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PaeExo IX: a unique deoxyribonuclease from Pseudomonas aeruginosa active in the presence of EDTA

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ABSTRACT

A new deoxyribonuclease, PaeExoIX, has been purified to electrophoretic homogeneity from extracts of Pseudomonas aeruginosa strain PAO. This enzyme, which is active in the presence of EDTA, is equally efficient in hydrolyzing native and heat-denatured DNA to acid-soluble products. The enzyme is partially or totally inhibited by the presence of several divalent cations. The active protein has a molecular weight of $1.6 \pm 0.1 \times 10^5$ and is composed of two nonidentical polypeptides with molecular weights of 78,000 and 69,000.

INTRODUCTION

In 1976, Miller and Clark (1) began a survey of the major deoxyribonucleases of Pseudomonas aeruginosa. This study was begun in an attempt to assess the similarities or dissimilarities between the mechanisms of genetic recombination found in P. aeruginosa and Escherichia coli. They assumed that enzymes which carry out similar metabolic tasks should have similar substrate specificities and modes of action. While not all the enzymes active in the pathways leading to genetic recombination have been identified, three exonucleases have been implicated as playing a role in recombination in E. coli. These enzymes are EcoExoI (2), EcoExoV (4, 5, 6), and EcoExoVIII (7, 8). Miller and Clark (1) searched for analogues of these three E. coli enzymes in extracts of P. aeruginosa. They identified three exonucleases in cellular extracts of P. aeruginosa. Two of the nucleases were purified and characterized. PaeExoI (1) was shown to have properties similar to EcoExoI (2). Both of these ExoI-type enzymes require Mg^{++} for maximal activity and are active only on heat-denatured DNA. Neither enzyme requires ATP for activity. The second exonuclease isolated from P. aeruginosa, PaeExoV (1), is similar to EcoExoV (4, 9) in its requirements for ATP and Mg^{++}, and its use of native DNA as a substrate. The activities designated ExoV do not appear to be identical in E. coli

and P. aeruginosa, however, EcoExoV has exonucleolytic and endonucleolytic activity on heat-denatured DNA (9), while PaeExoV does not (1).

Miller and Clark identified but did not purify an ATP-independent exonuclease active on native DNA. This exonuclease has several unique properties when compared to exonucleases which have been identified previously.

MATERIALS AND METHODS

Bacterial strains. P. aeruginosa strains used in this study were JC9006 [pur-600; (1)], RM40 [pur-600, trp-6, met-28, lys-56, str-901; (10)], and PAO303 [argB18; ch1-2; (11)]. They are all derivatives of strain PAO (11). A thymine-requiring strain of E. coli B (RM1003) was used for preparation of DNA. DNA was also extracted from the Pseudomonas phage F116 (12).

Preparation of DNA. Radioactively-labeled P. aeruginosa and E. coli DNA were prepared by the method of Lehman (13) or by the method of Mamur (14) except that labeling of P. aeruginosa DNA was performed as described by Pemberton and Clark (15). Labeling of phage F116 DNA was carried out as described by Miller, Pemberton, and Richards (12) and purified as described by Hinkle and Miller (16). ϕX174 DNA was purchased from New England Biolabs. Single-stranded bacterial DNA was prepared by heating native DNA in a boiling water bath for 10 min in a solution of 0.14 M NaCl and 0.01 M sodium citrate at a concentration of 50 µg of DNA per milliliter of solution. The heat-denatured DNA was then quickly cooled in an ice water-sodium chloride bath until the temperature had fallen below -10°C.

Enzymes and other materials. Lysozyme, catalase, aldolase, bovine serum albumin (BSA), streptomycin sulfate, thyroglobulin, sarkosyl, and trizma base were obtained from Sigma Chemical Company. [^{3}H]-adenine, and [^{3}H]-thymine were purchased from Amersham. Diethylaminoethylcellulose (DEAE-cellulose) was Cellex D from Bio-Rad Laboratories. Sepharose 6B was from Pharmacia Fine Chemicals, Inc. Yeast nucleic acid was from Calbiochem. Molecular weight standards for SDS-polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories.

Exonuclease assay. Exonuclease activity on native or heat-denatured DNA was measured by the release of acid-soluble fragments from DNA. The standard reaction mix (0.5 ml) included potassium phosphate buffer (30 mM, pH 7.0), 2-mercaptoethanol (10 mM), and 6.6 nmol of [^{3}H]-DNA (approximately

6-8 x 10^3 cpm/nmol from either P. aeruginosa or E. coli). Usually disodium ethylenediaminetetraacetate (EDTA) was added to the standard reaction mixture at a final concentration of 10 mM. Reactions were incubated for 30 min at 37°C. Reactions were then terminated by placing them on ice and adding 0.2 ml of a solution of yeast nucleic acid (2 mg/ml) dissolved in 50 mM tris(hydroxymethyl)aminomethane hydrochloride (tris-HCl; pH 8.0), followed by 0.7 ml of 10% cold tricholoracetic acid. This mixture was allowed to sit on ice for 5 min. The acid-precipitable material was sedimented by centrifugation for 15 min at 3,000 rpm in a Sorval GSL-R3 centrifuge with a type HL-4 rotor. A sample (0.2 ml) of the supernatant fluid was counted in a Searle scintillation counter. One unit of enzyme converts 1 nmol of native DNA to acid-soluble products in 30 min at 37°C.

Endonuclease assay. Endonuclease activity was measured in a 0.1 ml reaction mixture containing potassium phosphate buffer (30 mM; pH 7.0), 2-mercaptoethanol (10 mM), EDTA (10 mM), and 1 µg of ϕX174 closed-circular virion or RF1 DNA (in separate assays). The sample to be tested for enzyme activity was added to start the reaction which was allowed to proceed at 37°C for 30 min. The reaction was stopped by adding a 10 µl portion of 5% sarkosyl, 25% glycerol, and 0.025% bromphenol blue. The samples were loaded on an 0.8% agarose gel and electrophoresed at 65 volts for 16 h (16). They were then stained with a solution of 0.5 µg ethidium bromide/ml of H_2O for 30 min, destained in H_2O for 30 min and photographed with a Polaroid MP-4 camera using Polaroid type 57 film and a UV-filter (16). Enzymatic activity was assayed as the relaxation of the closed-circular ϕX174 DNA.

Glycercol gradient. Linear glycerol gradients (20 to 40%) prepared in 30 mM potassium phosphate buffer (pH 7.0) were centrifuged in a Beckman SW60 swinging-bucket rotor at 50,000 rpm (260,000xg average) for 12 h in a Beckman L5-75 ultracentrifuge.

Other methods. Radioactivity was determined by the addition of 4.5 ml of scintillation fluid {18.2 g of 2.5 diphenyloxayale, 1.22 g of 1,4-bis-[2-(5-phenyloroyalel)]benzene, 2,500 ml of Triton X-100, and 4,280 ml of toluene} to 0.2 ml of aqueous-phase sample and counting the sample in a Searle Isocap/300 liquid scintillation counter. Protein determinations were made by the method of Lowry et al. (17), using BSA as a standard.

Preparation of cell extracts; (i) Method 1 (survey method). Strains were inoculated and grown in 25 ml of Luria complete broth (LB; ref. 10) in a shaking water bath (37°C) until stationary phase had been reached. Cells were harvested by centrifugation (11,000xg) and resuspension in 2.5 ml

of 0.85% NaCl. The cells were repelleted and suspended in 1 ml of 10% sucrose prepared in 50 mM Tris-HCl (pH 8.0). The cells were then frozen in dry ice and ethanol and thawed in a 25°C water bath. One-tenth of one milliliter of a solution of lysozyme (2 mg/ml) dissolved in water was added to the suspension. The mixture was allowed to incubate at 0°C on ice for 60 min. Cleared lysates were prepared by centrifugation at 35,000xg for 30 min and decantation of the supernatant fluid.

(ii) Method 2 (preparative method). Strain RM40 was used to inoculate large stainless steel pans (total surface area: 3.8×10^3 cm^2) containing 3 liters of Luria agar (10). These pans were allowed to incubate overnight at 37°C. Cells were removed by washing the surface of the pans with LB, centrifuging the cells at 8,000xg for 15 min, resuspending the cells in 100 ml of 0.85% NaCl, and reharvesting them by centrifugation (wet weight of cells 16-20 g). Cells were suspended at a concentration of 0.5 g/ml of a 10% sucrose solution prepared in 50 mM Tris-HCl (pH 8.0). The suspension was then frozen in a dry ice-ethanol bath and thawed in a 25°C water bath. The volume was measured and 0.1 ml of a solution of lysozyme (2 mg/ml, prepared in water) was added for each milliliter of cell suspension. The mixture was allowed to incubate at 0°C for 60 min. At this point, the solution became quite viscous due to release of DNA from lysed cells. All subsequent operations in the purification of the enzyme were carried out at 4°C. Cell debris and DNA were removed by centrifugation for 30 min at 35,000xg. The supernatant fluid was decanted (fraction I).

Fractionation steps used in enzyme purification; (i) Streptomycin sulfate precipitation. Cell supernatant fluid prepared by cell lysis method 2 (fraction I) was treated with a 5% (wt/vol) solution of streptomycin sulfate prepared in water. The streptomycin solution was added in a proportion of 1 ml/425 optical density units at 260 nm. The mixture was stirred for 20 min, and the precipitate was separated by centrifugation for 20 min at 27,000xg. The precipitate was discarded and the supernatant fluid was dialyzed against 2 changes of 1.5 liters each of 50 mM Tris-HCl buffer (pH 8.0), 0.1 mM dithiothreitol, 0.1 mM EDTA and 10% (wt/vol) glycerol for 6 h to produce fraction II.

(ii) First DEAE-cellulose chromatography. A DEAE-cellulose column was equilibrated with 50 mM Tris-HCl (pH 8.0), 0.1 mM dithiothreitol, 0.1 mM EDTA, and 30% glycerol (buffer A). Fraction II was made 30% (wt/vol) glycerol and was layered directly onto the column, followed by a 35 ml wash of buffer A. A 300 ml gradient of 0.05 to 0.3 M NaCl in buffer A followed.

A flow rate of 40 ml/h was maintained. Nuclease activity on native DNA eluted as a single broad peak from 0.1 to 0.2 M NaCl. Active fractions were pooled and concentrated in an Amicon Pressure Concentrator to 7 ml. The concentrate was dialzyed against 2 changes of 1 liter each of 0.03 M potassium phosphate buffer (pH 7.0), 0.1 mM dithiothreitol, 0.1 mM EDTA, and 30% (wt/vol) glycerol (buffer B) for 6 h to make fraction III.

(iii) Sepharose 6B chromatography. Fraction III was layered onto a column of Sepharose 6B which had been equilibrated in buffer B. The column dimensions were 1.6 x 86 cm. The column was eluted with buffer B and fractions (25 drops) were collected. The nuclease activity eluted about five fractions after blue dextran. Active fractions were pooled to make fraction IV.

(iv) Second DEAE-cellulose chromatography. A second DEAE-cellulose column (0.8 x 7.5 cm) was equilibrated with buffer B. Fraction IV (12 ml) was layered directly onto the column, followed by a 3 ml wash of buffer B. A 50 ml gradient of 0.0 to 0.7 M KCl in buffer B followed. A flow rate of 30 ml/h was maintained. The nuclease activity on native DNA which eluted between 0.05 and 0.12 M KCl was pooled and concentrated with a Millipore Submersible Concentrator to make fraction V.

(v) Glycerol gradient. Fraction V was layered onto a preparative 20-40% (wt/vol) linear glycerol gradient and centrifuged in a Beckman SW 41 swinging-bucket rotor at 40,000 rpm (193,000xg average) for 16 h in a Beckman L5-75 ultracentrifuge. Gradients were fractionated and active fractions were pooled to make fraction VI.

Polyacrylamide gel electrophoresis. Nondenaturing polyacrylamide gel electrophoresis (18) and SDS-polyacrylamide gel electrophoresis (19) were carried out as previously described except that 7.5% (wt/vol) acrylamide gels were used in all cases.

RESULTS

Detection of nuclease activity in cell lysates. Cell extracts of RM40 were prepared by Method 1 and surveyed to detect nuclease activity on native DNA. (Table 1). In the absence of added Mg^{++}, the level of ATP-independent nuclease activity was stimulated two- to three-fold over the activity detected in the presence of added Mg^{++}. The activity was resistant to inhibition by the addition of 10 mM EDTA to the reaction mixture and may have been slightly stimulated.

Table 1. Nuclease activity in cell lysates of RM40 under various conditions.[a]

Other Additions to Standard Reaction Mixtures	ATP not added	ATP (40 μM) added
	(Units/mg protein)	
$MgSO_4$ (10 mM)	5.6	8.7
None	13.5	14.2
EDTA (10 mM)	15.0	ND[b]

[a]Native DNA isolated from E. coli was used as the substrate.
[b]ND: Not done.

Cleared lysates were prepared by Method 1 from two additional strains of P. aeruginosa (JC9006, and PA0303) and were assayed for nuclease activity on native DNA in the presence of EDTA. Each was found to exhibit EDTA-resistant nuclease activity. These findings encouraged us to try to purify this nuclease activity.

Purification of the EDTA-resistant nuclease. To begin the purification, cell extracts of P. aeruginosa strain RM40 were prepared by method II as described above. The crude cell extracts were subjected to a variety of fractionation procedures (Table 2) and purification was followed by assaying the degradation of native DNA in the presence of EDTA. This procedure gave a 71-fold purification with 3% recovery.

Properties of the nuclease in Fraction V. The activity of the purified enzyme was increased by one-third when EDTA (10 mM) was added to the reaction mixutre. When Mg^{++} or several other divalent cations were added to the reaction mixture the enzymatic activity was reduced. (Table 3). Ferrous ion inhibited the activity completely. Because of these effects, 10 mM EDTA was added to all subsequent assays reported in this study.

The nuclease has a pH optimum between pH 8.0 and 9.0 and retains greater than 80% of its maximal activity in the pH range of 6.5 to 9.5. At pH 5.0, however, the enzyme has only 40% of its maximum activity, whereas at pH 10.5, it is 70% as active.

The nuclease is equally active on native DNA isolated from P. aeruginosa, E. coli, and the Pseudomonas phage F116. One-hundred percent of the native-DNA substrate is rendered acid-soluble in reactions allowed to proceed to completion. The enzyme degrades heat-denatured, linear DNA with the same

Table 2. Purification of the nuclease.[a]

Fraction	Description	Volume (ml)	Total Protein (mg)	Specific Activity (units/mg Protein)	Recovery (%)
I	Cell supernatant fluid	50	205	18	100
II	Streptomycin SO_4 supernatant fluid	49	132	25	86
III	DEAE-Cellulose I	7	32	90	75
IV	Sepharose 6B	12	6	401	59
V	DEAE-Cellulose II	4	1.0	782	20
VI	Glycerol gradient	2	0.047	1281	3

[a]All reactions were carried out in the presence of 10 mM EDTA in the standard reaction mixture using native E. coli DNA as the substrate.

efficiency as native DNA. To insure that these multiple activities are properties of the same protein molecule, a sample of the enzyme preparation was sedimented through a 20 to 40% glycerol gradient and fractions were collected. Each fraction was assayed on both native and heat-denatured DNA in the presence of EDTA (Figure 1). A common peak of activity was observed. Fractions demonstrating similar enzymatic activity were then pooled from

Table 3. Effects of divalent cations on exonuclease activity.

Reaction Mixture[a]	Relative Activity (Percent)
30 mM Tris-maleate-NaOH (pH 7.0)	100
+ 5 mM $MgCl_2$	80
+ 5 mM $MnCl_2$	50
+ 5 mM $ZnSO_4$	80
+ 5 mM $FeSO_4$	0
+ 5 mM $CaCl_2$	87

[a]Tris-maleate-NaOH buffer was substituted for potassium phosphate buffer because Mn^{++} and Ca^{++} formed precipitates in the presence of potassium phosphate. The reaction mixture also contained 6.6 nmol of native DNA isolated from E. coli and 10 mM 2-mercaptoethanol.

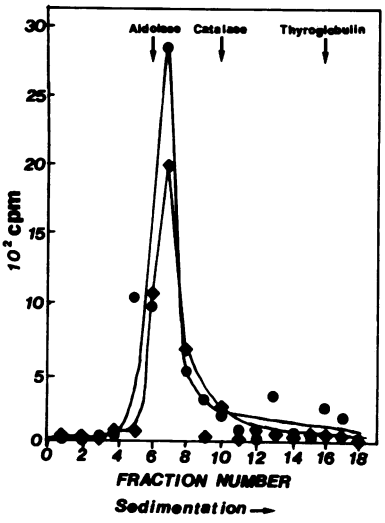

Figure 1. Sedimentation of the EDTA-stimulated deoxyribonuclease from fraction V in glycerol gradients (20 to 40%). The gradients were centrifuged in a Beckman SW60 rotor at 260,000xg as described in the text. Each fraction was assayed in the standard reaction mixture to which 10 mM EDTA and either native (●) or heat-denatured (◆) E. coli [^{3}H]-DNA had been added. Aldolase (7.35$S_{20,w}$; MW: 149,000), catalase (11.3$S_{20,w}$; MW: 247,000) and thyroglobulin (19.35 $S_{20,w}$; MW: 669,000) were sedimented in parallel gradients and used as sedimentation standards.

preparative glycerol gradients and electrophoresed on nondenaturing polyacrylamide gels. This pool contained only one discernible protein species.

Fraction V was tested for endonuclease activity as described in Materials and Methods. No enzymatic activity could be demonstrated when covalently-closed-circular ϕX174 RF1 DNA (double-stranded) or when closed-circular ϕX174 virion DNA (single-stranded) was used as a substrate in reaction mixtures containing 10 mM EDTA.

Molecular weight determination. The molecular weight of the enzyme in fraction V was estimated to be 1.6 ± 0.1 x 10^5 by determining its sedimentation coefficient in a linear 20 to 40% (wt/vol) glycerol gradient. (Figure 1). This molecular weight was calculated from the sedimentation coefficient (8S), using the method of Martin and Ames (20).

Fraction VI yielded a single band on nondenaturing polyacrylamide gels. Fraction VI was applied to a sodium dodecyl sulfate polyacrylamide gel and stained after electrophoresis as described in Materials and Methods. Two protein species were revealed——one with a molecular weight of 69,000 and the other with a molecular weight of 78,000 (Figure 2).

DISCUSSION

An EDTA-resistant deoxyribonuclease has been purified to electrophoretic homogeneity from P. aeruginosa strain RM40. This enzyme is

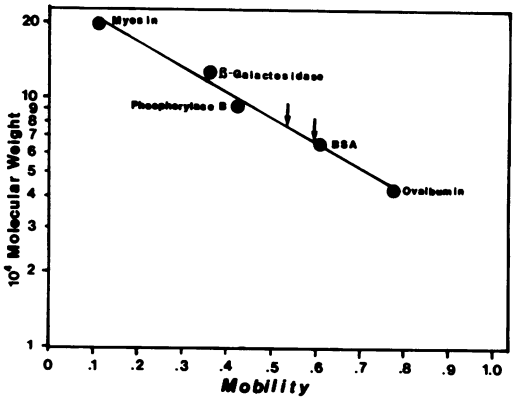

Figure 2. Molecular weight determinations of the denatured EDTA-stimulated nuclease from fraction VI by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Electrophoresis was carried out as described in the text using myosin (MW: 200,000), β-galactosidase (MW: 116,000), phosphorylase B (MW: 94,000), BSA (MW: 68,000), and ovalbumin (MW: 43,000) as standards. Two Coomassie Brilliant Blue-staining species were detected as indicated by the arrows.

equally active in the hydrolysis of native DNA and heat-denatured DNA. The enzyme cannot hydrolyze circular, single-stranded DNA or circular double-stranded DNA. Because a DNA molecule with a free terminus is required to initiate hydrolysis, we have classified the enzyme as an exonuclease and have named it Exonuclease IX (PaeExoIX) in accordance with the nomenclature proposed by Miller and Clark (1). This enzyme is different from other exonuclease activities described in P. aeruginosa (1) or E. coli (21).

PaeExoIX has been found in three different strains derived from the PAO strain of P. aeruginosa. Preliminary results using strain PAT, a second naturally occurring isolate of P. aeruginosa, indicate that PAT does not contain PaeExoIX. Whether this lack of activity is due to the absence of the nuclease or to the presence of an inhibitor is now being investigated. Mock purifications using PAT cell lysates through the first DEAE-cellulose column step do not reveal any EDTA-resistant activity (Scurlock and Miller, unpublished data).

The exonuclease PaeExoIX is most active when an agent capable of chelating divalent cations is added to the reaction mixture. This enzyme is unique among the exonucleases isolated from E. coli (20) and P. aeruginosa

(1). Of this group, only EcoExoVII shows any activity (albeit reduced) in the presence of EDTA (21). EcoExoVII is active only on heat-denatured DNA and is stimulated by Mg^{++} (21).

The data reported here indicate that PaeExoIX has a molecular weight of approximately 160,000 and is composed of two nonidentical polypeptides of slightly different sizes. The larger polypeptide has a mass of 78,000 daltons while the mass of the smaller subunit is 69,000 daltons.

PaeExoIX has some properties in common with EcoExoV in that it is active on native as well as heat-denatured DNA. However, it differs from EcoExoV in that it does not require Mg^{++} or ATP for activity and has no endonucleolytic activity. The ability of PaeExoIX to degrade both native and heat-denatured DNA exonucleolytically suggests the possibility that this enzyme may be a functional analogue of EcoExoV in the processes of recombination. Exonucleases which are active in degrading both native and heat-denatured DNA have been shown to have a function in the recombination of genetic material in several species of bacteria including E. coli (4, 6, 22, 23), Haemophilus influenzae (24, 25, 26), and Streptococcus pneumoniae (27, 28). While each of these enzymes also hydrolyzes ATP as an integral part of the hydrolysis of DNA, the function of this ATP hydrolysis is not clear. ATP hydrolysis can be uncoupled from the exonuclease activity of EcoExoV by presenting the enzyme with a nondegradable substrate such as crosslinked DNA (29). The process of recombination may not require that the hydrolysis of both ATP and DNA be a function of the same protein molecule. In fact, the ATP-independent exonuclease EcoExoVIII appears to substitute for the activities of EcoExoV associated with recombination in E. coli strains carrying recB or recC and sbcA mutations (30, 31) and LamExoI, the enzyme responsible for generalized recombination in coliphage lambda, is an ATP-independent exonuclease (32, 33). The role of PaeExoIX in the pathways of recombination which occur in P. aeruginosa is now under investigation.

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