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A Novel Endonuclease from Kinetoplastid Hemoflagellated Protozoan Parasite *Leishmania*[1]

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A nuclease activity has been purified from the nuclei-kinetoplast fraction of *Leishmania*. This enzyme, termed endonuclease M (Endo M), is shown by electrophoresis in a denaturing polyacrylamide gel to be associated with a single polypeptide of molecular mass 52 kDa. Physical analysis of the enzyme indicates that it has a sedimentation coefficient $S_{20,w}$ of 4.5S, a Stoke's radius of 32.5 Å, and a native molecular mass of 53 kDa. The final Mono Q purified Endo M possesses both DNase and RNase activities. It acts as an endonuclease by introducing random single-stranded nicks into the supercoiled DNA molecules, that often leads to its linearization due to nicking at the opposite strands, and subsequent degradation of the DNA with further incubation. Single-stranded DNA is twice preferred to double-stranded DNA as substrate. Single-stranded RNA is also degraded rapidly and is competitive as a substrate with single-stranded DNA. RNA:DNA hybrids, however, are largely resistant to the Endo M digestion.

Key words: endonuclease M, kinetoplastid protozoa, *Leishmania*, purification.

Leishmaniasis is one of the dreaded protozoan diseases that poses serious health problems worldwide. The causative organism *Leishmania* is a kinetoplastid parasite that has biphasic life cycle alternating between human and sand-fly as primary and secondary hosts respectively (*1*). The characteristic hallmark of the kinetoplastids is a mitochondrion-like organelle, the kinetoplast. The kinetoplasts consist of a unique DNA network, kinetoplast DNA (kDNA) of about 10^{10} Da, made up of thousands of topologically interlocked DNA circles. Because of this intercatenated structure, the replication of kDNA presents special topological problems. To circumvent this, the parasites have developed a typical strategy for kDNA replication, in which DNA topoisomerases play a pivotal role (*2-6*). Work in our laboratory has focussed primarily on the replication process of *Leishmania* kDNA and the DNA topoisomerases as potential targets for drug therapy. We have reported several forms of DNA topoisomerases from *Leishmania* (*7-9*). However, during the purification of topoisomerases, we have been encountering an enzyme whose activity is apparently confusing with that of topoisomerase I. Further purification and characterization of the enzyme showed it to be an endonuclease.

Endonucleases cleave single-stranded or double-stranded DNAs without involving the terminus. The prokaryotic restriction endonucleases cleave specific sites in the DNA. However, specific cleavage in eukaryotic endonucleases is much less common. There are several reports of isolation of endonucleases from lower eukaryotes like yeast. Of these, a mitochondrial endonuclease from *Saccharomyces cerevisiae* has been shown to have both RNase and DNase activity (*10*). Nuclease activities have also been identified in various trypanosomes. However, the three nucleases that have so far been isolated and characterized are all from *Crithidia fasciculata*. Of these, the *Crithidia* nicking enzyme introduces a single nick in either supercoiled DNA circles or relaxed circles containing a bent helical structure (*11, 12*). The second enzyme, designated Endo A, introduces single-stranded breaks at preferred sites on double-stranded DNA substrates (*13*). The third *Crithidia* nuclease is a DNase that exists in either a monomeric or a multimeric form and introduces single-stranded breaks into supercoiled plasmids. It preferentially attacks near the ends of linear double-stranded DNA molecules (*14*).

Another enzyme, a 3′-nucleotidase/nuclease capable of hydrolyzing both 3′-nucleotides and nucleic acids, has been reported in *Leishmania donovani* and related trypanosomatid protozoa. This externally oriented surface membrane-bound enzyme presumably plays a critical role in the salvage of purines essential for survival of the organisms (*15*).

We report here a novel endonuclease from a *Leishmania* sp. The enzyme, termed Endo M, has been purified to homogeneity. Biochemical and physicochemical studies suggest that the enzyme differs from the three nucleases

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Abbreviations: kDNA, kinetoplast DNA; EDTA, ethylene diamine tetra acetic acid; EGTA, ethyleneglycol-bis-(b-amino-ethyl ether)-*N,N′*-tetra acetic acid; PMSF, phenyl methyl sulfonyl fluoride; DTT, dithiothreitol; BSA, bovine serum albumin; TCA, trichloroacetic acid; PEG, polyethylene glycol; PIPES, piperazine-*N,N′*-bis(2-ethane sulfonic acid); HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; EtBr, ethidium bromide.

described in *Crithidia* and the 3′-nucleotidase/nuclease of trypanosomatid protozoa. The possible functions for the enzyme are discussed.

MATERIALS AND METHODS

Materials—α-^{32}P-labeled dATP, dCTP, and [γ-^{32}P]-ATP were purchased from Amersham International, UK. Electrophoresis grade acrylamide, *N,N,N′,N′*-tetramethyl ethylene diamine, ammonium persulfate, and hydroxyl apatite (HAP) were obtained from Bio-Rad Laboratories, Richmond, CA, USA. Brain heart infusion was purchased from Acumedia, Baltimore, MD, USA. Phosphocellulose P11 and DE 81 paper filter discs were obtained from Whatman, and DNA-cellulose (native) from Pharmacia Biotech. All other chemicals of highest purity and electrophoresis grade agarose were purchased from Sigma Chemical Company, St. Louis, MO, USA.

Nucleic Acids and Enzymes—Plasmid pGEM4Z was purchased from Promega, Madison, USA. Plasmid pLURkE3 contains an 830 bp major class minicircle cloned into the *Eco*RI site of pGEM4Z, and plasmid pLURkE3A contains a 630 bp variable region of the above minicircle cloned into the *Eco*RI/*Pst*I site of pGEM4Z (*16*). *Leishmania* DNA topoisomerase I was purified as described (*9*). T7 RNA polymerase, restriction enzymes, Nick-translation kit, and Klenow DNA polymerase were all obtained from Amersham International, UK.

Parasites and Culture Conditions—Leishmania strain UR6 (MHOM/IN/1978/UR6) was originally isolated from the bone marrow of a patient diagnosed with kala-azar on the basis of clinical manifestations and the identification of parasites in a bone marrow smear. *Leishmania* strain UR6 promastigotes were grown in Ray's modified media (*17*) and subcultured at intervals of 72 h. Cells were harvested in phosphate-buffered saline, washed several times with the same buffer, and kept frozen at −70°C.

Solubilization of the Endonuclease—The solubilization procedure was originally optimized for purification of DNA topoisomerase I of *Leishmania* (*9*). Unless otherwise stated, all procedures were carried out at 4°C. Aliquots of 10^{11} promastigotes (5 g wet weight) were suspended in buffer A (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 1 mM Benzamidine-HCl, and 0.5 mM DTT) and homogenized in a Sorvall Omnimixer. Cell disruption was checked under the microscope. The crude homogenate was centrifuged at 10,000 rpm for 15 min in a SS34 rotor in a Sorvall RC5B centrifuge. The pellet containing crude nuclei and kinetoplasts was further washed and resuspended in 15 ml of buffer A. To this an equal volume of buffer B (50 mM Tris-HCl, pH 7.5, 2 M NaCl, 1 mM PMSF, and 0.5 mM DTT) was added slowly with constant stirring. After about 30 min at 0°C, 15 ml of buffer C (50 mM Tris-HCl, pH 7.5, 1 M NaCl, 1 mM PMSF, and 0.5 mM DTT) containing 18% PEG was added with constant stirring. This solution was further incubated at 0°C for 30 min, then centrifuged at 10,000 rpm for 60 min in a Sorvall RC5B centrifuge using a SS34 rotor. The supernatant containing the endonuclease activity was either directly subjected to chromatography on a hydroxyl apatite column or stored at −70°C until further use.

Nuclease Assay—The nuclease was primarily assayed for its ability to convert the supercoiled form of plasmid DNA to the nicked relaxed form. The nicked form was differentiated from the supercoiled form by its decreased mobility in agarose gel containing ethidium bromide (EtBr). About 0.5 μg of supercoiled plasmid DNA was subjected to enzymatic digestion in 25 μl of solution containing 25 mM Tris-HCl, pH 7.9, 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol. Reactions were carried out at 30°C for 30 min and stopped by adding 5 μl of stop buffer containing 10 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol, and 50% glycerol. The products were analysed by electrophoresis in 1% agarose gel containing 0.5 μg/ml EtBr at 3 V/cm for 6 h in TAE buffer (40 mM Tris-acetate, pH 7.8, and 1 mM EDTA) and photographed under UV illumination.

Topoisomerase I Assay—The assay depends upon the decreased mobility in an agarose gel of supercoiled DNA after treatment with topoisomerase. The standard type I assay mix (25 μl) contained 25 mM Tris-HCl, pH 7.5, 5% glycerol, 50 mM KCl, 0.5 mM DTT, 10 mM $MgCl_2$, 30 μg/ml BSA, 0.5 μg of pGEM4Z DNA, and 2.5 μl of enzyme. The reaction was carried out at 37°C for 30 min and stopped by adding 1% SDS, 10 mM EDTA, 0.25 mg/ml bromophenol blue, and 15% glycerol. After electrophoresis in 1% agarose gel in TAE buffer at 1.5 V/cm for 12–14 h, the gels were stained with EtBr (5 μg/ml), destained in water, and photographed as above.

Polyacrylamide Gel Electrophoresis—Discontinuous polyacrylamide gel electrophoresis was performed on slab gel [10% (w/v) acrylamide] according to the method of Laemmli (*18*). Fractions containing low amounts of protein were concentrated by adding TCA to 10%. The precipitate was dissolved in sample buffer (10% glycerol, 5% β-mercaptoethanol, 3% SDS, and 0.001% bromophenol blue) and adjusted to neutral pH with 1 M Tris base. The samples were boiled for 2 min before loading on the gel. After electrophoresis, gels were silver stained as described (*19*). The relative mass of the protein bands was determined from their mobility relative to standard proteins. Electrophoresis in denaturing polyacrylamide gel was carried out as described (*20*).

Protein Determination—Protein concentrations were determined as described by Bradford (*21*) with BSA as a standard.

Gel Filtration—Gel filtration was performed on a Protein Pak I125 column (Waters) in the HPLC system (Waters). The column was pre-equilibrated with buffer D (50 mM Tris-HCl, pH 7.5, 0.5 mM DTT, 0.1 mM EDTA, and 0.2 mM PMSF) containing 10% glycerol and 100 mM NaCl. Concentrated sample of 100 μl in volume was loaded onto the column. The column was washed with the above buffer at a flow rate of 0.5 ml/min and fractions of 250 μl were collected. Alternate fractions were assayed for the nuclease activity. The column was standardized with BSA (66.7 kDa), ovalbumin (45 kDa), myoglobin (17 kDa), and cytochrome *c* (12.5 kDa). Ferritin and dextran blue were used to determine the void volume and total column volume respectively. Molecular weight and Stoke's radius were calculated according to published methods (*22*).

Glycerol Gradient Centrifugation—The purified endonuclease was subjected to centrifugation on a 5-20% linear gradient of glycerol in buffer D at 48,000 rpm for 7 h using a TST 60.4 rotor in a Sorvall OTD combi ultracentrifuge. Following the run, 0.25-ml fractions were collected from

the bottom of the tube and assayed for the nuclease. The sedimentation co-efficient ($S_{20,w}$) of the nuclease was determined as described (*23*) from the respective values of the standard marker proteins, aldolase (7.4S), BSA (4.41S), and cytochrome *c* (1.74S). The frictional ratio (f/f_0) was calculated from the $S_{20,w}$ value and the Stoke's radius of the enzyme.

Estimation of Single-Stranded and Double-Stranded DNA Degradation—Plasmid pLURkE3, linearized with restriction enzyme *Hin*dIII, was labeled with [^{32}P]dATP and [^{32}P]dCTP by nick translation (*20*). The ^{32}P-labeled DNA was used as double-stranded DNA substrate. Single-stranded DNA substrate was prepared by denaturing the labeled pLURkE3 DNA by boiling at 100°C for 15 min followed by quick chilling on ice. DNA degradation assay was the same as nuclease assay except that ^{32}P-labeled double-stranded or single-stranded DNAs were used as substrates in a volume of 10 μl. The reaction was terminated by adding BSA to 100 μg/ml and TCA to 10%. Acid-soluble radioactivity was measured by liquid scintillation counting of the supernatant.

Preparation of ^{32}P-Labeled RNA—Plasmid pLURkE3A and pLURkE3B were linearized with *Eco*RI. The linear DNAs were used as templates for preparation of run-on transcripts. Transcription assay mixture contained 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 50 mM NaCl, 1.5 mM spermidine, 10 mM DTT, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.1 mM UTP, 50 μCi of [^{32}P]UTP (Sp. activity 3,000 Ci/mmol), 50 units of T7 RNA polymerase, and 1-2 μg of template DNAs in a final volume of 25 μl. After incubation at 30°C for 10 min, the reaction products were extracted with phenol-chloroform and precipitated with ethanol. Pellets containing the labeled RNAs were dissolved in a buffer containing 100 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$ and treated with 10 units of RNase-free DNase at 37°C for 30 min to remove any contaminating template DNA. Labeled RNA was extracted with phenol-chloroform and precipitated with ethanol. RNA was generally labeled to $1\text{-}5\times10^7$ cpm/μg.

Measurement of RNase Activity—^{32}P labeled RNA prepared by transcribing the pLURkE3A DNA was used as substrate for RNase assay. The 630 nucleotide transcript was treated with the enzyme as described above. The reaction was terminated by adding BSA to 100 μg/ml and TCA to 10%. Acid-soluble radioactivity was measured as before.

Preparation of DNA:RNA Hybrid—The 200 nucleotide ^{32}P-labeled RNA, prepared by transcribing pLURkE3B DNA as described above, was hybridized with 830 bp minicircle from pLURkE3 in the hybridization buffer (40 mM PIPES, pH 6.4, 1 mM EDTA, 0.4 M NaCl, and 80% formamide, see Ref. *20*). Hybridization was carried out at 53°C for 10 h. DNA:RNA hybrid formation was checked by electrophoresis in 1.8% agarose gel.

Estimation of DNA:RNA Hybrid Degradation—DNA:RNA degradation was assayed in the buffer used as for the nuclease assay, with the ^{32}P-labeled DNA:RNA hybrid as substrate.

RESULTS

Purification of the Endonuclease—Purification of *Leishmania* endonuclease was monitored by assaying the nicking of the duplex circular DNA. The endonuclease was purified approximately 156-fold over the crude soluble fraction (Fraction I), to apparent homogeneity (Table I). Crude enzyme preparation (Fraction I, 45 ml) was loaded onto a hydroxyl apatite column (1.6×5 cm) pre-equilibrated with buffer C containing 6% PEG at a flow rate of 30 ml/h. The column was washed with 20 ml of the same buffer and eluted with an 80-ml linear gradient of 0-0.8 M potassium phosphate, pH 7, in buffer D (25% glycerol, 0.5 mM DTT, 5 mM β-mercaptoethanol, and 0.1 mM PMSF). Fractions of 4 ml were collected. The nuclease eluted with 100 to 250 mM phosphate, prior to the topoisomerase activities (lanes 2 and 3, Fig. 1). These active fractions which convert supercoiled DNA to nicked DNA and subsequently to linear forms were pooled and dialysed against buffer E (50 mM Tris-HCl, pH 7.5, 25% glycerol, 0.5 mM DTT, 5 mM β-mercaptoethanol, and 0.1 mM PMSF) containing 100 mM KCl. The dialysate (Fraction II, 16 ml) was then loaded onto a phosphocellulose P11 column (0.9×3.2 cm) pre-equilibrated with the above buffer containing 100 mM KCl at a flow rate of 15 ml/h. The column was washed with 15 ml of the same buffer, then eluted with a 30-ml linear gradient of 0.1-0.6 M KCl in buffer E. Fractions of 1 ml were collected. The active fractions eluting between 0.3 and 0.5 M KCl were pooled and dialysed against buffer E. This enzyme preparation (Fraction III, 8 ml) was further purified by chromatography on a DNA cellulose column (0.9×3.2 cm) that had been equilibrated with buffer E, at a flow rate of 12 ml/h. The column was washed with 10 ml of buffer E, and eluted with a 25-ml linear gradient of 0-1 M NaCl in the same buffer. Fractions of 1 ml were collected. The endonuclease eluted in the range of 0.25 to 0.35 mM NaCl. The pooled active fraction was then dialyzed against buffer E. The dialysate (Fraction IV, 6 ml) was concentrated 20-fold by centrifugation using centricon-10 filter units (Amicon) and further purified by chromatography on a Mono Q HR 5/5 FPLC anion exchange column (Pharmacia). The column was washed with buffer E containing 0.5 μg/ml leupeptin and 0.4 μg/ml pepstatin A for 20 min and eluted with a 15-ml linear gradient of 0-1 M NaCl in buffer E for 45 min (flow rate 0.3 ml/min, fraction size 0.5 ml). The Mono Q-purified active fractions were dialysed against buffer E containing 100 mM NaCl (Fraction V) and

TABLE I. **Purification table for *Leishmania* Endo M.**

Fractions	Total volume (ml)	Total protein (μg/ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
PEG-supernatant	45	370	16.65	ND	–	–	–
Hydroxyl apatite	16	350	5.6	3,200	571	100	–
Phosphocellulose	8	73	0.59	2,000	3,389	62.5	5.93
DNA cellulose	6	16	0.096	1,200	12,500	37.5	21.9
Mono Q	2.5	1.6	0.004	357	89,250	11	156

stored in small aliquots at −70°C.

Comments on the Purification—The purification was carried out as fast as possible in order to minimize proteolysis. In the initial phase of purification, most of the contaminating proteins were removed and the specific activity was increased considerably after chromatography on the phosphocellulose column (Table I). The specific activity of the endonuclease in the PEG supernatant could not be determined due to the presence of topoisomerase activities. There was gradual increase of specific activity up to the final purification step. Starting from hydroxyl apatite column purification step the endonuclease activity was purified 156-fold in Fraction V. The yield from 5 g of cells was 4 μg. The purified enzyme preparation is unstable at 0-4°C, but stable at −70°C in the presence of 25% glycerol for at least 6 months without significant loss of activity. At −20°C, the enzyme remains active for a maximum of 15 days. Glycerol concentration in the storage buffer is important as the activity of the enzyme is rapidly lost when stored in 10% glycerol at −70°C.

Physical Properties: Molecular Weight and Subunit Structure—Leishmania endonuclease migrates in 10% SDS-polyacrylamide gel under denaturing and reducing conditions as a single polypeptide band of 52 kDa (Fig. 2). This 52 kDa protein cofractionated with the endonuclease activity upon chromatography on hydroxyl apatite, phosphocellulose, double-stranded DNA cellulose, and MonoQ. The apparent molecular mass of the native *Leishmania* endonuclease determined by gel-filtration on a Protein Pak I125 column in the HPLC system was 53 kDa. These data suggest that this enzyme is a monomeric protein with approximate molecular mass of 50-53 kDa. Gel filtration data gave a Stoke's radius of 32.5 Å as calculated by the method of Laurant and Killander (*22*). A sedimentation coefficient ($S_{20,w}$) of 4.5S was estimated upon sedimentation through a 5-20% glycerol gradient according to method of Martin and Ames (*23*). The apparent molecular mass of the enzyme calculated from the experimental sedimentation coefficient and Stoke's radius by assuming a partial specific volume (υ) of 0.725 ml/g is 56 kDa. The frictional coefficient calculated from the equation $f/f_0 = a/(3\upsilon M/4\pi N)^{1/3}$ is 1.31, where a = Stoke's radius, M = molecular weight, υ = partial specific volume, and N = Avogadro's number.

Biochemical Properties—Purified *Leishmania* endonuclease (Fraction V) requires Mg^{2+} for its activity with the optimum at 10 mM (Fig. 3A). A low activity was detected with Ca^{2+} at very low concentration, but concentrations higher than 2 mM were inhibitory (data not shown). Mn^{2+} can efficiently be substituted for Mg^{2+} with optimum activity also at 10 mM (Fig. 3B). The enzyme also requires monovalent ions: activity is optimum with NaCl in the range of 100 to 150 mM and is inhibited with increasing concentration of NaCl (Fig. 3C). A similar pattern of activity is observed with KCl (data not shown). The enzyme remains active over a broad pH range of 6 to 8.8, with maximal activity at pH 7.5 (Fig. 3D). The enzyme is active over a wide temperature range of 20 to 65°C. A gradual increase of temperature beyond 37°C yields an increase in the population of linear DNA molecules. At about 50°C, the linear DNA is almost completely degraded. However, at 70°C and above, the enzyme activity is almost completely lost (Fig. 3E).

Catalytic Properties of the Enzyme: DNase Activity—At low concentrations, the enzyme introduces nicks in the supercoiled plasmid DNA, generating 3′-hydroxyl and 5′-phosphoryl ends. This is confirmed by resealing the nicked molecules with T4 DNA ligase, as T4 DNA ligase ligates nicks only when 3′-hydroxyl and 5′-phosphoryl groups are present. When a covalently closed circular plasmid is treated with increasing concentrations of the endonuclease under the standard reaction conditions, the supercoiled DNA is increasingly converted to the nicked form and

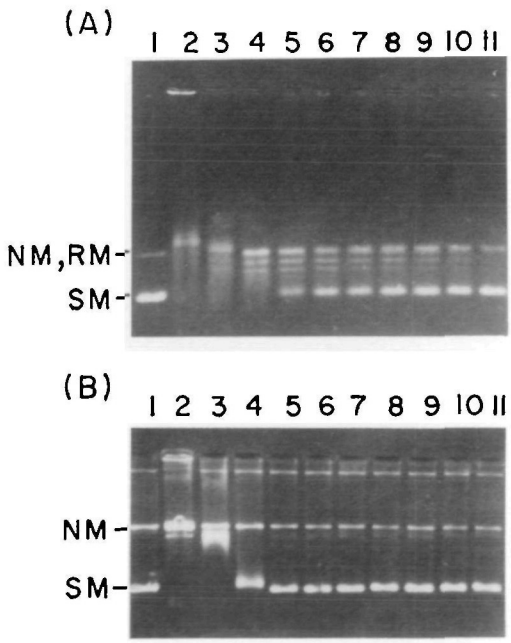

Fig. 1. **Hydroxyl apatite column chromatography of the PEG supernatant (Fraction I).** Aliquots of 5 μl of alternate fractions were assayed for Endo M activity as described in "MATERIALS AND METHODS." (A) Electrophoresis in 1% agarose gel in the absence of ethidium bromide, and (B) electrophoresis in 1% agarose gel in the presence of 0.5 μg/ml ethidium bromide. Position of supercoiled monomer (SM) and nicked or relaxed monomer (NM) are indicated.

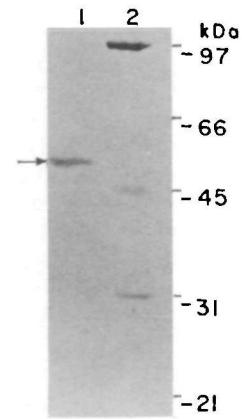

Fig. 2. **SDS-polyacrylamide gel electrophoresis of purified Endo M.** Mono Q-purified Endo M fraction was electrophoresed on a 10% SDS-polyacrylamide gel, which was then silver-stained. Lane 1, Fraction V, Mono Q, 1 μg; lane 2, molecular weight marker. The arrow indicates the 52 kDa polypeptide of Endo M.

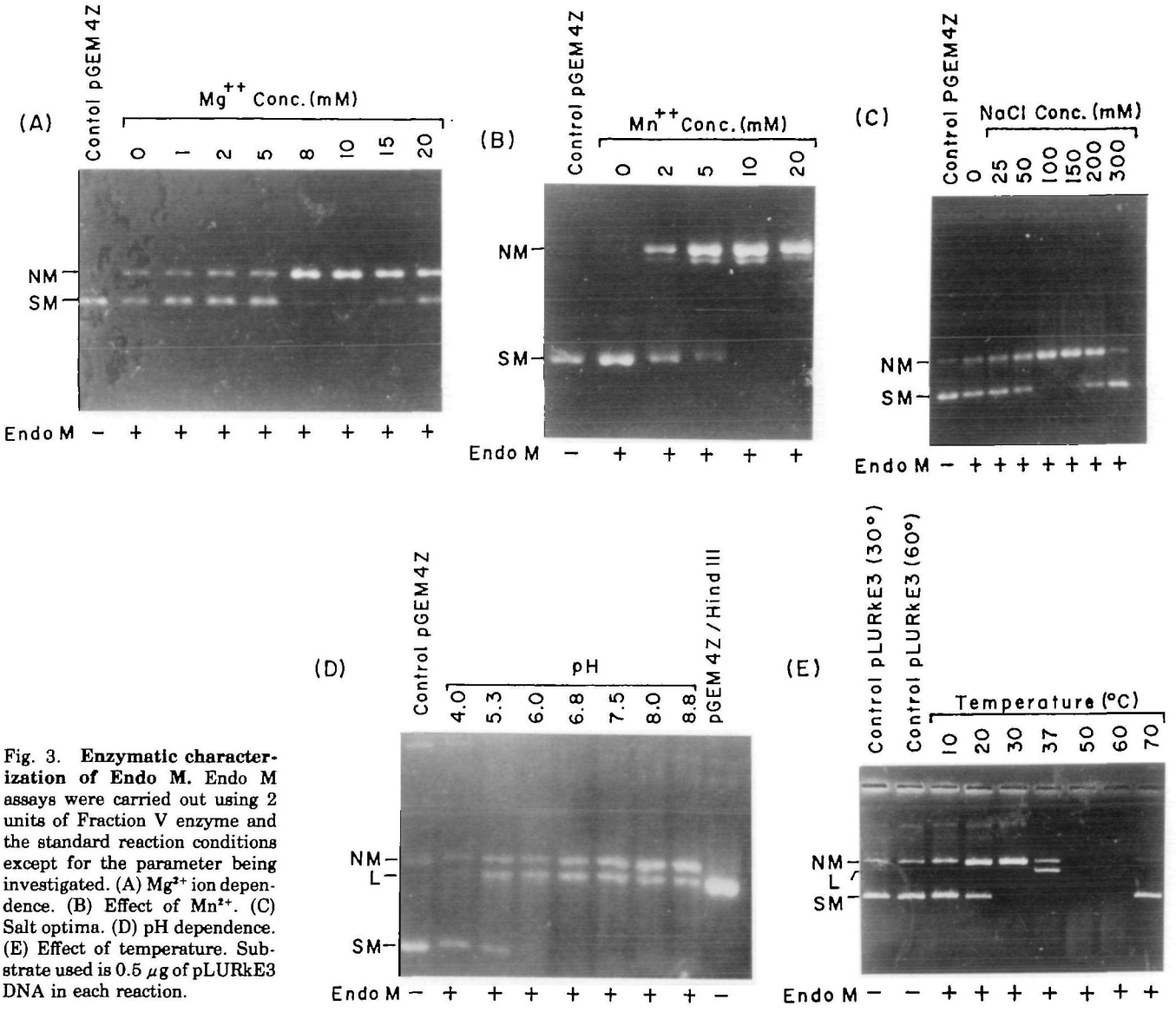

Fig. 3. **Enzymatic characterization of Endo M.** Endo M assays were carried out using 2 units of Fraction V enzyme and the standard reaction conditions except for the parameter being investigated. (A) Mg^{2+} ion dependence. (B) Effect of Mn^{2+}. (C) Salt optima. (D) pH dependence. (E) Effect of temperature. Substrate used is 0.5 μg of pLURkE3 DNA in each reaction.

subsequently to the linear form (Fig. 4). The appearance of the linear form may be due to the cleavage of a single strand opposite the nick in the circular DNA. The formation of the linear DNA raises the question of substrate specificity of the enzyme. As shown in Fig. 5, the enzyme shows a preference for single-stranded DNA over double-stranded DNA. The time course of production of acid-soluble radioactivity from labeled substrates, indicated that single-stranded DNA was degraded two times more efficiently than the double-stranded DNA (Fig. 5). The enzyme was also assayed for exonuclease activity. The minicircle insert in plasmid pLURkE3 was released by digestion with *Eco*RI, ^{32}P-labeled at the 3′ or 5′ end, and used as substrate. Figure 6 shows the kinetics of conversion to acid solubility of both radiolabels. There is only 20% release of radiolabel from either end even with prolonged incubation. These results suggest that the enzyme is predominantly an endonuclease which probably lacks sequence specificity.

RNase Activity—Next, we tested whether the enzyme has RNase activity in addition to its endonuclease activity.

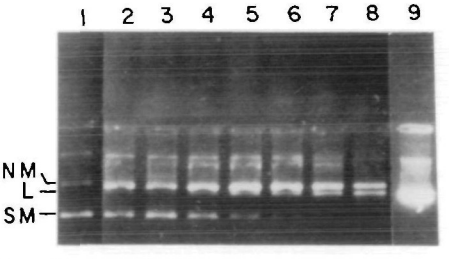

Fig. 4. **Linearization of covalently closed DNA substrates by Endo M.** Endo M reactions were carried out as described in "MATERIALS AND METHODS" in the presence of increasing concentrations of the enzyme. Lane 1, control supercoiled pLURkE3; lanes 2-8, incubations with 0.1, 0.2, 0.5, 1, 2, 3, and 5 units of the enzyme; lane 9, linear pLURkE3 obtained by digestion of the DNA with *Hin*dIII.

^{32}P-labeled run-on transcript was prepared by transcribing pLURkE3A using T7 RNA polymerase as described in "MATERIALS AND METHODS." The 630 nucleotide run-on

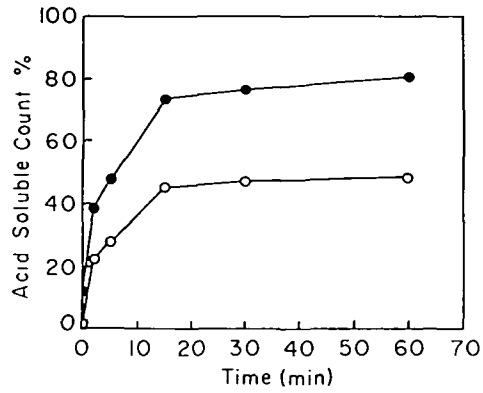

Fig. 5. **Comparison of single-stranded and double-stranded DNA as substrates for Endo M.** Labeled single-stranded (●) and double-stranded (○) DNAs were prepared as described in "MATERIALS AND METHODS" and incubated with 2 units of Endo M for different times. After incubation, the acid-soluble radioactivity was counted in scintillation fluid.

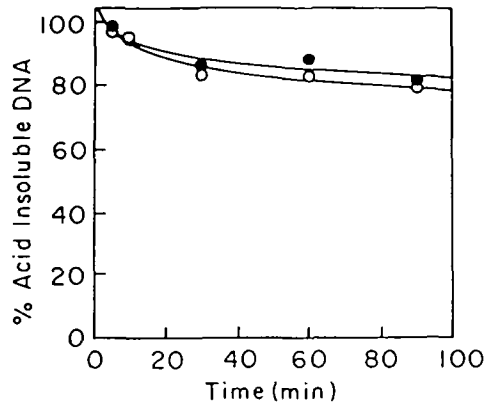

Fig. 6. **Measurement of exonuclease activity of Endo M.** The minicircle insert of pLURkE3 was labeled at either 5′- or 3′-end as described in "MATERIALS AND METHODS." Degradation of substrate DNAs was checked by incubating 0.25 μg of DNA (5′-labeled, 100,000 cpm; 3′-labeled, 200,000 cpm) with 2 units of Endo M in a total reaction mixture of 25 μl at 30°C for different times. (●), 3′-labeled DNA, (○), 5′-labeled DNA. Acid-insoluble DNA was measured in a scintillation counter.

transcript was used as the substrate for the endonuclease. Figure 7A shows that the enzyme readily degrades RNA. Initially the rate of degradation is very high, and within 30 min 80% of RNA is degraded, but no further degradation takes place beyond 30 min. The remaining 20% of acid-insoluble radioactivity may be due to formation of short oligonucleotides which are resistant to the enzyme. The RNase activity of the enzyme was reduced significantly when denatured calf thymus DNA was added to the reaction mixture. About 70% of the input RNA is protected by the addition of 250 ng of denatured calf thymus DNA (Fig. 7B). This experiment suggests that the DNA and RNA substrates are competitive and both RNase and DNase activity reside in the same polypeptide.

RNA:DNA Hybrid Is Resistant to the Endo M Digestion—Since both single-stranded and double-stranded DNA as well as RNA are susceptible to this endonuclease

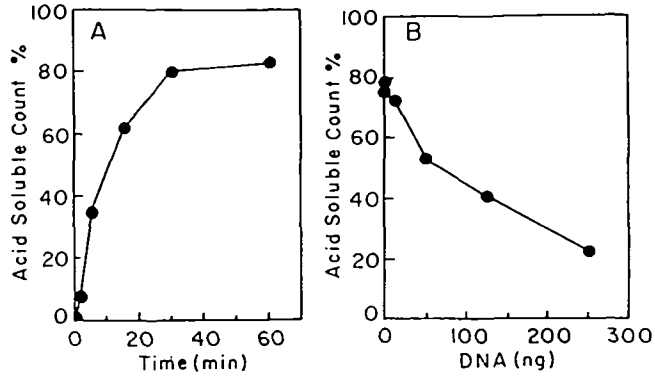

Fig. 7. **A single polypeptide of Endo M exhibits both RNase and DNase activity.** (A) ^{32}P-labeled 630 nucleotide RNA (100,000 cpm), prepared by transcribing pLURkE3 DNA as described in "MATERIALS AND METHODS," was incubated with 2 units of Endo M at 30°C in a reaction mixture of 10 μl for different times. The reaction was terminated by adding BSA to 100 μg/ml and TCA to 10%. Acid-soluble radioactivity was measured in a scintillation counter. (B) Labeled RNA, as described in (A), was incubated in 10 μl of solution with 2 units of Endo M in the presence of increasing concentrations of denatured calf thymus DNA. Acid-soluble count was measured as before.

digestion, we examined whether RNA:DNA hybrids are also susceptible. RNA:DNA hybrid was prepared by annealing the ^{32}P-labeled pLURkE3B transcript with the minicircle DNA of pLURkE3 as described in "MATERIALS AND METHODS" and used as the substrate for the Fraction V enzyme. RNA:DNA hybrid was incubated with the enzyme at a particular concentration (2 units) for different time periods. Figure 8A shows that RNA:DNA hybrid is resistant to degradation by the enzyme even after incubation for 90 min, whereas labeled RNA is degraded rapidly at the same enzyme concentration. When RNA:DNA hybrid is treated with increasing concentrations of the enzyme, the single-stranded DNA is hydrolyzed resulting in perfectly duplex DNA:RNA hybrid molecules (Fig. 8B, lanes 3-6). With excess enzyme, the perfectly duplex molecule also gets degraded (lane 6). Electrophoresis of the resulting hybrids in denaturing acrylamide gel further confirms the protection of labeled RNA in the RNA:DNA hybrid.

DISCUSSION

Endo M described in this paper is the first reported endonuclease from *Leishmania*. It is distinctly different from the three nucleases reported from the kinetoplastid parasite *C. fasciculata* (*11-13*). The *Leishmania* enzyme is unique in possessing both DNase and RNase activities. There are reports of several nucleases from other organisms. The well illustrated single-strand-specific nucleases like S1 nuclease (*24*), mung bean nuclease (*25*) or *Neurospora crassa* nuclease (*26*) possess RNase activity along with DNase activity. These enzymes act several thousand times more rapidly on single-stranded DNA than on native double-stranded DNA. On the other hand, the rate of degradation of single-stranded DNA by Endo M is only twice that of double-stranded DNA. Moreover, Endo M is active over a wide pH range of 6 to 8.8. In contrast, S1 and

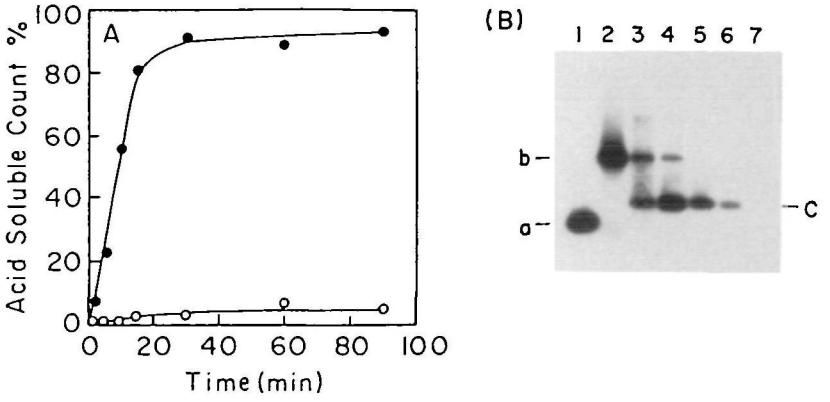

Fig. 8. **RNA:DNA hybrid is resistant to Endo M.** (A) ^{32}P-labeled RNA and ^{32}P-labeled RNA:DNA hybrid prepared as described in the "MATERIALS AND METHODS" were used as substrates. The reaction mixtures (10 μl) containing ^{32}P-labeled RNA (100,000 cpm) or ^{32}P labeled RNA:DNA hybrid (100,000 cpm) were incubated at 30°C with 2 units of Endo M for different periods. Reactions were terminated and acid-soluble count was measured. (○), RNA:DNA hybrid; (●), RNA only. (B) Autoradiogram of gel of Endo M-digested RNA:DNA hybrid. Electrophoresis was carried out in neutral 1.8% agarose gel. Lane 1, ^{32}P labeled RNA; lane 2, ^{32}P labeled RNA:DNA hybrid; lanes 3-6, ^{32}P labeled RNA:DNA hybrids incubated with 5, 10, 20, and 50 units of Endo M. Lane 7, ^{32}P labeled RNA incubated with 2 units of Endo M. Approximately 100,000 cpm was loaded in each lane. a, ^{32}P labeled RNA; b, RNA:DNA hybrid with unlabeled single-stranded DNA overhanging end; c, perfect RNA:DNA duplex.

mung bean nucleases are active at acidic pH. These single-strand-specific endonucleases have wide applications in quantitation of nucleic acid hybridization, analysis of heteroduplex DNA, and analysis of superhelical DNA. The *Leishmania* Endo M is closer to the membrane-bound endonuclease purified from the mitochondrial fraction of *S. cerevisiae* (*10*) in its enzymatic properties. The activity of *Saccharomyces* endonuclease toward denatured DNA is only five times higher than that toward native DNA substrate. The activity toward RNA is 40% of that toward denatured DNA, and the two substrates are found to be competitive. The *Saccharomyces* enzyme in addition possesses a 5′-exonuclease activity toward double-stranded DNA. The *Saccharomyces* endonuclease gene was cloned and a mutant of this gene was isolated. Since the mutant showed no phenotypic change, this enzyme probably does not play any significant role in the survival of the yeast.

Endo M was purified from the nuclei-kinetoplast fraction. However, its exact subcellular location remains undetermined. *C. fasciculata* DNase (*13*) has been reported to have a general distribution in the nucleus and in the kinetoplast. Endo M may also have a general distribution.

The physiological function of Endo M is also unclear, although our data indicate that it is responsible for the majority of nuclease activity within the cell. This suggests that it may participate in general nucleic acid degradation within the cell. Moreover, *Leishmania* is a purine auxotroph and thus cannot synthesize purine bases *de novo* (*27*). It depends for the supply of purines on the host AMP pool (*28*). The general nucleic acid degradation by Endo M may also serve as a source of purines for survival of the parasite. Work is in progress to determine the function(s) of this unique endonuclease. At the same time, our data suggest that Endo M may also serve as a tool in DNA:RNA mapping studies.

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