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Characterization of a novel endonuclease from *Crithidia fasciculata*

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ABSTRACT

A new endonuclease activity has been identified in whole cell lysates of the trypanosomatid *Crithidia fasciculata*. This activity, termed endonuclease A (Endo A), introduces single-strand breaks at highly preferred sites in double stranded DNA substrates Physical analysis of this enzyme indicates that it has a sedimentation coefficent $S_{20,w}$ of 4.9 and a Stokes radius of 59Å and thus, a native molecular weight of 125,000 and a frictional coefficient of 1.8. A monomeric structure is suggested for the enzyme based on the recovery of Endo A activity associated with a polypeptide with a molecular weight of 116,000−120,000, following electrophoresis on sodium dodecyl sulfate polyacrylamide gels. Endo A shows an absolute requirement for Mg^{2+} or Mn^{2+} and exhibits activity over a broad pH and temperature range, with optimal conditions for activity at pH 8.0 and 30°C.

INTRODUCTION

Endonucleases are characterized by their ability to cleave either single stranded or double stranded DNA without the involvment of a terminus (1). In general, endonucleases cleave at non-random sites, but often with low specificity. The most common site-specific endonucleases are the prokaryotic restriction endonucleases. These enzymes function *in vivo* to degrade unmethylated foreign DNA and generally recognise a symmetric sequence four or six nucleotides in length (1). Other endonucleases of high specificity are associated with the replication and packaging of bacteriophage DNAs (1). The ΦX174 gene A protein makes a single incision (nick) in a decameric recognition site to initiate rolling-circle replication. The bacteriophage lambda terminase makes staggered scissions at a specific site (cos) to yield twelve nucleotide cohesive ends.

Site-specific cleavage by eukaryotic endonucleases is much less common. Three site-specific endonucleases have been isolated from *Saccharomyces cerevisiae*. YZ endonuclease introduces a double strand break in the ZI region near the YZ junction of the mating locus (2). This site-specific endonuclease is essential for mating-type recombination. Endo.*Sce*II has a distinct cleavage site, but the cellular function of this enzyme is unknown (2). Endo.*Sce*I introduces a double stranded break at a 26 bp consensus sequence (3). The physiological function of this enzyme is also unknown. Other examples of eukaryotic site-specific endonucleases include a site-specific single strand endonuclease from *Chlamydomonas* (4) and a bovine mitochondrial endonuclease which cleaves a conserved sequence in the displacement loop of mitochondrial DNA and has been implicated in the regulation of DNA replication (5).

Nuclease activities have also been identified in various trypanosomes. However, there is only one example of an endonuclease activity which has been purified from a trypanosome (*C.fasciculata)* (6). This enzyme, designated *Crithidia* nicking enzyme introduces a single

nick in either supercoiled DNA circles, or relaxed circles containing a bent helical structure. Structural features of the DNA rather than the primary sequence appear to specify the cleavage sites for this enzyme (7).

A second endonuclease activity from *C. fasciculata* is described here. This enzyme introduces single strand breaks at highly preferred cleavage sites in double stranded DNA.

MATERIALS AND METHODS

Materials

Labeled [γ-^{32}P] ATP was purchased from ICN Dupont. Enzymes used include T4 polynucleotide kinase (Boehringer-Mannheim), *E.coli* DNA polymerase I (United States Biochemicals), HinfI (Promega), MluI and PstI (Toyobo), BamHI and BglII (Pharmacia), and AluI, EcoRI and TaqI (Bethesda Research laboratories).

Nucleic acids

A 249 bp probe was derived from the *Crithidia* half-minicircle clone M13CFK120-H6(8) after subcloning of the half-minicircle into pUC8 to yield the plasmid pCFK14M. The plasmid DNA was cleaved at the unique MluI site followed by 5′ end-labeling as described (9). The 5′ end-labeled 249 bp probe was obtained by cleavage of the linear DNA with HinfI and isolation of the 249 bp MluI-HinfI fragment by gel electrophoresis (9). A 71 bp probe was derived from the 249 bp sequence by oligonucleotide mutagenesis (10) to insert BamHI, BglII and XhoI sites as shown in Fig. 1. The 75 bp BamHI-XhoI fragment was sub-cloned into the BamHI and SalI sites of pUC19 to yield the plasmid pOriB-6. The plasmid DNA was then cleaved at the unique BamHI site and 5′end-labeled as described (9). The 71 bp probe was obtained by cleavage of the labeled DNA with BglII followed by isolation of the 71 bp BamHI-BglII fragment by gel electrophoresis as described (9).

A 542 bp probe was derived from the carrot extensin gene clone pDC5A1 (11), which had been subcloned into pUC8. In this case the plasmic DNA was cleaved at the unique EcoRI site and either 5′endlabeled with [γ-^{32}P]ATP or 3′ end-labeled with [α-^{32}P]dTTP as described (9). Single end-labeled probes were obtained by cleavage of the linear DNA with PstI and isolation of the 542bp EcoRI-PstI fragment by gel electrophporesis (9).

Purification

C. fasciculata was cultured as described (12) and stored at -75°C. All purification steps were carried out at 0$-$4°C. Fifty grams of frozen *C. fasciculata* were lysed by thawing in 125ml 50mM KPO_4 pH 7.5, 5mM Na_2 EDTA, 300mM NaCl, 0.5% (v/v) Brij 58, 5mM β-mercaptoethanol, 2μg ml^{-1} leupeptin, 1mM benzamidine, 1mM pepstatin, 1mM PMSF. The suspension of lysed cells was homogenized with two strokes of a Potter-Elvehjem tissue grinder. The resulting crude lysate was clarified by centrifugation in a Sorvall SS34 rotor at 17,000rpm for 30min. The supernatant was filtered through one layer of siliconized rayon (Miracloth) and the NaCl concentration of the filtrate adjusted to 2.3M. Nucleic acids were precipitated by the addition of PEG 6000 to a final concentration of 7.5%. The extract was centrifuged in a Sorvall GSA rotor at 12,500rpm for 30min. The supernatant was decanted, the NaCl concentration of the supernatant adjusted to 4M and applied to a 7.5cm×5cm column of Phenyl Sepharose (Pharmacia), previously equilibrated in Buffer A (20mM KPO_4 pH 7.5, 20% (v/v) glycerol, 1mM Na_2EDTA, 0.05% (v/v) Brij 58, 5mM β-mercaptoethanol, 1μg ml^{-1} leupeptin, 1mM benzamidine, 1mM pepstatin, 1mM PMSF) containing 4M NaCl. After sample application, the column was washed sequentially with two column volumes of Buffer A containing 4M NaCl, three

column volumes of Buffer A containing 2M NaCl, and three column volumes of Buffer A. Endo A was eluted from the column with Buffer A containing 1% (v/v) Brij 58. Endonuclease-containing fractions were pooled, $MgCl_2$ added to a final concentration of 10mM and applied to a 16cm×2.5cm column of Heparin Sepharose, previously equilibrated in Buffer B (30mM KPO_4 pH 7.5, 10mM $MgCl_2$, 20% (v/v) glycerol, 0.05% (v/v) Brij 58, 1mM Na_2EDTA, 5mM β-mercapto-ethanol, 1μg ml^{-1} leupeptin, 1mM benzamidine, 1mM pepstatin). Following sample application the column was washed with two column volumes of Buffer B. Endo A was eluted from the column with Buffer C containing 75mM KCl and precipitated with $(NH_4)_2SO_4$ at 42% saturation followed by centrifugation in a Sorvall SS34 rotor at 17,500rpm for 30min. The resultant pellet was carefully drained and resuspended into 4.75ml Buffer D (50mM Tris-HCl pH 8.0, 20% (v/v) glycerol, 50mM NaCl, 1mM Na_2EDTA, 3mM β-mercaptoethanol, 1μg ml^{-1} leupeptin, 1mM benzamidine, 1mM pepstatin). The solution was clarified by centrifugation in a Sorvall SS34 rotor for 20min at 17,500rpm and stored on ice at 0°C.

Endo A assays

Standard reactions contained (in 15μl) 50mM Tris pH 8.0, 10mM $MgCl_2$, 1mM DTT, 100mM NaCl, 0.2 ng ^{32}P labeled 71 bp probe (5,000 acid precipitable cpm) and 4 units partially purified Endo A. Reactions were incubated at 30°C for 30min followed by the addit ion of 85μl of 0.3M NaOAc, 10mM Na_2EDTA, 0.6% (w/v) SDS, 5μg tRNA. The DNA was extracted with phenol/ chloroform/isoamyl alcohol (25: 24:1), ethanol precipitated and resuspended into 4μl gel loading buffer [99% formamide, 0.1×TBE, 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue]. Samples were heated at 100°C for 5min and run on 8% polyacrylamide (acrylamide:bisacrylamide ratio 19:1) 8M urea gels (20cm×20cm×0.3cm) at 800V for 35min in TBE (89mM Tris pH 8.0, 89mM borate, 2mM Na_2EDTA). In each of the characterization assays the standard reaction conditions were maintained except for the parameter being tested. Gels were dried and exposed to Kodak XAR film at −70°C with a Cronex Quanta III intensifying screen for 36h. Endo A activity was quantitated by densitometric analysis of autoradiographs on a Model 620 video densitometer (Bio Rad). One unit of Endo A activity is defined as the amount of activity required for conversion of 50% of the 71 bp probe to a 30 nucleotide species using the standard reaction conditions.

Determination of the Stokes radius

Gel filtration of 200μl of fraction V (4,000 Endo A units) was carried out on a Pharmacia Superose 12 (10/30) column equilibrated in 50mM Tris pH 8.0, 900mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) Brij 58, 0.1mM Na_2EDTA, 3mM β-mercaptoethanol. All samples were chromatographed in this buffer at a flow rate of 0.5ml min^{-1}. The column was calibrated with individual runs of yeast alcohol dehydrogenase, β -thyroglobulin, apoferritin and bovine serum albumin. Benzamidine was included in each run as a common marker. Marker proteins were monitored by absorbance at 280 nm. Endo A containing fractions (300ml) were detected using the standard reaction assay.

Glycerol gradient sedimentation

Partially purified Endo A (fraction V) was sedimented through a 4.4 ml linear (10−30% v/v) glycerol gradient containing 50mM Tris pH 8.0, 1mM DTT,0.1mM Na_2EDTA, 0.1% Brij 58, 900mM NaCl and 2μg ml^{-1} leupeptin. A 100 μl sample containing 2,000 units of enzyme was applied and run in the same gradient with 500 μg catalase and 10 units *E.coli* DNA polymerase I. The sample was centrifuged in a Beckman SW50.1 rotor at 42,000rpm at 2°C for 20h. Fractions (100 μl) were collected from the bottom of the

Table 1. Partial purification of Endo A.

Fraction	Step	Protein (mg)	Activity (units × 10^5)	Specific Activity (U/mg)	Recovery (%)
I	0.3 M supernatant	4100	23.5	1.7×10^2	–
II	PEG supernatant	1240	52.7	4.2×10^3	100
III	Phenyl Sepharose eluate	472	47.3	1.0×10^4	90
IV	Heparin Sepharose eluate	56.4	21.9	3.9×10^4	42
V	$(NH_4)_2SO_4$ precipitate	4.75	7.1	1.5×10^5	13

tube. Each fraction was assayed for catalase by measuring absorbance at 405 nm, for DNA polymerase using a standard nick translation assay (9) and for Endo A.

Recovery of Endo A activity following electrophoresis

Partially purified Endo A was electrophoresed at 4°C on 8% acrylamide resolving gels (acrylamide:bisacrylamide ratio 30: 0.8) and 5% stacking gels according to Laemlli (13). Protein bands were visualized by silver staining as described by Wray *et al* (14). Recovery of enzymatic activity from the gel following electrophoresis was as described (15) with the following modifications. Gel slices were crushed and eluted overnight at room temperature. The guanidine concentration of the sample was reduced by dialysis into buffer containing 50mM Tris-HCl pH 8.0, 20% (v/v) glycerol, 100μg ml^{-1} BSA, 150mM NaCl, 1mM DTT, 0.1mM Na_2EDTA.

Protein Determination

Protein concentrations were determined by the Bradford method (16) using the Bio Rad assay reagent. Bovine serum albumin was used as a protein standard.

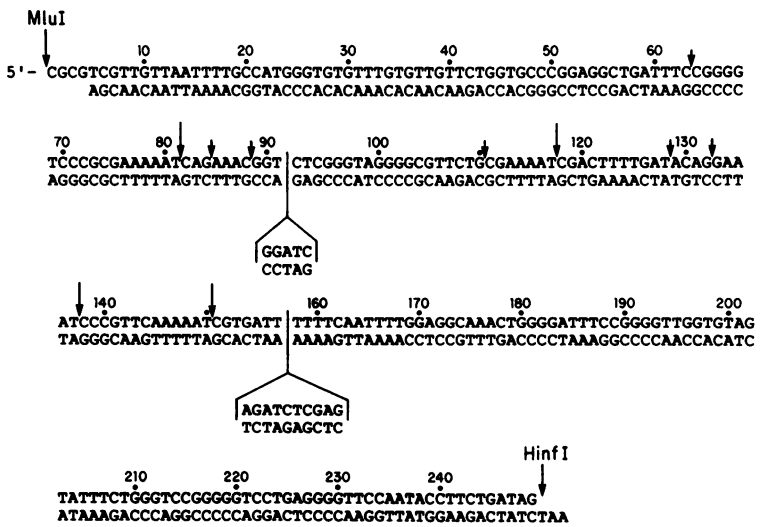

Figure 1. *Nucleotide sequence of DNA probes.* The nucleotide sequence of the 249 bp MluI-HinfI minicircle fragment is shown with the indicated sites of oligonucleotide insertion used in the preparation of the 71 bp probe. Location of the nicks are indicated by arrows. The frequency of cleavage are indicated by the length of the arrows.

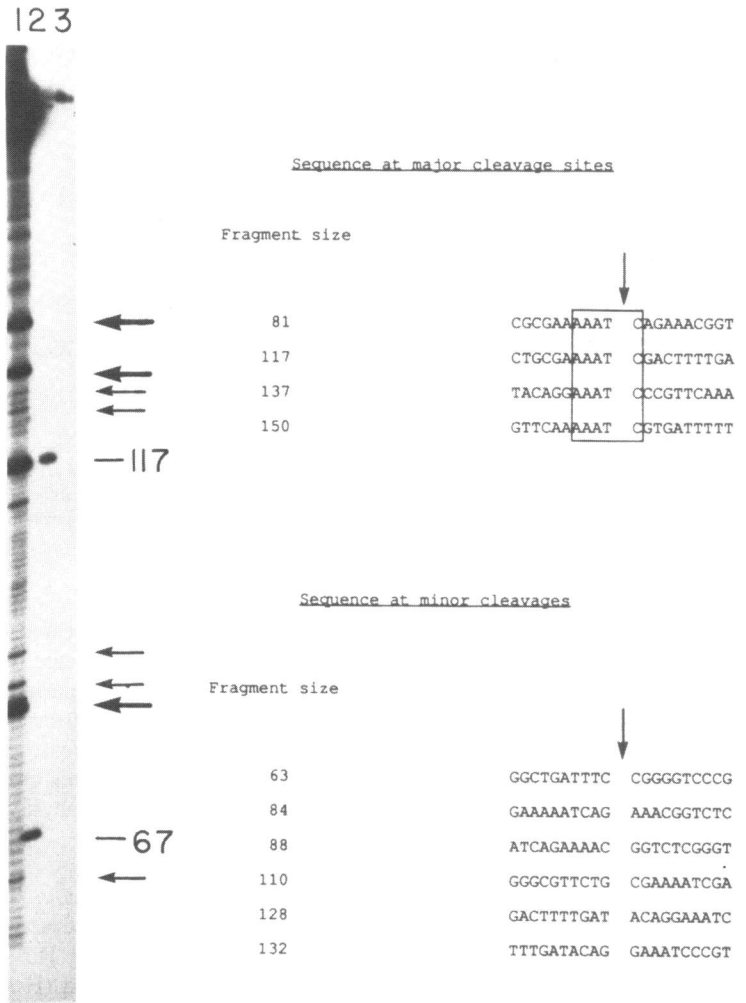

Figure 2. *Endo A cleavage at highly preferred sites.* The standard Endo A reaction was carried out using the 5′-end-labeled 249 bp probe. Lane 1, 249 bp probe digested with 1μl whole cell lysate of *C. fasciculata* ; Lane 2, 249 bp probe digested with AvaII to give a 67 nucleotide fragment; Lane 3, 249 bp probe digested with Taq I to give a 117 nucleotide fragment. The products of digestion were separated on 8% denaturing gels and detected by autoradiography. Bold arrows indicate major cleavage sites and thin arrows indicate the more prominant minor cleavages. The sequences at the major and minor cleavage sites are shown in the adjacent panel.

RESULTS

Partial purification of Endo A

Purification was carried out at 0−4°C in the presence of protease inhibitors in order to minimize proteolytic degradation. Results of the partial purification are summarized in Table 1. The value given for the number of Endo A units in the starting material is only

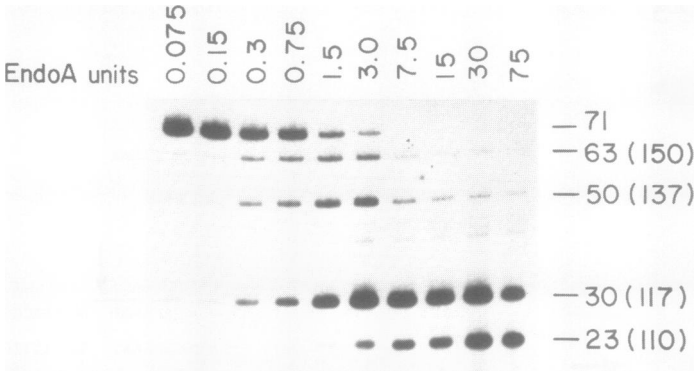

Figure 3. *Titration of Endo A activity.* Standard Endo A reactions were carried out in the presence of increasing amounts of Endo A and 0.2ng 5'-end-labeled 71 bp probe. Digestion products were separated on 8% denaturing gels. Gels were dried and subjected to autoradiography as described. Numbers on the right indicate the size of the cleavage products from the 71 bp probe. Numbers in parentheses indicate the corresponding position of cleavage on the 249 bp probe.

an approximation, due to the presence of inhibitors in the crude lysate. Consequently, the recovery of Endo A is calculated on the basis of the PEG supernatant. Endo A activity was eluted from the hydrophobic matrix, Phenyl Sepharose, in the presence of 1% Brij 58. This step efficiently removes nucleic acids before application to the Heparin Sepharose column. Endo A was eluted from the Heparin Sepharose column in the presence of 75mM KCl. The final purification step involved the precipitation of endonuclease-containing fractions by $(NH_4)_2SO_4$ at 42% saturation. The overall purification of this enzyme was estimated to be ~500-fold. This partially purified enzyme was used for the physical analysis and biochemical characterization of Endo A.

Endo A introduces a single-strand break at highly preferred sites

Endo A activity was originally identified in whole cell lys-ates of *C. fasciculata.* The 249bp and 71bp DNA probes used in this analysis were derived from the conserved sequence region around one of the kinetoplast minicircle DNA replication origins (8). The sequences of these fragments are shown in Fig.1. Endo A cleaves the upper strand (H strand) of the 249 bp probe at four major sites at positions 81, 117, 137 and 150 (Fig.2). Each cleavage is within the TpC dinucleotide at the sequence AAATC(Fig.1). Six minor cleavage sites are also observed at positions 63, 84, 88, 110, 128 and 132.

Endo A (Fraction V), was characterized further using the smaller 71 bp probe which incorporated three of the most preferred Endo A sites contained within the original 249 bp probe. In this case, the major cleavages occur only at the AAATC sequence at positions 63, 50 and 30 until relatively high concentrations of the enzyme are used (Fig.3). At these higher concentrations Endo A generates mainly the 30 nucleotide species but an additional cleavage at the sequence TCTGC within the GpC dinucleotide occurs, generating a further 23 nucleotide species corresponding to the 110 nucleotide fragment produced from the 249 bp probe.

A third probe was used to analyse the specificity of cleavage in an unrelated fragment of DNA and to determine whether Endo A cleaves one or both strands of DNA. Individual

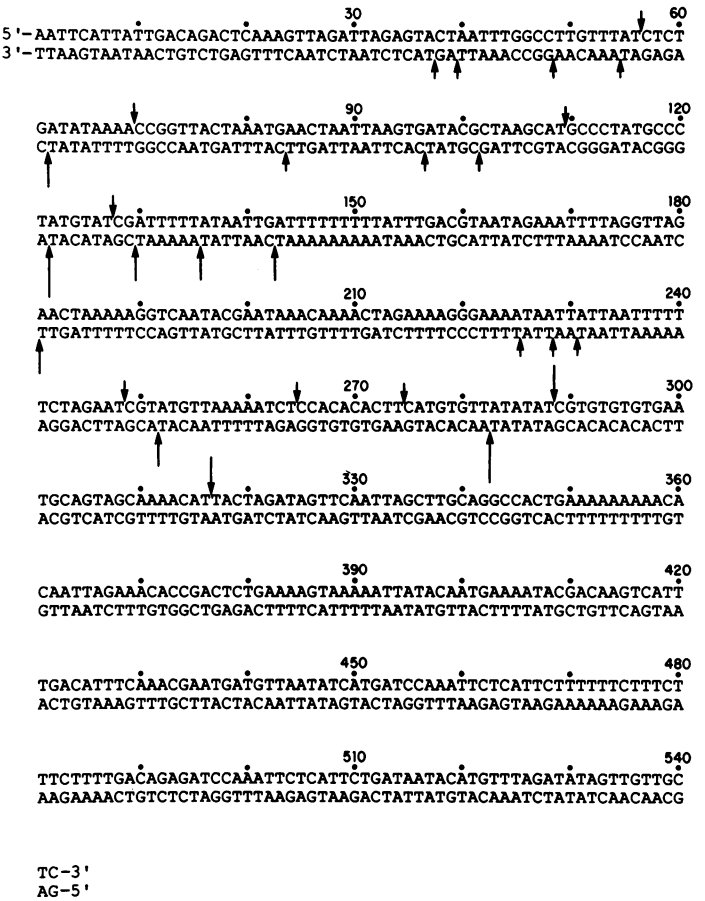

Figure 4. *Nucleotide sequence of the 542 bp probe.* Location of the sites of cleavage within the first 334 base pairs of the fragment are indicated by arrows. The frequency of cleavage is indicated by the length of the arrow.

preparations of the DNA fragment were either 5′ or 3′ end-labeled. The sequence of the probe and the Endo A cleavage sites are shown in Fig.4. Digestion products from the reactions were resolved on either 4%, 6%, or 8% denaturing gels to determine the exact cleavage sites. Only the first 334 bp of the probe were analysed due to the difficulty of assigning exact cleavage sequences on larger fragments of DNA. In this case, the most preferred cleavage site is ACATA in the lower strand at position 121−125, cutting within the TpA dinucleotide (Fig.5 Lanes 8 and 10). The sequence AAATC at position 129−133 and 142−146 was also cleaved at a high frequency on the 3′ end-labeled strand in addition to other similar sequences at positions 50−54, 81−85, 135−139, 281−285. The most preferred cleavage site on the 5′ end-labeled strand is ATATC, at position 285−289, cleaving within the TpC dinucleotide (Fig.5 Lanes 2 and 4). On this strand the sequence AAATC, at position 259−263, is only cleaved at a very low frequency. Note that the most preferred sites cleaved here do not occur in the 249 bp and 71 bp probes. Also,

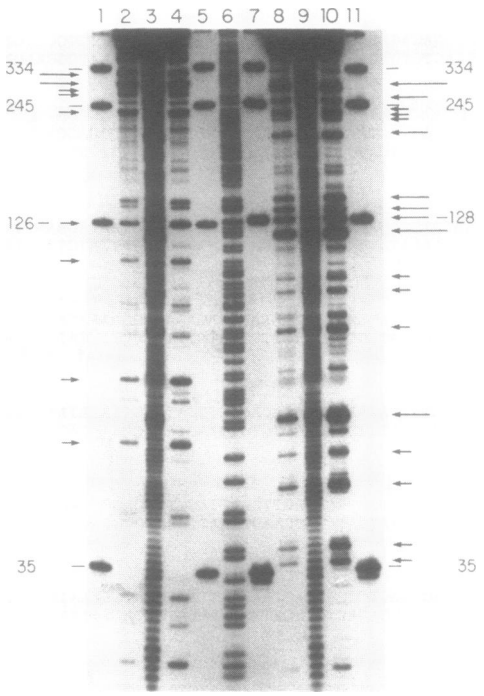

Figure 5. *Endo A introduces a single stranded break at highly preferred sites.* The 542 bp EcoRI-PstI fragment was either 5′ or 3′ end labeled at the EcoRI site and digested with Endo A (Fraction V) under standard reaction conditions. Cleavage products were separated on 8% denaturing gels. Gels were dried and subjected to autoradiography as described. Lanes 1 and 5, combined digests of the 5′ endlabeled probe with AluI, XbaI, TaqI and RsaI; Lanes 2 and 4, digestion of the 5′ end-labeled probe with 0.75 units and 3.0 units of Endo A respectively; Lane 3, DNAse I digest of 5′ end-labeled probe; Lane 6, G+A ladder of the 5′end-labeled probe; Lanes 7 and 11, combined digests of the 3′ end-labeled probe with AluI, XbaI, TaqI and RsaI; Lanes 8 and 10, digestion of the 3′end-labeled probe with 0.75 and 3.0 units of Endo A respectively; Lane 9, DNAse I digest of the 3′end-labeled probe. The frequency of cleavage at the major cleavage sites is indicated by the length of the arrows. Numbers indicate the size of the nucleotide fragment generated from digestion of the probe by the restriction enzymes.

it should be noted that certain sequences are not cleaved with the same frequency e.g. compare cleavage of the sequence ACATA at positions 121−125 and 251−255 on the 3′ end-labeled strand. Other sequences are not cleaved in every case e.g. AAAAC is cleaved at postion 67−70 but not at position 208−211 on the 5′ end-labeled strand. These results indicate that Endo A cleavage sites depend on the sequence context, as well as the nucleotide sequence at the cleavage site.

However, analysis of the frequency of nucleotide residues at, or flanking the cleavage site show that there is a highly preferred sequence for cleavage (Table II). The nucleotide usage at each postion relative to the cleavage site was calculated on the combined data from the 249 bp and 542 bp probes. These results indicate that ANATC represents a highly preferred sequence for cleavage.

Table II. Nucleotide usage (%) at major cleavage sites

	−4	−3	−2	−1	+1	+2	+3	+4
A	65	30	76	14	24	35	35	24
T	24	35	18	70	8	14	30	30
C	0	24	3	5	55	19	19	24
G	11	11	3	11	13	32	16	22
consensus:	A	N	A	T	C			

Comparison of the cleavage products from the 5′and 3′end-labeled 542 bp probe following digestion by Endo A, show that at the major cleavage sites there is not a corresponding cleavage on the opposite strand of DNA, indicating that this enzyme introduces single strand breaks into double stranded DNA (Fig.5). How ever, this enzyme cannot cleave single stranded oligonucleotides (data not shown). Digestion of the plasmid pUC19 with Endo A further confirms the nicking activity of the enzyme. When a covalently closed circular plasmid is cleaved by Endo A under standard reaction conditions, approximately 80% of the supercoiled plasmid is converted into a nicked form (Form II) before linear molecules (Form III) begin to be produced (Fig.6).

Requirements for endonuclease activity

Endo A activity occurs over a wide pH range, with optimal activity being at pH 8.0 (Fig.7a). There is no change in the basic cleavage pattern from pH 6.0−pH 9.0 (data not shown). Endo A activity also occurs over a wide range of temperatures with optimal activity at 30°C (Fig.7b). Optimal enzyme activity is observed with 100mM NaCl (Fig.7c). At higher salt concentrations Endo A activity is reduced. A similar pattern of activity is observed with KCl (data not shown). Endo A has an absolute requirement for Mg^{2+} (Fig.7d). Optimal levels of Mg^{2+} are in the range 5−10mM. This Mg^{2+} requirement can be substituted by Mn^{2+}. At similar concentrations the characteristic pattern of cleavage is observed, although the extent of cleavage is much reduced. However, at lower Mn^{2+} concentrations (0.25mM − 1mM) Endo A exhibits a changed sequence specificity, so

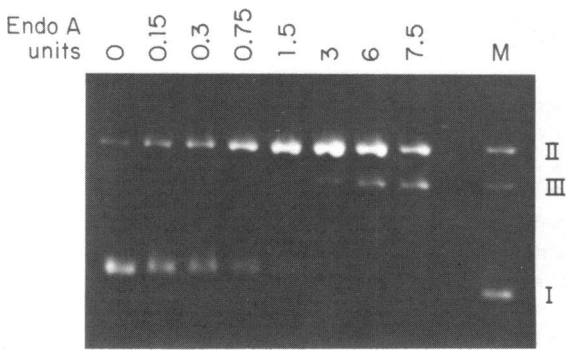

Figure 6. *Endo A nicks covalently closed DNA substrates.* Standard Endo A reactions were carried out in the presence of increasing amounts of Endo A and 150ng of pUC19. Digestion products were resolved on 0.6% agarose gels in the presence of ethidium bromide. M, pUC19 treated with purified topoisomerase IImt (19). I, covalently closed circles; II, nicked circles; III, linears.

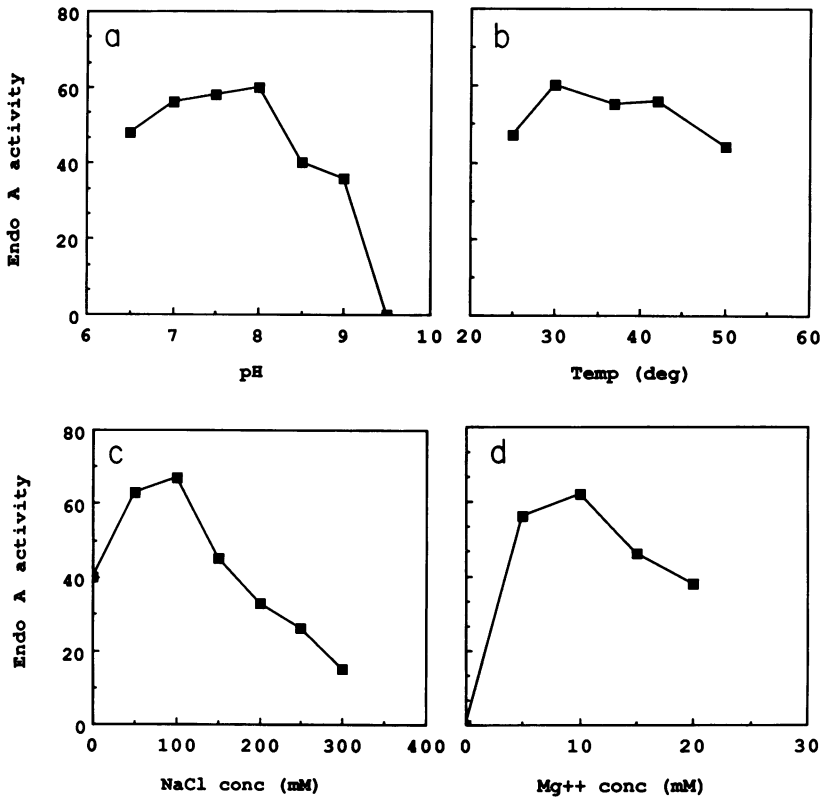

Figure 7. *Enzymatic characterization of Endo A.* Endo A assays were carried out using four units of Endo A and the standard reaction conditions except for the parameter being investigated. Endo A activity is given as the percentage conversion of the 71 bp probe to the 30 bp species. (a) pH dependence, (b) Temperature dependence, (c) Salt dependence, (d) Metal ion dependence.

that the major cleavage product is that of the 23 nucleotide species (data not shown) instead of the characteristic cleavage pattern, where the major product of digestion is the 30 nucleotide species. Ca^{2+} and Zn^{2+} were unable to substitute for the Mg^{2+} cofactor.

Size determination of Endo A

The sedimentation coefficient (S value) of the partially purified enzyme (fraction V) was estimated by sedimentation through a linear 10−30% (v/v) glycerol gradient. Catalase (monomer 4.2S and tetramer 11.6S) and *E. coli* DNA polymerase I (5.6S) were run in the same gradient as internal sedimentation markers. The results of the gradient are shown in Fig.8a. Endo A sediments with an $S_{20,w}$ value of 4.9. Gel filtration analysis on a Superose 12 column incorporating yeast alcohol dehydrogenase (116Å), β thyroglobulin (85Å), apoferritin (61Å) and bovine serum albumin (35.5Å) as calibration markers, indicate that Endo A has a Stokes radius of 59Å (Fig. 8b). Based on these results, the native molecular weight of the enzyme was calculated as descibed by Siegel and Monty (17) to be 125,000 with a frictional ratio of 1.8.

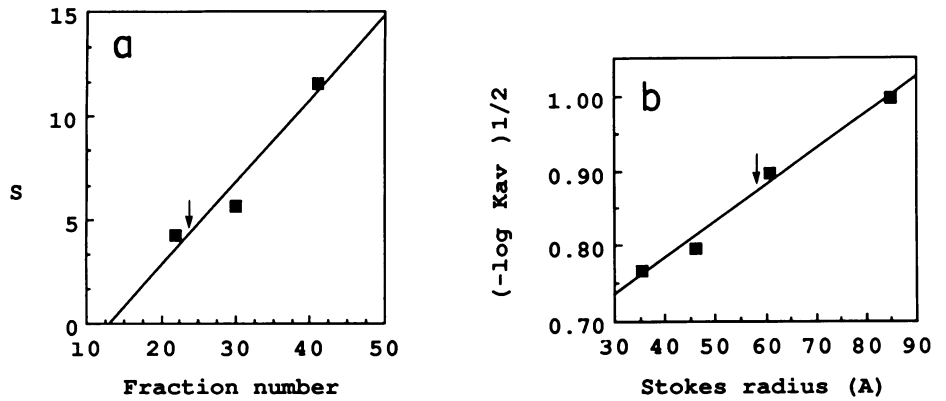

Figure 8. *(a) Glycerol gradient sedimentation.* Partially purified Endo A (100μl of fraction V) and internal marker proteins were sedimented as described. The plot of S values of catalase(monomer 4.2S and tetramer 11.6S) and *E.coli* DNA polymerase I(5.6S) are shown. The arrow indicates the position of Endo A (4.9S).(b) *Gel filtration analysis.* Gel filtration analysis on a Superose 12 column was used to determine the Stokes radius of Endo A. Yeast alcohol dehydrogenase (116Å), β-thyroglobulin (85Å), apoferritin (61Å), and bovine serum albumin (35.5Å) were used as marker proteins. The arrow indicates the elution position of Endo A (59Å).

The monomeric molecular weight was determined by recovery of Endo A activity following separation on an SDS gel. The majority of Endo A activity was recovered from gel slice 4 (Fig. 9a, Lane 4). SDS gel analysis of the renatured proteins, in the presence of BSA, from this gel slice shows the presence of a major protein species with a molecular weight of approximately 120,000 (Fig. 9b Lane 4). This protein species probably represents the intact enzyme. Endo A activity is also observed with proteins renatured from the gel slice corresponding to proteins in the molecular weight range 102,000−110,000 (Fig. 9a, Lane 5) and the gel slice corresponding to proteins in the molecular weight range 62,000−69,000 (Fig. 9a, Lane 13). The Endo A activity observed in these portions of the gel may result from the enzyme retaining its enzymatic activity, despite being partially degraded.

DISCUSSION

This paper describes the identification of a novel endonuclease activity from *C. fasciculata.* This enzyme, designated Endo A, introduces single-strand breaks (nicks) into double stranded DNA at highly preferred sites. The most preferred site in the 249 and 71 bp probes derived from *C.fasciculata* is cleavage of the TpC dinucleotide in the sequence AAATC. Endo A also cleaves this DNA at other sites, albeit at a much lower frequency. It is likely that Endo A is responsible for these minor cleavages as this cleavage activity is coincident with Endo A recovered following SDS gel electrophoresis.

Further analysis of Endo A cleavage specificity was carried out using an unrelated piece of DNA. In this case the most preferred cleavage site occured at the sequence ACATA between the TpA dinucleotide on the 3′ end-labeled strand and at the sequence ATATC between the TpC dinucleotide on the 5′ end-labeled strand. The sequence AAATC was also cleaved at a high fequency in addition to other related sequences. Analysis of the

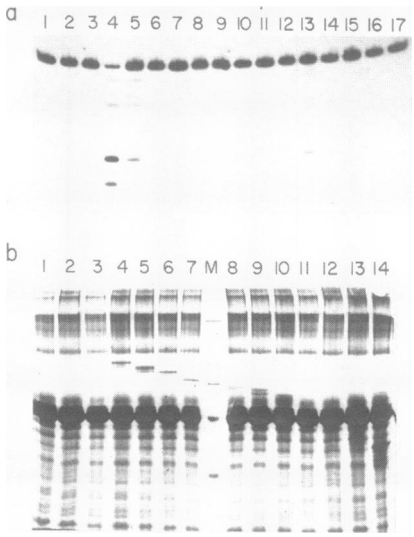

Figure 9. *Renaturation of Endo A activity from SDS polyacrylamide gels.* Partially purified Endo A (100mg) was separated on an 8% SDS polyacrylamide gel. Sequential slices of either 1, 0.5, or 0.25cm were taken. The enzyme was eluted and renatured from each slice as described. Endo A activity was identified using the standard reaction and 5μl of each sample. In Panel a and b lane numbers correspond to analysis of renatured proteins from the same gel slice. Lane 1 refers to the first slice taken from the top of the gel. Panel (a) Autoradiograph of Endo A assays. Lanes 1, 2, 15, 16 and 17, proteins derived from 1cm slices; Lanes 3−8, 0.25cm slices; Lanes 10−14, 0.5cm slices. Panel(b) Silver stained SDS-gel of renatured proteins from the gel slices above. Renatured proteins from gel slices 13−17 were not resolved on SDS-PAGE. Marker proteins: myosin [H chain] (200,000 Da), β-galactosidase (116,000 Da) phosphorylase B (97,400 Da), bovine serum albumin (66,000 Da), ovalbumin (45,000 Da), carbonic anhydrase (29,000 Da). The major protein contamination observed in each of the sample lanes results from the presence of bovine serum albumin in the renaturation reactions.

nucleotide usage flanking the cleavage site revealed a highly preferred sequence for cleavage, ANATC.

Gel filtration and velocity sedimentation analysis indicate that Endo A has a Stokes radius of 4.9S and sedimentation coefficient of 59Å. Based on these results, the native molecular weight of Endo A was calculated to be 125,000. The frictional ratio of the enzyme is 1.8, which is unusually large, suggesting that the enzyme has a very elongated structure. Determination of the monomeric molecular weight of the enzyme was facilitated by the ability to recover Endo A activity following gel electrophoresis in the presence of SDS and β-mercaptoethanol. Based on this analysis, the Endo A polypeptide has a molecular weight in the range 116,000−120,000, consistent with the enzyme having a monomeric structure. However, in addition to the intact enzyme, Endo A activity was recovered from other gel slices which correspond to parts of the gel containing lower molecular weight proteins. This activity may represent degradation products of the intact enzyme which have retained Endo A activity. Partial purification of the enzyme was carried out at 0−4°C in the presence of a range of protease inhibitors, to minimize proteolytic degradation. However, the very elongated structure may make it particularly prone to proteolytic cleavage.

Endo A is active over a broad range of pH and temperatures. High salt concentrations (above 150mM NaCl) start to inhibit Endo A activity, but at concentrations below 100mM NaCl the extent of cleavage at the TCTGC site relative to the AAATC site on the 71 bp probe is increased. Thus, salt concentration affects the specificity of cleavage to a small extent. The cleavage specificity of Endo A is also altered in the presence of Mn^{2+}. In this case at low Mn^{2+} concentrations the major cleavage product is the 23 nucleotide fragment. An altered sequence specificity is sometimes observed with prokaryotic restriction enzymes under sub-optimal conditions (18).

There is only one report of an endonuclease activity from *C. fasciculata*, the *Crithidia* nicking enzyme (6). It is proposed that the cleavage of covalently-closed DNA by this enzyme is not sequence directed *per se*, but is directed by the tertiary topology of the DNA substrate. Although the bent region of the *C. fasciculata* minicircle kDNA is required for nicking of relaxed covalently closed circles it is not a preferred cleavage site, rather other structural features determine the cleavage site (7). An AAATC site within this region of the minicircle kDNA, was not cleaved by the *Crithidia* nicking enzyme. Likewise, the other major sites at which Endo A cleaves are not cleaved by this enzyme. In addition, *Crithidia* nicking enzyme cannot cleave linear fragments of DNA. The *Crithidia* nicking enzyme can also be distinguished from Endo A based on its molecular weight and biochemical requirements for activity. This enzyme exists as a dimer of 60,000 Da subunits and unlike Endo A, *Crithidia* nicking enzyme exhibits a narrow pH optimum between 7.50−8.25 and is inhibited by salt concentrations above 10mM.

The physiological function of Endo A is unknown. It is unlikely that Endo A is involved in DNA replication due to the moderate specificty of the cleavage reaction. However, the enzyme may be involved in some aspect of DNA repair or in general DNA degradation. It will be necessary to purify and immunolocalize Endo A before it is possible to suggest a physiological function for this enzyme.

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