ml) were layered on the gel and electrophoresis was carried out at 8 mA per tube for 6 hours. The gels were then immersed for 18 hours in a Coomassie brilliant blue R (0.05%) dissolved in methanol/acetic acid/water (5/1/5, v/v/v), soaked 30 min in 7.5% acetic acid, 5% methanol, and destained electrophoretically in the same solvent for 3 hours.

**Sucrose Gradient Centrifugation**

Samples (0.2 ml) containing cytochrome c as a internal marker were layered on a linear gradient (4 ml) of sucrose (15 to 20% w/v) in 100 mm sodium acetate 10 mm EDTA, pH 5.0, and then were centrifuged at 63,000 rpm for 16 hours in a Spincos SW65 rotor. Two-drop fractions were collected and diluted to 0.5 ml in 100 mm sodium acetate buffer, pH 5.0. Cytochrome c was determined by absorption at 406 nm and acid DNase activity as described above.

**Other Measurement**

Protein was determined either according to Lowry et al. (19) using bovine serum albumin (Fraction V) as a standard, or spectrophotometrically at 280 nm.

**RESULTS**

**Purification of Acid DNase**

A summary of the procedure is presented in Table I. All procedures were carried out at 4°C.

**Step I. Preparation of Crude Extract**—Frozen beef liver (1 kg) was thawed, miniced, freed from connective tissue, and homogenized in 5 liters of 100 mm NaCl. Unbroken cells and organelles were disrupted by a 10-min sonication in a Branson
S 125 sonic oscillator (the temperature was kept below 6°C). The extract was centrifuged 15 min at 11,000 × g and the supernatant was saved (crude extract).

**Step II: Ammonium Sulfate Fractionation**—Solid ammonium sulfate was slowly added with stirring to the crude extract fraction to 30% saturation. After 30 min the suspension was centrifuged at 11,000 × g for 10 min and the supernatant was brought to 100% saturation with ammonium sulfate. After 30 min, the solution was centrifuged, the pellet dissolved in 1 liter of distilled water, dialyzed overnight against 10 liters of 50 mM NaCl solution, then concentrated in a bed of polyethylene glycol (Carbowax 20,000) for 24 hours. The concentrated material was then dialyzed against four changes of 1 liter of 50 mM potassium phosphate, pH 6.0. The precipitate formed during dialysis was eliminated by centrifugation and the supernatant (about 300 ml) was saved.

**Step III: CM-Sephadex Chromatography**—The ammonium sulfate fraction was applied to a column of CM-Sephadex C-50 (20 cm × 10 cm) equilibrated with 50 mM potassium phosphate, pH 6.0. The column was washed with 1.5 liters of the same buffer, then eluted at a flow rate of 2 ml/min with a 2-liter linear gradient: 50 mM potassium phosphate, pH 6.0, to 350 mM potassium phosphate, pH 7.0. The fractions containing the acid DNase, eluted after 250 mM phosphate, were pooled. They are contaminated with the protein inhibitor and other proteins interacting with the acid DNase.

**Step IV: Affinity Chromatography**—In order to separate DNase from its inhibitor and other interacting proteins, Fraction III was applied to a column (1.75 cm × 10 cm) of immobilized acid DNase (DSOS) which had been equilibrated with 300 mM potassium phosphate, pH 6.8. The column was then washed with 100 ml of the same buffer. Unadsorbed proteins were collected and dialyzed for 1 hour against three changes of 1 liter of 50 mM potassium phosphate, pH 6.8.

**Step V: Hydroxylapatite Chromatography**—Fraction IV was applied to a hydroxylapatite column (5 cm × 10 cm) previously equilibrated with 50 mM potassium phosphate, pH 6.8. The column was eluted at a rate of 50 ml/hour, successively with 200 ml of 100, 200, and 300 mM potassium phosphate, pH 6.8. Fractions of 3 ml were collected. Acid DNase was eluted with 300 mM potassium phosphate following a peak of inactive proteins. The fractions with the highest specific activities were pooled, dialyzed for 1 hour against three 1-liter changes of 100 mM sodium phosphate, pH 7.1 and concentrated with Carbowax to reduce its volume to about 2 ml.

**Step VI: Gel Filtration on Sephadex G-100**—Fraction V was applied to a column of Sephadex G-100 (5 cm × 100 cm) previously equilibrated with 100 mM sodium phosphate, pH 7.1. The column was then eluted with the same buffer at a flow rate of 10 ml/hour using a pressure head of 10 cm. Fractions (3 ml) were collected. Peaks of phosphodiesterase and DNase activities were eluted at 190 and 210 ml, respectively, resulting in a partial separation of the two activities (Fig. 1). The peak fractions which contain acid DNase without phosphodiesterase activity were pooled, concentrated with Carbowax and stored in 50% glycerol at −20°C.

**Purification of the Acid DNase Inhibitor**

A summary of the procedure is presented in Table II. Steps I, II, and III of the purification of the acid DNase and its inhibitor were identical. In the CM-Sephadex chromatography (Step III), it was not possible to measure quantitatively the specific inhibitor because of the interference with nonspecific inhibitors of the acid DNase (histones and other basic proteins). Therefore the presence of specific inhibitor was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The fractions containing inhibitor, usually eluted between 200 and 250 mM phosphate, were pooled (Fraction III).

**Step IV: Hydroxylapatite Chromatography**—Fraction III, previously dialyzed four times against 2 liters of 50 mM potassium phosphate, pH 6.8, was applied to a hydroxylapatite column (28 cm × 7.5 cm) equilibrated with the same buffer. The column was washed with 100 ml of the same buffer and the proteins eluted with a linear gradient of potassium phosphate 50 to 400 mM, pH 6.8 (total volume 1.5 liters). The flow rate was 70 ml/hour and fractions of 20 ml were collected. The contaminating acid RNase was eluted between 75 and 200 mM potassium phosphate while the acid DNase was eluted between 250 and 350 mM potassium phosphate. The amount of specific inhibitor contained in the fractions may be determined by microdensitometry of sodium dodecyl sulfate-polyacrylamide gels. Therefore, the presence of specific inhibitor was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The fractions located between 170 and 280 mM potassium phosphate were pooled, concentrated with Carbowax to about 70 ml and dialyzed for 1 hour in three 1-liter changes of 300 mM potassium phosphate, pH 6.8.

**Step V: Affinity Chromatography**—The Fraction IV was applied to an insoluble acid DNase (DSOS) column (5 cm × 10 cm) equilibrated with 300 mM potassium phosphate, pH 6.8. The column was washed with 100 ml of the same buffer and the adsorbed proteins were eluted successively (Fig. 2) by the following solutions: 200 mM guanidine-HCl, 140 mM sodium acetate, pH 7.1, and 750 mM guanidine-HCl, 500 mM sodium acetate, pH 7.1, and 750 mM guanidine-HCl, 500 mM sodium acetate.
activity was only 40%. Of incubation at this temperature the percentage of remaining stable up to 50°C but lost 95% of its activity at 60°C. After 5 min in 100 mM sodium phosphate, pH 7.1. The enzyme was determined by preincubation at various temperatures for 15 min in 50% glycerol at -20°C. To stabilize the enzyme during incubation at 37°C at pH 7.4. The protein pattern determined spectrophotometrically at 280 nm showed two peaks; the first one contained the pure inhibitor while the second one contained a small amount of inhibitor along with eight other proteins (interacting directly or indirectly with the insolubilized acid DNase). Both peaks were dialyzed for 1 hour in three 1-liter changes of 100 mM sodium phosphate, pH 7.1. The inhibitor was concentrated with Carbowax and stored in the same buffer in 50% glycerol at -20°C.

**Polyacrylamide Gel Electrophoresis**

The purity of acid DNase and its inhibitor was followed by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis. As shown on Fig. 3, A and B, both acid DNase and its protein inhibitor appeared as single bands. The gel pattern of proteins eluted from the insoluble acid DNase with 750 mM guanidine-HCl/500 mM sodium acetate solution, is presented on Fig. 3 C. It shows nine molecular species which were able to interact directly or indirectly with acid DNase in the chromatographic system, one of these proteins being the specific inhibitor, as checked by its influence on acid DNase kinetics (see below). Another protein (molecular weight, 59,000) possesses the phosphodiesterase activity (see below).

**Properties of Acid DNase**

Tests for Contaminating Enzymes—One milligram of pure acid DNase (which represents about 50,000-fold the amount commonly used in an enzymatic assay) contained practically undetectable activities of the following enzymes: acid phosphatase (less than 0.0025 unit), alkaline phosphatase (less than 0.001 unit), acid RNase (less than 0.002 unit), alkaline RNase (less than 0.002 unit), and neither exonuclease activity nor phosphodiesterase activity.

Stability—There was no detectable loss of activity for over 6 months when the acid DNase was stored in 50% glycerol at -20°C. To stabilize the enzyme during incubation at 57°C at pH 5, the addition of 100 μg of bovine serum albumin per ml of assay is required. The heat sensitivity of the acid DNase was determined by preincubation at various temperatures for 15 min in 100 mM sodium phosphate, pH 7.1. The enzyme was stable up to 50°C but lost 95% of its activity at 60°C. After 5 min of incubation at this temperature the percentage of remaining activity was only 40%.

**Molecular weight**—The molecular weight was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18). A value of 26,500 was obtained. This result is in good agreement with the estimation of molecular weight by gel filtration on Sephadex G-75 (27,000), and by sucrose density gradient zone centrifugation (2.85 S) according to Martin and Ames (20).

Effect of Different Ions, RNA, and Other Reagents—As observed with all acid DNases, divalent cations, and sulfate exhibit inhibitory effects. Hydrolysis of DNA was completely inhibited by 20 mM of either Ca²⁺, Mg²⁺, or Mn²⁺. Total yeast RNA was a potent competitive inhibitor of beef liver acid DNase (Kᵢ = 0.33 × 10⁻⁸ M), as already described for other acid DNases and RNAs (24, 25). Sodium p-hydroxymercuribenzoate (3 mM) and sodium iodoacetate (1 mM) are both strongly inhibitory (97% inhibition). On the other hand, 2-mercaptoethanol (2%) and EDTA (5 mM) did not affect enzyme activity.

Catalytic Properties—The optimal conditions for acid DNase activity were determined. The enzyme exhibited maximum activity at pH 4.9 to 5.1 in reaction mixture containing 100 mM sodium acetate buffer. At lower and higher ionic strength the rate of DNA hydrolysis was reduced. The effect of the secondary structure of DNA on enzymatic rate was studied. The initial velocity of DNA degradation was four times higher for native than for heat-denatured DNA. Experiments were carried out in order to control whether beef liver enzyme, as all acid DNases, yields oligonucleotides bearing 3'-P end groups (21). After extensive hydrolysis of fish sperm DNA (200 mg) during 23 hours at 37°C with acid DNase the reaction products were loaded to a Dowex 1 column then eluted with a stepwise gradient of ammonium acetate (pH 4.5). Many small oligonucleotides were produced but not mononucleotides. All these oligonucleotides were good substrate for spleen phosphodiesterase but not for snake venom phosphodiesterase. These experiments show that beef liver acid DNase produces endonucleolytic breakages giving oligonucleotides with 3'-phosphomonooester end groups.

The kinetics of the purified acid DNase obey Michaelis-
Properties of the Inhibitor

The protein nature of the inhibitor has been documented previously using an impure preparation (5).

Purity and Molecular Weight—The inhibitor (Fraction V) shows a single band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3B) and its molecular weight determined in the same way was 21,500. The results obtained after centrifugation in a sucrose density gradient in nondenaturing conditions have confirmed this value and showed consequently that the molecule does not have subunits. The Alcian blue staining used to reveal glycoproteins in acrylamide disc electrophoresis (22) was negative. Contaminating enzyme activities in 1 mg of purified inhibitor (which represents 400 times the amount normally used for inhibitor determinations) were either undetectable (phosphodiesterase, acid RNase, acid DNase), or extremely weak: acid phosphatase (less than 0.001 unit), alkaline phosphatase (less than 0.002 unit), exonuclease (less than 0.0001 unit), and alkaline RNase (less than 0.0004 unit).

pH Optimum and Ionic Strength Requirement—The inhibitor exhibited maximum activity in 100 mM sodium acetate buffer, 10 mM EDTA at pH 5.0. At pH 5.0, 5.3, and 5.7 the inhibitor exhibits, respectively, 50, 25, and 10% of its activity at pH 5.0. At pH 5.0 but in 0.2 M and 0.25 M sodium acetate the inhibitor exhibits, respectively, 90 and 40% of its maximum activity.

Influence of Inhibitor on Enzyme Kinetics—We have previously shown that the acid DNase inhibitor modifies the kinetics of pure acid DNase (5). In the presence of the inhibitor, a plot of V against S gave a sigmoid-shaped curve at pH 5.0. With increasing inhibitor concentration, the sigmoidal shape became more pronounced. Moreover, the enzyme-inhibitor interaction disappeared progressively with a small pH shift from 5.0 to 5.2 when the inhibitor exhibits, respectively, 50, 25, and 10% of its activity at pH 5.0. At pH 5.0 but in 0.2 M and 0.25 M sodium acetate the inhibitor exhibits, respectively, 90 and 40% of its maximum activity.

Evidence for Acid DNase: Inhibitor Interaction—As long as the inhibitor was not obtained in a highly purified form, the existence of a specific complex between the acid DNase and its inhibitor could not be ascertained, because the association of the molecules could be mediated by other proteins. The affinity chromatography experiments described above revealed that in addition to the inhibitor, eight proteins were able to bind to insoluble acid DNase. This ambiguity was resolved by rechromatography of the highly purified inhibitor on an insoluble acid DNase column, the strong binding of inhibitor, only eluted by a guanidine-HCl solution, attests clearly to the direct and specific interaction between acid DNase and its inhibitor. The latter was monitored by sodium dodecyl sulfate-acrylamide gel electrophoresis (Fig. 3D).

The stoichiometry of the acid DNase-inhibitor system was determined by gel filtration of both molecules on Sephadex G-75 according to Andrews (23). A molecular weight of 45,000

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the absence and in the presence of inhibitor. Fig. 7 shows that the inhibitor is also able to inhibit the phosphodiesterase. This result will be discussed below.

Other Proteins Interacting with Acid DNase

As shown in Fig. 3C the sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins eluted from the insoluble acid DNase column with a 750 mM guanidine-HCl/500 mM sodium acetate solution, pH 7.4, revealed that nine molecular species were able to interact in the chromatographic system. Among these proteins, only two were identified. One is the inhibitor, as checked by its influence on acid DNase kinetics, the other is a protein (molecular weight, 59,000) which exhibits phosphodiesterase activity towards bis(p-nitrophenyl) phosphate, as suggested by its chromatographic behavior (Fig. 1) and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis experiments (see below). The molecular weights of the seven other proteins were the following: 1, 17,000; 2, 46,000; 3, 71,000; 4, 79,000; 5, 89,000; 6, 100,000; 7, 112,000. Their other properties are unknown.

![Graph](https://example.com/graph1.png)

**Fig. 6.** Acid DNase kinetics as a function of substrate concentrations. Without inhibitor (○); with inhibitor (Fraction V), 50 μg/ml (■); with inhibitor (Fraction V), 50 μg/ml and adding of yeast total RNA, 1 μg/ml (■). Acid DNase (Fraction V), 0.3 unit. The assay was performed as described under "Experimental Procedures" with a 10-min incubation at 37°.

**Fig. 7.** Effect of the inhibitor on the unspecific phosphodiesterase activity at different concentrations of substrate. Acid DNase (Fraction V), 12.5 μg, and inhibitor (Fraction V) were used. The assay was performed as described under "Experimental Procedures", except that a 2-hour incubation at 37° was made.
Fig. 8. Identification of phosphodiesterase as a protein distinct from acid DNase. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out on samples from the acid DNase peak obtained after gel filtration on Sephadex G-100. (Fig. 1). This peak was divided into three parts and the samples (100 µl) contained, respectively, A, 305 units of acid DNase, 17 units of phosphodiesterase; B, 800 units of acid DNase, 4.5 units of phosphodiesterase; C, 610 units of acid DNase, no phosphodiesterase. For other experimental details see under “Experimental Procedures.”

### TABLE III

Effects of denaturing reagents on acid DNase and phosphodiesterase activities

The assays were performed on acid DNase (Fraction V) as described under “Experimental Procedures.”

| Reagents                | Concentration | Activity after |        |
|-------------------------|---------------|----------------|--------|
|                         |               | 30-min incubation at 37°C (acid DNase) | 120-min incubation at 37°C (phosphodiesterase) |
| Control                 |               | 100            | 100    |
| Sodium p-hydroxymercuribenzoate | 5 µM         | 3              | 8      |
| Sodium iodoacetate      | 1 µM          | 3              | 88     |
| Sodium iodoacetate      | 5 µM          | 3              | 74     |

Bis(p-nitrophenyl) phosphate, further work is obviously required because several enzymes are able to degrade this substrate as mentioned by Bernardi and Bernardi (31). According to our results, it does not look as if it is the acid exonuclease because no such activity was detectable in our DNAse preparation before the elimination of phosphodiesterase activity (Fraction V).

The properties of beef liver acid DNase are comparable with those described for enzymes extracted from various sources.

Concerning the inhibitor, the requirements for the highest efficiency have been defined and the stoichiometric measurements conclude that the enzyme-inhibitor complex consists of one molecule of acid DNase and one molecule of inhibitor. On the other hand the determination of interaction parameters in vitro indicates that the acid DNase possesses a higher affinity for the substrate than for the inhibitor.

These results, as well as the kinetic data, are consistent with the model of an enzyme-inhibitor complex composed of catalytic and regulatory subunits as already proposed (5). According to this hypothesis one molecule of inhibitor would be bound to one molecule of acid DNase. The quaternary structure of the latter is not yet completely understood. The peptide mapping of hog spleen acid DNase seemed to indicate that the enzyme was composed of two identical subunits (32) but molecular weight determination of the acid DNase under a variety of dissociating conditions (6 M guanidine with or without β-mercaptoethanol) failed to show any dissociation into subunits (33). These results could be reconciled if the assumption could be checked that the acid DNase is either made up of identical subunits bound through covalent linkage or contains large portions of the polypeptide chain in duplicate. The human serum transferrin raises similar problems (34). In all cases the model we suggest for the acid DNase-inhibitor complex would be suitable. Although the structure of the inhibitor is unknown, it can be suggested that, like the acid DNase, the inhibitor molecule might exhibit a second order symmetry. Upon binding to the enzyme, the inhibitor would induce a conformational change to a new state in which the catalytic sites would have less affinity for the substrate. The fact that RNA is able to reverse the action of the inhibitor and, like the DNA substrate, shows a high affinity for catalytic sites (24, 25), provides further support to this model.

The new procedure described herein for the extensive purification of beef liver acid DNase and its inhibitor leads to the availability of large quantities of these proteins. Furthermore affinity chromatography is a convenient means for the isolation of several proteins which directly or indirectly exhibit interacting properties towards acid DNase. The existence of such a system will facilitate studies concerning the role of acid DNase in the cells and the regulation of their activity. In this prospect a lot of various investigations are obviously required. The comparison of specificity of acid DNases in the absence and in the presence of inhibitor would be of a great interest, as mentioned by Lehman (35), concerning the possible involvement of this enzyme in other biological events than DNA degradation (DNA replication, recombination, cell division, etc...). Studies on purification and identification of a number of proteins able to interact with acid DNase are still necessary. The subcellular localization of all the components of the system will be attractive and might give preliminary indications on their physiological role. Inside the cell, they might be part, with acid DNase, phosphodiesterase, and inhibitor, of a multimeric complex including other enzymatic activities as well as structural proteins (36-38). This could explain the action of the inhibitor on the phosphodiesterase. This hypothesis is suggested by the fact that it is impossible to separate these proteins by polyacrylamide gel electrophoresis under nondenaturing conditions or by gel filtration on Sephadex G-100, the group of proteins being excluded as a large complex. According to another hypothesis, these proteins could be receptors of the enzyme located in subcellular organelles, membranes, or nucleus.

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