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Purification and Characterization of a Deoxyriboendonuclease from *Mycobacterium smegmatis*

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A deoxyriboendonuclease has been purified to near homogeneity from a fast growing mycobacterium species, *M. smegmatis* and characterized to some extent. The size of enzyme is about 43 kDa as determined by a denaturing gel analysis. It shows optimum activity at 32°C in Tris-HCl buffer (pH 7.2) containing 2.5 mM of $MgCl_2$. Both EDTA and K^+ but not Na^+ inhibit its activity. Evidences show that the enzyme is not a restriction endonuclease but catalyzes the endonucleolytic cleavage of both the double- as well as the single-strand DNA non-specifically. It has been shown that the cleavage by this enzyme generates DNA fragments carrying phosphate groups at 5' ends and hydroxyl group at the 3' ends, respectively. Analysis reveals that no endonuclease having size and property identical to our deoxyriboendonuclease had been purified from *M. smegmatis* before. The property of our enzymes closely matches with the deoxyriboendonucleases purified from diverse sources including bacteria.

Keywords: Deoxyriboendonuclease, Mycobacteria, *M. smegmatis*

Introduction

Deoxyriboendonucleases perform various crucial jobs inside the living cells by catalyzing the cleavage of DNAs either at the specific sites or randomly. While some of them actively participate in the recombination and repair of DNA molecules (Miller *et al*., 2003; Schofield and Hsieh, 2003; Krogh and Symington, 2004), majority of the others protect the genomic DNAs of the cellular organisms from degradation by viruses (Dryden *et al*., 2001; Pingoud and Jeltsch, 2001). A very special kind of deoxyriboendonucleases designated homing endonucleases catalyze the lateral transfer of an intron/intein containing intervening sequence from one gene to a homologous allele (Chevalier and Stoddard, 2001). To date, hundreds of endonucleases are commercially available, which not only expedite the research in molecular biology but also profoundly enrich the fields like agriculture, medicine, dairy, poultry industries, etc. (Kishi *et al*., 2001; Suri, 2005).

Several deoxyribonucleases including endonucleases have also been purified from mycobacterial systems and characterized to some extent. A nucleotide triphosphate-dependent deoxyribonuclease was purified partially from *M. smegmatis* cells grown under iron-limited condition and has been suggested that this enzyme degrades DNA mostly exonucleolytically (Winder and Lavin, 1971). A couple of restriction endonucleases purified from different species of mycobacteria have been shown to be the isochizomers of *Pst*I (Shankar and Tyagi, 1992), *Sau*3AI (Shankar and Tyagi, 1993a), *Not*I and *Hae*III (Shankar and Tyagi, 1993b). A homing endonuclease from *Mycobacterium tuberculosis* has also been reported (Guhan and Muniyappa, 2002). The genes encoding recB, recC, and recD deoxyriboendonucleases were detected in both *M. tuberculosis* and *M. smegmatis* (Griffin *et al*., 1999; Hatfull, 2000). Thus far, no deoxyriboendonuclease has been purified from mycobacterial system which is involved in non-specific endonucleolytic cleavage of DNA. In this communication we report the purification and partial characterization of such deoxyriboendonuclease from *Mycobacterium smegmatis*.

Materials and Methods

Growth of bacteria and chemicals. *E. coli* 594 and *M. smegmatis* mc^26 cultures were routinely grown in TB (Sambrook and Russell, 2001) and in 7H9 broth (Chaudhuri *et al*., 1993), respectively, at 37°C. *M. smegmatis* mc^26 was obtained from Dr. B. Bloom, Albert Einstein College of Medicine. The fine chemicals were purchased from Sigma Co. and [^{3}H]-thymidine, from BRIT.

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Assay of deoxyriboendonuclease. It was carried out by quantifying the acid (Perchloric acid)-soluble radioactivity that is formed by the action of the enzyme on [^{3}H]-labeled *E. coli* DNA (fragmented by limited sonication) (Radding, 1964). To prepare the [^{3}H]-labeled *E. coli* DNA, log phase *E. coli* 594 cells were grown with [^{3}H] thymidine (2 μCi/ml, 1000 Ci/mmol) and 2-deoxyadenosine (250 μg/ml) for 3 h followed by isolation of the DNA by the method of Murmur (1961). Radioactive counts were determined in a Beckman Liquid Scintillation Counter Model LS 5000CE using aqueous or non-aqueous cocktail as required.

Purification of deoxyribonuclease. Cell pellet harvested from 6 liter saturated culture of *M. smegmatis* mc^{2}6 was suspended in cold Buffer A (20 mM Tris-HCl, pH 7.0, 1 mM 2-mercapto ethanol; 20 ml/g of cells) followed by the preparation of cell extract by sonication. To supernatant (called crude extract) yielded after centrifugation of the above extract at 100,000 g for 2 hr, streptomycin sulphate solution (10% in Buffer A) was added to a final concentration of 1% over a period of 30 min with constant gentle stirring. The mixture was centrifuged at 10,000 g for 20 min and the resulting pellet suspended in 20 ml Buffer A (called streptomycin sulphate fraction). To this solution, 7 ml of 30% (w/v) PEG 8000, 3 ml of 20% (w/v) Dextran solutions and solid sodium chloride (to a final concentration of 4 M) were added successively and the mixture was stirred for 2 h and centrifuged at 10,000 g for 20 min. The top aqueous phase containing the enzyme was collected and extensively dialyzed against Buffer A. The solution was then centrifuged at 10,000 g for 20 min, and the pellet was suspended in 7 ml of Buffer A. To this solid sodium chloride was added to a final concentration of 0.2 M and mixed well to dissolve the salt. This was then homogenized for 5 min at room temperature followed by centrifugation at 10,000 g for 10 min. The supernatant containing the enzyme was dialyzed against Buffer B (Buffer A + 10% glycerol) and was called PEG-Dextran phase fraction. This fraction was then loaded onto a heparin-agarose column (10 ml bed volume, pre-equilibrated with Buffer B). The enzyme activity was detected in flow-through and washings. The fractions containing the enzyme were pooled (Heparin-agarose fraction). This was then loaded onto a QAE-Sephadex column (100 ml bed volume) pre-equilibrated with Buffer C (Buffer B at pH 7.4) and the column was washed with Buffer C containing 0.2 M NaCl. The enzyme was eluted with Buffer C containing 0.5 M NaCl. The fractions showing enzyme activity were pooled (QAE-Sephadex fraction) and concentrated by dialyzing against 20% PEG in Buffer D (Buffer C containing 10 mM EDTA) to around 1 ml. This concentrated protein was then loaded onto a DNA-Cellulose column (6 ml bed volume) pre-equilibrated with Buffer D and washed with the same buffer. The proteins were eluted step-wisely using varying concentrations of NaCl in Buffer D. The enzyme eluted with 0.2 M NaCl. The fractions containing the enzyme activity were pooled (DNA-Cellulose fraction) and concentrated by dialysis against 20% PEG in Buffer D. This was then subjected to gel-filtration chromatography on Superdex-75 column (95 ml bed volume; 35 cm × 0.8 cm) pre-equilibrated with Buffer D containing 0.2 M NaCl. The elution was made with the same buffer. All the individual fractions showing enzyme activity were stored on ice. Protein content in each sample was estimated by the method of Bradford (1976) using bovine serum albumin as standard.

Results and Discussion

Purification of a deoxyribonuclease from *M. smegmatis*. A deoxyribonuclease was purified from *M. smegmatis* cells according to the procedure as described in Materials and Methods. The progress of the enzyme purification was monitored by estimating the amount of deoxyribonuclease activity (Table 1) as well as by SDS-PAGE analysis in small protein aliquots taken from each of the above steps (Fig. 1A). It was found that there were enzyme activities in all the six fractions (1.5 ml each) collected around the void volume region of gel filtration (Superdex 75) chromatography. Of the six fractions, fifth and sixth fractions showed only one protein band having size around 43 kDa (Fig. 1A). The final purification from Superdex Gel was around 521 fold with a total recovery of 3.8% compared to the crude extract (Table 1).

Conditions for maximum activity of the enzyme. The purified enzyme in Tris-HCl buffer (pH 7.4) containing 10

Table 1. Summary of the purification of deoxyriboendonuclease from *M. smegmatis*

Treatment	Yield			Sp. Activity	Fold Purification
	Protein (mg)	Units	% (Units)		
Crude extract	1,225	22,192	100.0	18	
Streptomycin ppt	210	6,098	27.5	29	1.6
PEG-Dextran	82	2,604	11.7	32	1.7
Heparin Agarose	6	1,928	8.7	321	17.7
QAE-Sephadex	5.2	1,873	8.4	360	19.7
DNA-Cellulose	0.9	1,220	5.5	1,355	79.8
Gel Filtration (Superdex 75)	0.09	850	3.8	9,444	521.2

One unit of the enzyme was defined as the amount of protein which could liberate 1 nmole of TCA soluble nucleotide from [^{3}H]-labeled DNA in 30 min under the conditions of the assay. *Specific activity* of the enzyme has been expressed in Units/mg protein. *Specific activity* of substrate [^{3}H] DNA was 1,800 cpm/nmole of nucleotide.

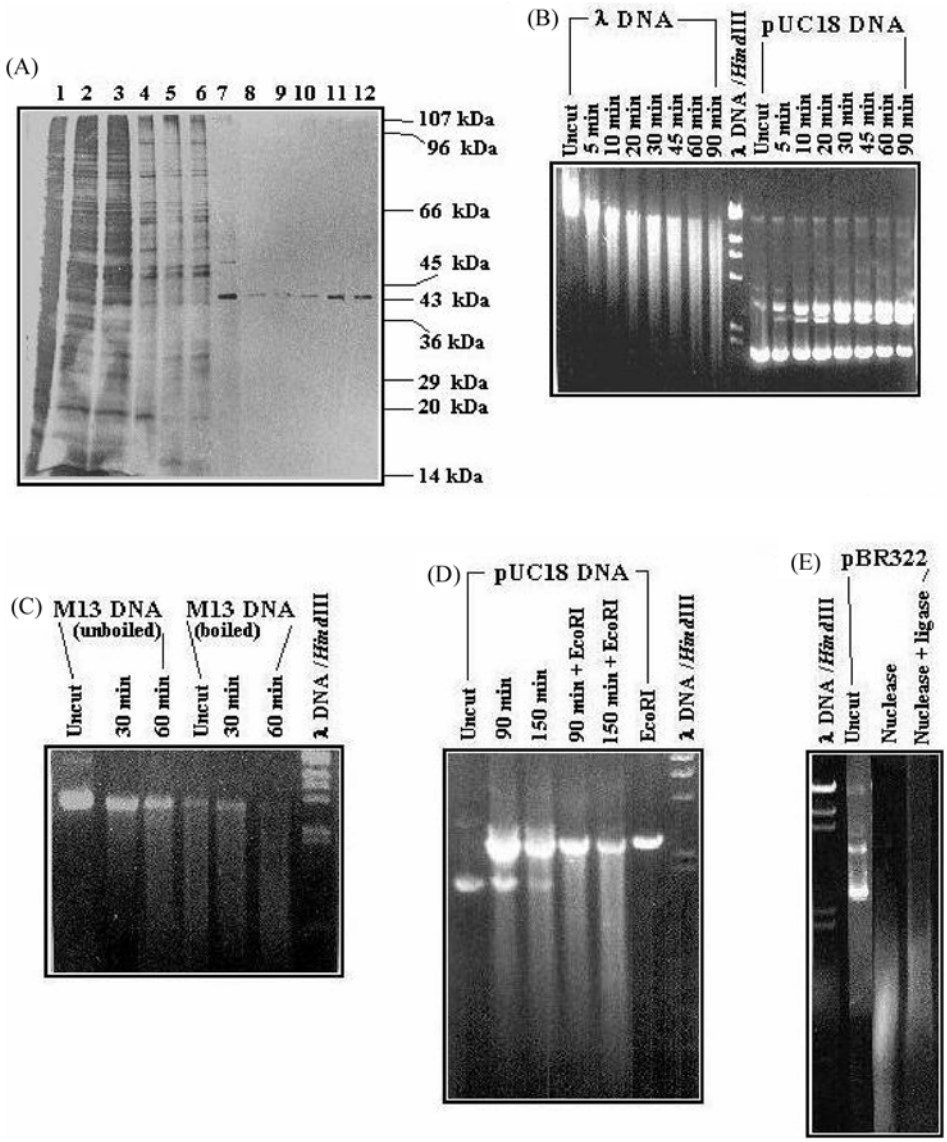

Fig. 1. Purification and characterization of a deoxyriboendonuclease from *M. smegmatis*. (A) Purity of the enzyme at different steps of purification. Suitable aliquots from different fractions during purification of the deoxyribonuclease was electrophoresed in SDS-PAGE as described in Methods. The protein bands were stained with silver nitrate and photographed. Lanes: 1. Crude extract (Ultra sup); 2. Streptomycin sulphate fraction; 3. PEG-Dextran phase fraction; 4. Heparin-Agarose fraction; 5, QAE-Sephadex fraction; 6, DNA-Cellulose fraction; 7-12, Superdex G-75 fractions 14 to 19. Sizes of the protein markers were shown at the right side of the gel picture. For other details, see Text and Methods. All the experiments reported in this paper were repeated 2-3 times, each showing reproducible results. Unless otherwise stated, the results of one set of experiments are presented. (B-C) Kinetics of action of deoxyribonuclease on (B) linear (lambda) and covalently closed circular (pUC18 plasmid) DNAs and (C) Single-stranded M13 DNA. About 0.01 unit enzyme was incubated with 1 µg lambda DNA or 0.5 µg pUC18 or 0.5 mg of M13 (unboiled or boiled for 10 min at 100 C) DNA in a total volume of 175 µl containing 20 mM Tris-HCl, pH 7.2, 10 mM Mg^{++} at 32°C. At different times, aliquots of 20 µl were taken out and electrophoresed on 0.7% agarose. Lanes in (B) & (C) as indicated. (D) Action of *Eco*RI restriction endonuclease on the linear form of pUC18 DNA generated by the deoxyriboendonuclease. Lanes as indicated. *Eco*RI-digested DNA products were obtained after 90/150 min reaction with deoxyriboendonuclease. (E) Action of T4 DNA ligase on the fragments of pBR322 DNA generated by the mycobacterial deoxyriboendonuclease. The fragments of pBR322 DNA purified by phenol - chloroform extraction and alcohol precipitation were treated with T4 DNA ligase for 2 h at 16°C. Lanes as indicated.

mM EDTA and 10% glycerol remains fully active for at least 15 days when stored in ice. To maintain this stability, the presence of 10 mM EDTA was found essential (data not shown). The enzyme shows maximum activity at a pH of 7.2 (Tris-HCl buffer was used) at 32°C in the presence of Mg^{++} (2.5 mM). Manganese (Mn^{++}) can replace Mg^{++} showing

better activity. The activity of enzyme is inhibited by EDTA (90% of the activity shown in the absence of EDTA is inhibited at 2.5 mM EDTA) as well as by K^+ but not by Na^+. The inhibition of enzyme activity by EDTA may possibly be explained by the fact that the enzyme requires Mg^{++} for its activity, and EDTA chelates this divalent cation. It has been shown that increasing the Mg^{++} concentration can restore the activity of the enzyme even in the presence of 2.5 mM EDTA (data not shown). This property of inhibition of the enzyme activity by EDTA has been exploited for the purification of this enzyme by affinity chromatography on DNA-Cellulose in the presence of 10 mM EDTA, when the degradation of the matrix-bound DNA by the enzyme was completely prevented. It is interesting to note that the enzyme activity is inhibited also by monovalent cation like K^+ but not by Na^+ while the activity of bacteriophage λ-encoded exonuclease is inhibited by Na^+ but not by K^+ (Radding, 1964).

Deoxyribonuclease is an endonuclease. To see whether this enzyme is an exo- or endonuclease, its action on both covalently closed circular (CCC) DNA (pUC18) and linear DNA (λ DNA heated at 60°C for 10 min) was tested. The experiment was done as described in Fig. 1B. The results show that at an extremely limiting concentration of the enzyme (around 0.01 unit enzyme per reaction), the CCC form of the plasmid (pUC18) DNA was first converted to its relaxed circular form and then to the linear form. When λ DNA was used as the substrate, size of the linear DNA fragments was gradually reduced with the increase of the time of reaction. With relatively high concentration of the enzyme (as used in routine assay of the enzyme activity, see Methods), acid soluble products were formed. It could be seen that during the initial period of reaction, the relaxed circular form accumulated faster than the linear form of the same plasmid DNA. So we conclude that at a very limiting concentration of the enzyme (very low enzyme: substrate DNA molar ratio and at > 0.01 unit of the enzyme per reaction) first one strand of the double-stranded DNA is cleaved, and this is followed by the cleavage of the opposite strand and possibly the cleavage of the second strand occurs as a random process but not by the same event as of the first strand cleavage. Results in Fig. 1C show that the enzyme cleaves also the single-stranded circular DNA (M13 DNA). When the M13 DNA was boiled at 100°C for 10 min and then used immediately as a substrate in the enzyme reaction, the DNA band disappeared faster than that of the unboiled DNA under exactly identical conditions of reaction.

The enzyme is not a restriction endonuclease. Production of acid-soluble nucleotides upon digestion of the substrate DNA with this endonuclease suggests that the site of cleavage on the DNA is randomly selected by the enzyme. To get further support in favor of the above, the linear pUC18 DNA produced by the digestion of its CCC form by cleavage with the above enzyme was gel purified and then digested with *Eco*RI (*Eco*RI has only one cleavage site in pUC18 DNA) and the products analyzed on agarose gel. The results show that no fragment having a definite size was produced and the fragment sizes reduced with the increase in the time of digestion (Fig. 1D) and by prolong digestion, those were converted to acid-soluble forms. These results suggest that the enzyme cleaves the DNA randomly with respect to all possible phosphodiester bonds.

The enzyme cleaves on the 3' side of the phosphodiester bonds in the DNA. To decipher whether our purified mycobacterial enzyme cleaves on the 3' side of the phosphodiester bonds in the DNA, pBR322 DNA was digested with the above enzyme and the resulting smaller fragments were used as the substrate for T4 DNA ligase. As shown in Fig. 1E, smaller fragments were ligated to generate larger fragments. This result suggests that this mycobacterial enzyme like all other deoxyribonucleases so far reported also cleaves the 3' phosphodiester bond in the DNA to generate fragments with 5' phosphate and free 3' OH ends.

The deoxyriboendonuclease reported here shows that it cleaves both the double and single stranded DNAs non-specifically and cleavage occurs at the 3' side of the phosphodiester bond in DNA. The size and properties exhibited by this endonuclease did not match with either of *M. smegmatis*-specific recA - recD proteins involved in recombination (Papavinasasundaram *et al*., 1997; Griffin *et al*., 1999; Ganesh and Muniyappa, 2003). Database searching shows that in addition to above rec proteins expressed by *M. smegmatis*, *M. tuberculosis* encodes 5 more putative endonucleases (http://genolist.pasteur.fr/TubercuList). Putative orthologous proteins of these 5 proteins are also present in *M. smegmatis* (http://tigrblast.tigr.org/ufmg). It was found that size of none of the 5 *M. smegmatis*-specific proteins matches with that of deoxyriboendonuclease purified by us. Our enzyme also differs from one isolated by Winder and Lavin (1971). Taken together, the data suggest that our deoxyriboendonuclease is a new protein and was not purified before from *M. smegmatis*. Further experimentation is needed to prove that it is present in mycobacterial systems other than *M. smegmatis*. However, the properties of our enzyme have somehow matched with the deoxyriboendonuclease enzymes expressed by different living organisms including bacteria (Starosciak and Dobrzanski, 1980; Rama *et al*., 1987; Martinez-Canamero *et al*., 1991; Baranovskii *et al*., 2004; Brnakova *et al*., 2005). It was suggested that the enzymes like deoxyriboendonucleases are utilized for carrying out number of biological functions such as DNA replication, recombination, protection of chromosome from integration of foreign DNA, apoptosis etc. It is not clear at this moment for what purpose exactly *M. smegmatis* needs to synthesize this non-specific endonuclease.

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