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Purification and characterization of an endo-exonuclease from adult flies of *Drosophila melanogaster*

Ke Shuai+, Chanchal K.Das Gupta, R.Scott Hawley, John W.Chase*, Kathryn L.Stone[1] and Kenneth R.Williams[1]
Department of Molecular Biology and Genetics, Albert Einstein College of Medicine, Bronx, New York, NY 10461 and [1]Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, CT 06520, USA

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ABSTRACT

An endo-exonuclease (designated nuclease III) has been purified to near homogeneity from adult flies of *Drosophila melanogaster*. The enzyme degrades single- and double-stranded DNA and RNA. It has a sedimentation co-efficient of 3.1S and a stokes radius of 27Å The native form of the purified enzyme appears to be a monomer of 33,600 dalton. It has a pH optimum of 7 – 8.5 and requires Mg^{2+} or Mn^{2+} but not Ca^{2+} or Co^{2+} for its activity. The enzyme activity on double-stranded DNA was inhibited 50% by 30 mM NaCl, while its activity on single-stranded DNA required 100 mM NaCl for 50% inhibition. Under the latter conditions, its activity on double-stranded DNA was inhibited approximately 98%. The enzyme degrades DNA to complete acid soluble products which are a mixture of mono- and oligonucleotides with 5'-P and 3'-OH termini. Supercoiled DNA was converted by the enzyme to nicked and subsequently to linear forms in a stepwise fashion under the condition in which the enzyme works optimally on single-stranded DNA. The amino acid composition and amino acid sequencing of tryptic peptides from purified nuclease III is also reported.

INTRODUCTION

The study of DNA metabolic proteins in the fruit fly, *Drosophila melanogaster*, has advanced over the last several years with the isolation of DNA polymerase α (1) and β (2), DNA topoisomerase I and II (3–6), RNA polymerase (7), RNase H (8), histones (9), nonhistone chromosomal proteins (10), DNA ligase (11 & 12), and many others. In fact, the detailed genetic characterization of this highly differentiated and complex organism makes it an ideal system for studying a seminal biological process like DNA metabolism.

Studies with many deoxyribonucleases from both prokaryotes and eukaryotes have shown that a number of these enzymes are involved in DNA recombination, repair, and replication (13–15). In *D. melanogaster*, strains having reduced activity of two deoxyribonucleases, DNase I and DNase II, have been identified (16). However, neither of these have been purified or characterized beyond the level of crude extracts. In this study, we report the purification, biochemical characterization and amino acid sequence of tryptic peptides of a nuclease (designated nuclease III), which is different from DNase I and DNase II in terms of its pH and cationic requirements, from wild type adult flies of *Drosophila melanogaster*. In its mechanism of action nuclease III resembles a number of endo-exonucleases which have been purified and characterized recently from *N. crassa* and yeast (17 & 18; 27). The implication that nuclease III is a member of this class of nucleases and the possible function of nuclease III are discussed.

MATERIAL AND METHODS

Collection of *Drosophila melanogaster* adult flies

Wild type *Drosophila melanogaster* adults were collected about seven days after the embryos hatched to larvae and immediately stored at −70°C.

Nucleotides

Unlabeled deoxy- and ribonucleoside triphosphates were from PL Biochemicals. [γ-^{32}P] ATP, [α-^{32}P]ddATP and [^{3}H] uracil were from Amersham. [Methyl-^{3}H] thymidine was from New England Nuclear.

Nucleic acids

Salmon testes DNA was from Sigma. T7 [^{3}H]DNA was prepared as described (19). *E. coli* [^{3}H]RNA was made by labeling growing cells with [^{3}H] uracil followed by hot phenol

* To whom correspondence should be addressed at Department of Biochemistry, Case Western Reserve University, School of Medicine, Cleveland, OH 44121, USA

+ Present address: Laboratory of Molecular and Cellular Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10021-6399, USA

extraction and ethanol precipitation. When necessary, the terminal phosphates from T7 [^{3}H]DNA were removed and 5'- and 3'-ends were labeled with ^{32}P as described (20). Plasmid pGEM-3 was kindly provided by Daniel Diaz of this department.

Chromatography media

DEAE-52 cellulose was purchased from Whatman; PBE-94 and Sepharose CL-6B were from Pharmacia; Bio-Rex 70 was from Bio Rad. Single-stranded DNA cellulose was prepared as described (21).

Enzymes and protein standards

Snake venom phosphodiesterase and calf intestine alkaline phosphatase were from Boehringer Mannheim. Spleen phosphodiesterase and terminal transferase were from United States Biochemical Corporation. Bacterial alkaline phosphatase was from Sigma. T4 polynucleotide kinase was from PL Biochemicals. Bovine serum albumin (BSA) was from Miles; Cytochrome C was from Worthington.

Chemicals

Tris (ultra pure) was from Schwarz Mann. Imidazole (grade III), phenylmethylsulfonyl fluoride (PMSF), benzamidine and agarose were from Sigma. Sodium bisulfite (analytical grade) was from Baker. Dithiothreitol (DTT) was from Boehringer Mannheim. Acrylamide, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylene diamine, Coomassie Brilliant Blue G-250 and sodium-dodecyl sulfate (SDS) were from Bio-Rad.

Assay of nuclease III

The standard reaction mixture contained 20 mM Tris HCl (pH 7.5), 60 μM [^{3}H]DNA or [^{3}H]RNA, 1 mg/ml BSA, 5 mM $MgCl_2$, 1 mM DTT and the enzyme in a total volume of 100 μl. Incubation was at 30°C for 30 min. The reaction was stopped by adding 50 μl of 300 μg/ml salmon testes DNA and 50 μl of 40% trichloroacetic acid. After 10 min. on ice, the solution was centrifuged at 9000 rpm for 10 min. in a Sorvall SM-24 rotor. All of the supernatant fluid was removed and the radioactivity was determined by counting in Hydrofluor (National Diagnostics). The enzyme was diluted in a buffer containing 50 mM Tris-HCl (pH 7.5), 10% glycerol, 0.1 mM EDTA, 1 mg/ml BSA and 1 mM DTT. A unit of enzyme is defined as the amount which makes 1 nmole (nucleotides) of single-stranded DNA acid soluble in 30 min. at 30°C at pH 7.5.

SDS-polyacrylamide gel electrophoresis

Polyacrylamide gels containing SDS were prepared and run using the buffer system of Laemmli (22). The stacking gel contained 3% polyacrylamide and was 1.5 cm in height. The resolving gel contained 12% acrylamide. Gels were silver stained as described (23).

Agarose gel electrophoresis

DNA samples were analyzed on 0.8% agarose gels containing 36 mM Tris-base, 30 mM NaH_2PO_4, 1 mM Na_2EDTA. DNA was stained with ethidium bromide.

Amino acid analysis

Amino acid analysis of nuclease III was carried out on a Beckman 121M analyzer following hydrolysis in 6 N HCl, 0.2% phenol for 24 hrs at 110°C.

Amino acid sequencing of tryptic peptides

Tryptic peptides were obtained from 175 pmol nuclease III that had been dissolved in 25 μl 8 M urea, 0.4 M NH_4HCO_3, pH 8.0. After adding 5 μl of 45 mM dithiothreitol, the sample was incubated at 50°C for 20 min prior to carboxamidomethylating with 5 μl of 100 mM iodoacetamide. After incubating for 20 min at room temperature, the sample was diluted with water to a final urea concentration of 2 M and then digested for 24 hrs at 37°C with a 1:25 (w:w) ratio of trypsin. The digest was stopped by injecting onto a 5 micron particle size Vydac C-18 column (4.6 mm×25 cm) that had been equilibrated in 0.05% trifluoracetic acid containing 2% buffer E (0.05% trifluoracetic acid, 80% acetonitrile) at a flow rate of 0.50 ml/min. The resulting peptides were eluted from a Waters Associates HPLC system with increasing concentrations of buffer E as follows: 0–60 min (0–37.5% E), 60–90 min (37.5–75% E), 90–105 min (75–98% E) and were collected in 1.5 ml Eppendorf tubes using an ISCO Model 2150 peak separator and an ISCO 'Foxy' fraction collector. Those absorbance peaks selected for sequencing were loaded directly onto an Applied Biosystems Model 470A Proteins/Peptide Sequencer in volumes ranging from 0.2–0.4 ml.

Other methods

Protein was determined by the method of Bradford (24). Bovine serum albumin was used as the protein standard. All pH measurements were made at a buffer concentration of 0.05 M at room temperature. Freshly prepared protease inhibitors were added to buffers (at 4°C) immediately before using. The ionic strength of buffers was determined with a Radiometer conductivity meter.

RESULTS

Purification of *Drosophila Nuclease III*

The results of a typical purification are summarized in Table I. Unless otherwise indicated, all operations were performed at 4°C.

Preparation of Crude Extract. Adult flies (40 g, frozen at −70°C overnight) from wild type *D. melanogaster* were crushed with a mortar and pestle and suspended in 190 ml Buffer A containing 30 mM Tris-HCl (pH 8.1), 10 mM EDTA, 0.25 M sucrose, 0.5 mM DTT, 5 mM KCl, 0.5 mM magnesium acetate, 1 mM PMSF, 10 mM sodium bisulfite and 2 mM benzamidine. The suspension was homogenized six times with a loose-fitting pestle and then six times with a tight-fitting pestle in a glass Dounce homogenizer and filtered though Nitex screen (75 μm). The filtrate was kept on ice. To the residue was added 50 ml of Buffer A and it was homogenized as before. After filtering through Nitex

Table I. Purification of nuclease III from adult flies of wild-type *Drosophila melanogaster*

Fraction	Activity Units	Protein (mg/ml)	Specific Activity (Units/mg)	Yield
I. Crude Extract*	–	11.6	–	–
II. High Speed Spin*	–	6.9	–	
III. DEAE-52 Cellulose & Bio-Rex-70	1.1×10^5	7.9×10^{-2}	6.9×10^3	100
IV. PBE-94 & Single-Stranded DNA Cellulose	6.0×10^4	7.5×10^{-3}	8.0×10^6	54.5

*The activity of nuclease III could not be assayed in these fractions.

screen, the two filtrates were pooled together and centrifuged at 9,000 rpm for 30 min. in a Sorvall SS-34 rotor. The supernatant was filtered through Nitex screen. The final volume of the filtrate was 210 ml (Fraction I).

Preparation of the high speed supernatant fraction. Fraction I (210 ml) was centrifuged in a Beckman 60 Ti rotor at 40,000 rpm for 60 min. The supernatant was filtered through Nitex screen. The final volume of filtrate was 190 ml (Fraction II). We could not assay the enzyme activity in Fraction I and II, presumably because of the presence of large amounts of nucleic acids or inhibition of nuclease activity.

DEAE-52 cellulose and Bio-Rex 70 chromatography. Fraction II was applied to a DEAE-52 column (2.5×31 cm) equilibrated with 30 mM Tris-HCl (pH 8.1), 0.1 mM EDTA, 1 mM DTT, 10% glycerol (Buffer B). The column was washed with 450 ml Buffer B containing 1 mM PMSF, 10 mM sodium bisulfite and 2 mM benzamidine. The flow through and wash were pooled and directly applied to a Bio-Rex 70 column (2.5×20 cm) equilibrated with 50 mM imidazole (pH 6.8), 0.1 mM EDTA, 1 mM DTT and 10% glycerol (Buffer C). The column was washed with Buffer C containing 150 mM NaCl, 1 mM PMSF, 10 mM sodium bisulfite and 2 mM benzamidine. An 800 ml gradient of 0−1 M NaCl in Buffer C was applied to the column and 11 ml fractions were collected. The enzyme activity eluted at about 0.5 M NaCl and was pooled in a total volume of 200 ml (Fraction III).

PBE-94 and single-stranded DNA cellulose chromatography. Fraction III was dialyzed against 2 liters of 30 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM DTT, 10% glycerol (Buffer D) with 1 mM PMSF and 10 mM sodium bisulfite added for seven hours and applied to a PBE-94 column (0.9×11 cm) equilibrated with Buffer D. The column was washed with 24 ml of Buffer D. The flow through and wash were pooled and directly applied to a single-stranded DNA cellulose column (2.0×6.5 cm). The column was washed with 60 ml of Buffer D containing 0.2 M NaCl and a 200 ml linear gradient of 0−1.5 M NaCl in Buffer

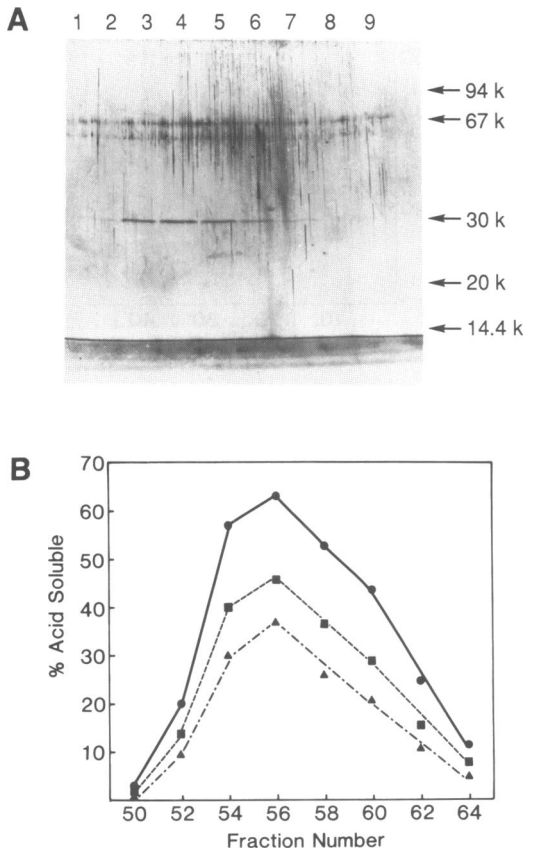

Figure 1. (A) SDS-polyacrylamide gel analysis of nuclease III. Eight active fractions from the DNA-cellulose column were analyzed on a SDS-polyacrylamide gel (40 μl each, lane 1−8 corresponding to fraction number 50, 52, 54, 56, 58, 60, 62, 64, respectively). 10 μl sample buffer alone was loaded on lane 9. The migration and size of molecular weight standards are indicated on the right. (B) Activity profile of fractions from DNA cellulose column. Activities of each fraction noted above were assayed. Assays were done as described under 'Materials and Methods'. ●--●, ssT7 [^{3}H]DNA; ■--■, dsT7 [^{3}H]DNA; ▲--▲, *E. coli* [^{3}H]RNA.

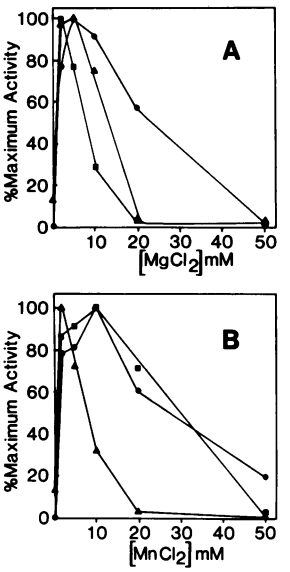

Figure 2. Effect of the concentration of divalent cations on nuclease III activity. Assays were done as described under 'Materials and Methods' varying the concentrations of $MgCl_2$ and $MnCl_2$ as indicated. 37 mM NaCl was included in each assay. ●--●, ssT7 [^{3}H]DNA; ■--■, dsT7 [^{3}H]DNA; ▲--▲, *E. coli* [^{3}H]RNA. A, Mg^{2+}; B, Mn^{2+}.

Table II. Requirements for nuclease III activity. Nuclease III was assayed under standard condition at pH 7.5 using single-stranded T7 or double-stranded T7 [^{3}H]DNA or *E. coli* [^{3}H]RNA except for the additions and deletions noted.

Condition	Relative Activity (%) on ss T7DNA	ds T7DNA	*E. coli* RNA
Complete system	100	100	100
−$MgCl_2$	0	0	13
+5 mM $CaCl_2$	5	7	0
+5 mM$CoCl_2$	0	0	0
+10 mM EDTA	4	0	0
−DTT+1 mM N-ethylmaleimide	12	6	14
+1 mM ATP	96	90	105
+1 mM dATP	100	100	100
−BSA	12	7	1
−Enzyme	0	0	0

D was applied to it. Fractions (2.5 ml) were collected. The enzyme activity eluted at about 700 mM NaCl and was pooled in 25 ml. The enzyme was then concentrated by Amicon filtration to a final volume of 1 ml (Fraction IV).

Polyacrylamide Gel Electrophoresis. The active peak fractions from the single-stranded DNA cellulose column were analyzed by 10% SDS-polyacrylamide gel electrophoresis (Fig. 1A). Bands just ahead of the 67,000 molecular weight marker were two artifactual bands of silver-staining material as indicated by using sample buffer as a control (Lane 9). The major polypeptide present in the peak fractions migrated slightly above the Mr 30,000 standard. The active peak fractions were assayed for nuclease activities on single- and double-stranded DNA and RNA (Fig. 1B). The correlation of activities with intensities of the major protein band across the peak fractions on the SDS gel strongly suggests that this protein is responsible for all three activities of nuclease III.

Characterization of *Drosophila* nuclease III

Reaction requirements. The purified enzyme required divalent cations, Mg^{2+} or Mn^{2+}, for activity (Fig. 2). The maximum stimulation of the activity on single-stranded DNA occurred at concentrations of 2–10 mM for Mg^{2+} and 2–15 mM for Mn^{2+}. For double-stranded DNA the maximum stimulation occurred at concentrations of 2–5 mM for Mg^{2+} and 2–15 mM for Mn^{2+}, whereas for *E. coli* RNA optimal activity occurred at concentrations of 2–10 mM for Mg^{2+} and 2–5 mM for Mn^{2+}. Ca^{2+} and Co^{2+} could not fulfill the divalent cation requirement (Table II). All three activities were inhibited by 10 mM EDTA. A sulfhydryl reagent, such as dithiothreitol, was apparently required for all three activities since addition of 1 mM N-ethyl-maleimide and the absence of DTT from the standard reaction mixture lead to the inhibition of all three activities. 1 mM ATP or 1 mM dATP did not alter the enzyme activity significantly. BSA was necessary to prevent enzyme inactivation. In the absence of BSA, the enzyme was almost immediately inactivated after dilution in the reaction mixture (Table II). The enzyme has been stored in Buffer D in the presence of BSA (0.5 mg/ml) at −70°C for 7 months with only 10% loss of activity.

All three activities of nuclease III were found to be optimally active in the range of pH 7–8.5 (data not shown). Activity on double-stranded DNA was inhibited by low concentrations of NaCl, whereas 50% of the activity on both single-stranded DNA and *E. coli* RNA was still observed at 100 mM NaCl (Fig. 3).

Heat Inactivation. Nuclease III was incubated at 30°C and 37°C for 40 min. and its activity was assayed on DNA and RNA at various times. As shown in Fig. 4 the rates of heat inactivation were identical with DNA and RNA. The fact that nuclease activity was equally affected on single- and double-stranded DNA and RNA at 30°C and 37°C further suggests that the activity on each substrate was due to a single enzyme.

Determination of molecular weight and frictional coefficient. The Stokes radius of nuclease III was determined to be 27Å by Sepharose CL-6B gel filtration chromatography utilizing standards of known Stokes radii (data not shown). The sedimentation coefficient of the enzyme was determined to be 3.1S by glycerol gradient (10–30%) sedimentation including

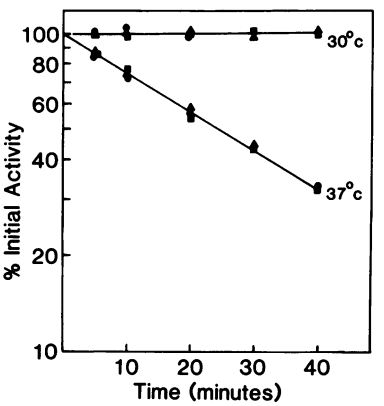

Figure 4. Heat inactivation of nuclease III. Nuclease III was incubated at 30°C and 37°C in dilution buffer. Aliquots were withdrawn and assayed to measure activity on single- and double-stranded DNA and RNA as described under 'Materials and Methods'. ●--●, ssT7 [^{3}H]DNA; ■--■, dsT7 [^{3}H]DNA; ▲--▲, *E. coli* [^{3}H]RNA.

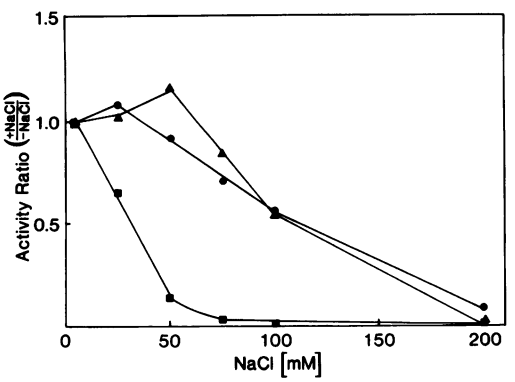

Figure 3. Effect of NaCl on the activity of nuclease III. Assays were performed as described under 'Materials and Methods' varying the concentrations of NaCl as indicated. ●--●, ssT7 [^{3}H]DNA; ■--■, dsT7 [^{3}H]DNA; ▲--▲, *E. coli* [^{3}H]RNA.

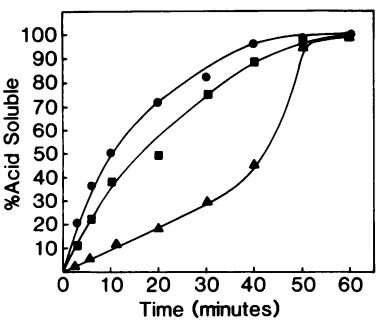

Figure 5. Activity of nuclease III toward different substrates. Standard reactions were set up at pH 7.5 in 100 µl with 80 µM [^{3}H]DNA and 57 µg/ml *E. coli* [^{3}H]RNA and 11 units of nuclease III at 30°C. 10 µl aliquots were withdrawn at times indicated to determine acid soluble radioactivity. At 30 min., 11 and 22 units of additional enzyme were added to [^{3}H]DNA and [^{3}H]RNA reaction mixtures, respectively. ●--●, ssT7 [^{3}H]DNA; ■--■, dsT7 [^{3}H]DNA; ▲--▲, *E. coli* [^{3}H]RNA.

appropriate standards of known sedimentation coefficient (data not shown). The molecular weight calculated from the Stokes radius and sedimentation coefficient using the method of Siegel and Monty (25) assuming a partial specific volume of 0.725 cm^3/g was 33,600. The calculated frictional ratio f/f_0 was 1.25. From SDS-polyacrylamide gel analysis, the molecular weight of the protein was estimated to be about 32,000. The native form of the protein, therefore, is probably a monomer. It was noted that the activity of nuclease III on DNA and RNA could not be separated by either gel filtration or sedimentation (data not shown). This again supports the notion that the activity on each substrate was due to a single enzyme.

Activity of nuclease III on various substrates. Purified nuclease III completely hydrolyzed native T7 DNA, sonicated and denatured T7 DNA and *E. coli* RNA into acid soluble products (Fig. 5).

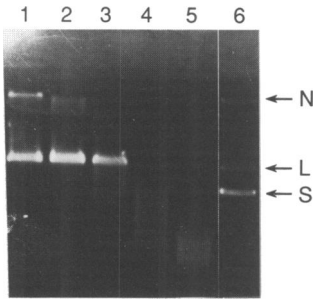

Figure 6. Activity of nuclease III on supercoiled DNA. pGEM-3 plasmid DNA (6.7 μg) in 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 75 mM NaCl, 1 mM DTT and 1 mg/ml BSA was incubated with 8 units of fraction V at 30°C in a reaction volume of 50 μl.. At 1, 3, 6, 10, 20 min., 10 μl were withdrawn and applied to lane 1, 2, 3, 4, 5 of a 0.8% argarose gel. Lane 6 contained untreated pGEM-3 plasmid DNA. Abbreciations: N, nicked; L, linear; S, supercoiled.

Table III. Analysis of DNA ends generated by nuclease III

DNA	Enzymes Added	% Acid Soluble
Sonicated and Denatured [^{3}H]T7	Nuclease III[1]	50
	Nuclease III[2]	50
	Nuclease III+ Snake Venom Phophodiesterase[3]	100
	Nuclease III+ Spleen Phosphodiesterase[4]	53
	Nuclease III+ Bacterial Alkaline Phosphatase + Spleen Phosphodiesterase[5]	100

Reaction mixture (100 μl) containing 63 μM sonicated and denatured T7 [^{3}H]DNA was treated with 10 units of nuclease III for 10 min. The enzyme was inactivated by heating the reaction mixture for 10 min. at 68°C. It was then divided into 5 equal parts. [1]One part was kept on ice. [2]The second part was incubated for another 90 min. at 30°C. [3]The third part was incubated for 30 min. at 37°C after adding 4 μl of 0.5 M sodium acetate (pH 6.5), 2.5 μl of 100 mM $MgCl_2$ and 1 μl (5.7 units/ml) of snake venom phosphodiesterase. [4]5 μl of 0.5 M sodium acetate (pH 6.5) and 0.5 μl of spleen phosphodiesterase (10 units/ml) were added to the fourth part and incubated for 30 min. at 37°C. [5]2 μl of 0.5 M Tris-HCl (pH 8.5) and 10 μl of bacterial alkaline phosphatase (1 unit) were added to the fifth part. After 60 min. at 37°C, 0.6 μl of 2 M sodium acetate (pH 4.5) and 0.5 μl (10 units/ml) of spleen phosphodiesterase were added and incubated for another 30 min. Acid soluble radioactivity was measured for all the samples.

Superhelical plasmid DNA was incubated with nuclease III under conditions where the enzyme worked most efficiently on single-stranded DNA. Various time points were analyzed by agarose gel electrophoresis (Fig. 6). The DNA was quickly converted to linear DNA before it was completely degraded. It appeared from the electrophoretic pattern of the DNA on the agarose gel that one strand of the DNA was preferentially nicked before it was linearized (Fig. 6, lane 1).

Purified nuclease III was also able to degrade single-stranded M13 circular DNA (data not shown). The fact that this enzyme could degrade supercoiled plasmid DNA and single-stranded M13 circular DNA suggests that nuclease III has an endonucleoytic mechanism of action.

Hydrolysis of the 5′ and 3′ termini of DNA. Both native and denatured T7 [^{3}H, 5′-^{32}P] DNA and native and denatured T7 [^{3}H, 3′-^{32}P] DNA were treated with nuclease III. Both 5′ and 3′ termini were preferentially hydrolyzed compared to the rest of the DNA molecule in the case of both double-stranded and single-stranded DNA. This suggests that nuclease III has preference for termini of DNA molecules and thus functions exonucleolytically as well. The preference of nuclease III for 5′ termini was somewhat greater than that of 3′ termini and this difference was more pronounced on single-stranded DNA (data not shown).

Product distribution. Limit digests of both single- and double-stranded DNA yielded mixtures of mono- and oligonucleotides (data not shown).

Analysis of DNA ends generated by nuclease III. Sonicated and denatured [^{3}H] T7 DNA was hydrolyzed to render 50% of the radioactivity acid soluble. The products were rendered totally acid soluble by subsequent treatment with snake venom phosphodiesterase. Spleen phosphodiesterase, however, did not

Table IV. Amino acid analysis of nucleae III. The following data are based on triplicate or in the case of threonine, serine and glutamic acid, duplicate amino acid analyses. The value cited for glycine is the lowest obtained. It was assumed that the higher levels of glycine found in the other two amino acid analyses resulted from background contamination. The standard deviations given below are higher than expected due to the relatively small amount of protein, from 0.4 to 1.0 μg, analyzed. For this latter reason it was also not possible to quantitate proline.

	Number of Residues	
	Predicted[a]	Std. Dev.
Asp	26.7	1.3
Thr	12.6	1.9
Ser	18.8	2.3
Glu	29.9	0.5
Pro	N.D.[b]	
Gly	46.6	–
Ala	21.8	1.9
Val	16.7	2.3
Met	3.6	0.6
Ile	12.6	2.6
Leu	21.6	2.2
Tyr	9.2	0.4
Phe	11.6	2.2
His	9.5	0.4
Lys	24.2	3.2
Arg	12.1	1.0

[a]Calculated assuming a molecular weight of 33,600.
[b]N.D. = Not determined.

hydrolyze the products unless they were pretreated with bacterial alkaline phosphatase (Table III). The data support the conclusion that the enzyme leaves 5'-P and 3'-OH termini on the DNA.

Protein chemistry studies of nuclease III. The amino acid composition of nuclease III is shown in Table IV. Table V summarizes the amino acid sequencing results obtained from the four absorbance peaks that are numbered in Fig. 7. Peaks numbered 1 and 3 each contained a single major tryptic peptide whose partial sequence is shown in Table V. The remaining two peaks sequenced proved to consist of a mixture of two peptides. In the case of peak 2, both peptides were present in similar amounts and so a primary sequence could not be called. Data from this sequencing run is included in Table V as it should help to confirm any cDNA clone that might be isolated and that is thought to correspond to nuclease III. Although peak 4 also contained two peptides, the ratio of the yield of the sequence corresponding in Table V to 4a to that corresponding to 4b was sufficiently high, approximately 3, that a major and minor sequence could be called with confidence. After correcting for an anticipated coupling yield on sequencing of 50%, the overall recovery of the peptides whose sequences are given in Table V averaged about 10%. While this extent of recovery was lower than the average recovery of about 33% that we previously obtained with another protein (26) from a 555 pmol digest (as compared to the 175 pmol of nuclease III that was digested), it is sufficiently high to ensure that the sequences given in Table V derive from the single Coomassie blue-staining band present in the purified nuclease III preparation.

DISCUSSION

We have purified nuclease III from adult wild type *Drosophila melanogaster* to near homogeneity. The enzyme appears to be a single polypeptide of molecular weight 33,600.

Nuclease III degrades single- and double-stranded DNA and RNA to complete acid solubility (Fig 5). The results presented in this paper show that all three activities are similar in their optimal pH and heat inactivation. All three activities co-sediment in a glycerol gradient and co-chromatograph on a molecular sieving column (data not shown). Taken with the fact that all three activities co-purify in our purification procedure, these results strongly support the conclusion that all three activities are due to a single enzyme molecule.

Under standard reaction conditions an increase in NaCl concentration inhibited nuclease III activity on double-stranded DNA, whereas its activity on single-stranded DNA was not inhibited with concentrations of NaCl up to 50 mM. It is interesting to note that at ionic strengths in a range expected to be physiologically significant, nuclease III is much more active on single-stranded DNA than on double-stranded DNA. Thus its activity on single-stranded DNA may reflect its *in vivo* activity.

Our results suggest that nuclease III degrades DNA by an endo-exonucleoytic mode of action. Its 5' exonuclease activity on both double- and single-stranded DNA is greater than that of its 3' exonuclease activity. A number of endo-exonucleases which are similar in many properties have been reported recently, including the *N. crassa* ss-DNA-binding endo-exonuclease (17), the yeast endo-exonuclease (18) and the yeast major mitochondrial nuclease (27). All these endo-exonucleases have RNase activity. They all cross react with an antibody raised against the *N. crassa* endo-exonuclease, suggesting they all belong to a class of related proteins. Nuclease III resembles this class of nucleases in its properties. Nuclease III is a polypeptide of molecular weight of 33,600. The *N. crassa* endo-exonuclease is a polypeptide of 31,000–33,000 daltons. Both enzymes have endo- and exonuclease activity on single- and double-stranded DNA. Their DNA digestion products have 5'-P and 3'-OH termini. Both enzymes require either Mg^{2+} or Mn^{2+} and have pH optimum of approximately 7.0–8.5. The yeast major mitochondrial nuclease has an apparent molecular weight of 39,000 and has a pH optimum of 7.0–7.5. It also has endonuclease activity on both single- and double-stranded DNA and 5' exonuclease activity on double-stranded DNA. Its digestion products also have 5'-P and 3'-OH termini. The yeast endo-exonuclease has a molecular weight of 72,000. It has endonuclease activity on single-stranded DNA and exonuclease activity on double-stranded DNA. The enzyme has a pH optimum of 7.5 and requires either Mg^{2+} or Mn^{2+} for its activity.

It was suggested (28) that in eukaryotes there is widespread presence of a mitochondrial DNase/RNase. Drosophila nuclease III is a DNase/RNase and shows many similarities with mitochondrial nucleases from yeast and *N. crassa* as described

Table V. Amino acid sequence of tryptic peptides from nuclease III

Peak[a] No.	Sequence
1	X-His-Ser-Asp-Tyr-Val-Leu-Ser-Tyr-Asp
2[b]	(*Phe*)-(*Ala*)-(*Phe*)-(*Asn*)-(*Asp*)-(*Thr*)-(*Glu*)-(*Ala*)-(*Gly*)-(*Val*)-(*Asp*)-Val-His-Lys
	(X) (X) (Glu) (Tyr) (Thr) (Leu) (Asp) (His) (His) (Phe) (Arg)
3	X–X–Val–Pro–His–X–Val–Phe–Glu–X–Leu
4a	X-Ala-Gly-Leu-Leu-Phe-Phe-Asp-Gln-Ile-Asn-(Ser)-Lys
4b	X-Ile-Glu-Asn-Gln-Tyr-Glu-Thr

[a]Peak number refers to the corresponding peaks in the HPLC chromatogram shown in Figure 7.

[b]This peak contained an approximately equimolar mixture of two peptides so that for the first 11 cycles either of the amino acids shown could derive from either of the two peptides.

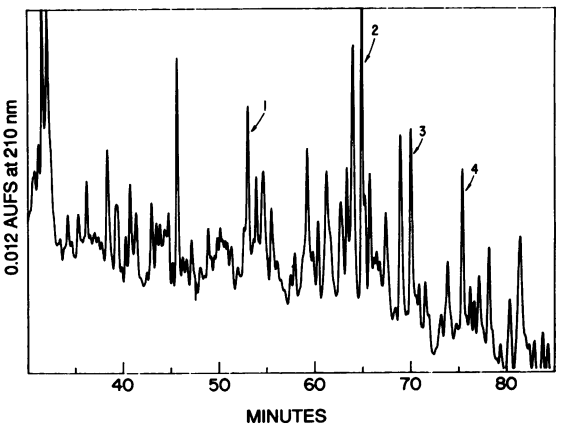

Figure 7. Reverse phase HPLC chromatogram of peptides isolated from a trypsin digest of 175 pmol of nuclease III. The digest was stopped by injecting directly onto a 4.6 mm×25 cm Vydac C-18 column that was equilibrated with 0.05% trifluoracetic acid, 1.6% CH_3CN and then eluted by increasing the concentration of CH_3CN as described in Material and Methods. The numbered absorbance peaks were subjected to amino acid sequencing as described in more detail in 'Material and Methods' and Table V.

above. It will be interesting to determine in the future whether nuclease III is a *Drosophila* mitochondrial nuclease.

There are also important differences among these endo-exonucleases. The *N. crassa* endo-exonuclease was found to be inhibited by ATP, while the *Drosophila* nuclease, like the yeast nucleases, was not affected by ATP. In *Neurospora*, an inactive precursor of the ss-DNA-binding endo-exonuclease was detected, while no inactive precursors were found for both yeast nucleases. We did not find any evidence for an inactive precursor of nuclease III. The yeast major mitochondrial nuclease gene, *Nuc1*, has recently been cloned and sequenced (28). Extensive homology has been found between the yeast mitochondrial nuclease sequence and the carboxy-terminal one-quarter of the *E. coli recC* polypeptide sequence (29). By computer analysis we have compared our peptide sequences with the predicted amino acid sequence of *Nuc1* protein and found no significant homology; however, no conclusion can be drawn until the complete sequence of *Drosophila* nuclease III is obtained. Further investigations must be carried out in order to determine whether these endo-exonucleases truly belong to a class of related proteins.

The *in vivo* functions of all these nucleases are not clear, although there are suggestions that the *Neurospora* and yeast endo-exonucleases might be involved in DNA replication and repair. The function of *Drosophila* nuclease III is purely speculative at this point. Nuclease III digestion products of DNA have 5′-P and 3′-OH termini. These ends make DNA an appropriate substrate for enzymes like DNA ligase, DNA polymerase, etc., which are involved in filling and sealing the discontinuities in DNA intermediates in DNA repair and recombination. Supercoiled DNA could be converted by nuclease III to nicked and subsequently to linear DNA in a stepwise fashion (Fig. 6). Such activities have been proposed to be important in DNA recombination (30). All these facts suggest that nuclease III may be involved in genetic recombination in *Drosophila melanogaster*. Detailed genetic and biochemical studies must be done to confirm this.

Because we have been able to obtain several peptide sequences from nuclease III, it will now be possible to make antibodies and/or oligonucleotides which can be used to clone the gene which encodes nuclease III. By taking advantage of the genetic system provided by *Drosophila melanogaster*, it will be possible to investigate the biological function of nuclease III in detail.

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