Single-strand-specific DNase activity is an inherent property of the 140-kDa protein of the snake venom exonuclease

Stoyno S. Stoynov, Anastassia T. Bakalova, Svetoslav I. Dimov, Atanaska V. Mitkova, Luben B. Dolapchiev*

Institute of Molecular Biology, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria

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Abstract Polyclonal antibodies against the exonuclease from *Crotalus adamanteus* venom (the 140-kDa protein) inhibit both the exonucleolytic and the single-strand-specific endonucleolytic activities, present in the exonuclease preparation. The antibodies also diminish the ability of the enzyme to split the negatively supercoiled Bluescript KS$^+$ in the AT-rich fragment near-by the transcription termination site of the Ampicillin gene. Therefore the single-strand-specific endonucleolytic activity was attributed to the protein molecule of the exonuclease. The processivity of the exonucleolytic action was found to be less than 3 monomers as indicated by the heparin trapping method.

Key words: Exonuclease; Bluescript II KS$^+$ plasmid; Single-strand-specific endonuclease; Processivity; *Crotalus adamanteus*

1. Introduction

The phosphodiesterase isolated from different snake venoms, often referred to as exonuclease I is known to split nucleoside 5′-monophosphate one at a time strictly from the 3′-end of single and double stranded DNAs as well as from polyribonucleotides bearing non-phosphorylated 3′-OH ends. After its successful purification from the venom of *Crotalus adamanteus* [1] the exonucleolytic activity was attributed to one polypeptide chain of 140 kDa [2]. However, even the most purified exonuclease preparations always showed several low molecular weight bands on SDS-PAGE with unknown properties.

Pritchard et al. [3] suggested that the venom exonuclease possesses endonuclease activity towards single-stranded DNA. The argument used in favour of this suggestion was the similar mobility of the both activities during non-denaturating PAGE. Because of well-known protein-protein interactions in non-denaturating conditions, this result may simply be caused by non-specific association of the two activities.

In the studies on the initiation of DNA replication the single-strand-specific endonucleases such as mung bean or P1 nucleases are extremely important for mapping of the DNA unwinding elements (DUE) [4,5]. But in all cases these single-strand-specific endonucleases, on account to lower enzyme activity, have to be used in higher than their pH-optimum pH. The attribution of the single-strand-specific endonucleolytic activity to well defined protein in the venom exonuclease preparation, which has an alkaline pH-optimum, would make it very convenient for such studies.

The occurrence of endonuclease in the preparation or the ability of the venom exonuclease to hydrolyze endonucleolytically single-stranded regions of DNAs raise the question of the processivity of the exonucleolytic mode of action of this enzyme. Significantly different results would be obtained as a consequence of parallel exonuclease and endonuclease activities in the case of non-processive or processive types of hydrolysis.

Here we describe experiments which assign the endonucleolytic activity towards single-stranded DNA to the 140-kDa venom exonuclease.

2. Materials and methods

2.1. Isolation and purification of Crotalus adamanteus venom exonuclease

The enzyme was purified according to the published procedure [1] with the modifications described later [2].

2.2. Preparation of polyclonal rabbit antibodies

An electrophoretically purified fraction of the 140-kDa SVE, containing 0.05 mg of the enzyme was excised from 7.5% SDS-PAGE after Coomassie Brilliant Blue staining. It was homogenized in 0.2 ml of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4{\cdot}7H_2O$, 1.4 mM KH_2PO_4, pH 7.3), suspended (1:1 v/v) with complete Freund's adjuvant (Difco, USA) and used for immunization. IgG fraction of the serum was precipitated by 35% ammonium sulfate and dialyzed against PBS.

2.3. Western Blots

The Western blotting was performed as described [6]. The protein samples were separated by SDS-PAGE and transferred on nitrocellulose membrane (Electrophoresis grade, 0.45 μm, Sigma, USA) with Semi-Dry-Blotting Apparatus (W.E.P. Co., USA). The membrane was washed with PBS and blocked with 0.5% BSA, (Pentex, USA) for 2 h at 37°C. It was incubated with antibodies for 2 h at 37°C (1:1000 dilution). The visualization of the protein-antibody complexes was done with anti-rabbit IgG conjugated with horse-radish peroxidase (Sigma, USA).

2.4. Detection of the single-strand-specific endonucleolytic activity

Venom exonuclease (0.005 U/ml) was incubated either with BSA (1 mg/ml) or with anti-SVE antibodies (1 mg/ml) for 1 h at 37°C. The enzyme was analyzed for its endonucleolytic activity in the following incubation mixture: 0.1 M Tris-HCl, pH 9.0, containing 0.01 M $MgCl_2$, 0.005% Triton X-100 and 50 μg/ml of Bluescript II KS$^+$ supercoiled DNA for 15 min. Four microliters of the DNA digest were run in 1% agarose gel containing 0.5 μg/ml of ethidium bromide [7]. The SVE hydrolysed plasmid was subjected to *Kpn*I or *Kpn*I and

*Corresponding author. Fax: (359) (2) 72-35-07.
E-mail: lbd@obzor.bio21.acad.bg

Abbreviations: SVE, snake venom exonuclease; *p*-NPh.pT, thymidin 5′-monophospho-*p*-nitrophenyl ester; *bis-p*-NPhP, *bis-p*-nitrophenyl phosphate

Enzymes: snake venom exonuclease (oligonucleate 5′-nucleotidohydrolase, EC 3.1.15.1).

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*Sac*I digestion and the fragments obtained were visualized electrophoretically [7].

2.5. Heparin inhibition of SVE

The inhibitory effect of the heparin on SVE was measured according to Kunitz method by following the amount of acid soluble nucleotides, released from thymus DNA and spectrophotometrically using p-NPh.pT and *bis-p*-NPhP – both from Sigma, USA. The activity in the presence and absence of heparin was measured as described [1].

2.6. Heparin trapping experiments

Two double-stranded DNA fragments of 448 and 2512 bp with blunt ends were prepared from Bluescript II KS$^+$ plasmid isolated as described [7] after digestion with *Pvu*II restriction endonuclease. Twelve micrograms of these fragments were incubated in 50 μl of 0.1 M Tris-HCl, pH 9.0, containing 0.01 M $MgCl_2$ with 8 μg of SVE. Heparin (Sigma, USA) was added to final concentration of 12 μg/μl immediately after the addition of exonuclease [8]. Four-microliter aliquots were run in 5% PAGE in Tris-borate buffer.

3. Results and discussion

The SDS-PAGE of the purified phosphodiesterase is presented on Fig. 1A. Although insignificant as contaminating proteins the fractions lower than 140 kDa could account for the single-strand-specific endonucleolytic activity. Therefore, to analyze this activity further, it was of importance to prepare specific antibodies against the exonuclease. For this purpose the immunization was accomplished with a cut-off of the single 140-kDa band after the SDS-PAGE.

The specificity of the generated anti-exonuclease antibodies was checked against the proteins, obtained after the first step of the exonuclease purification procedure, that is the acetone

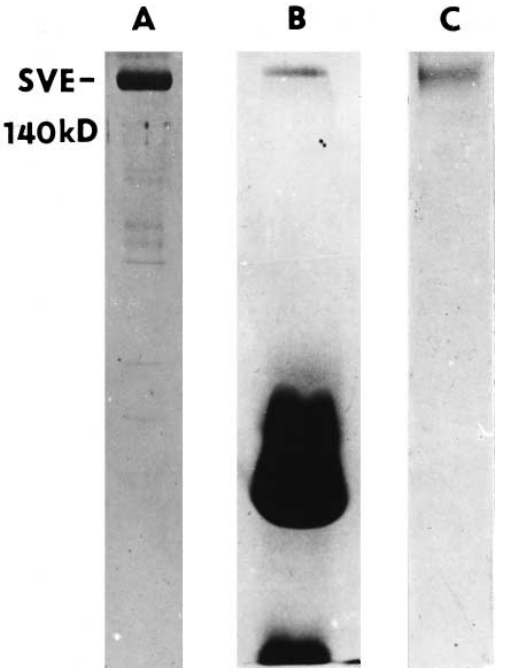

Fig. 1. Specificity of the obtained antibodies. (A) SDS-PAGE (12%) of highly purified preparation of exonuclease from the venom of *Crotalus adamanteus*. (B) SDS-PAGE (10%) of the acetone step of the purification of the exonuclease. (C) Western blot of the electrophoresis of B.

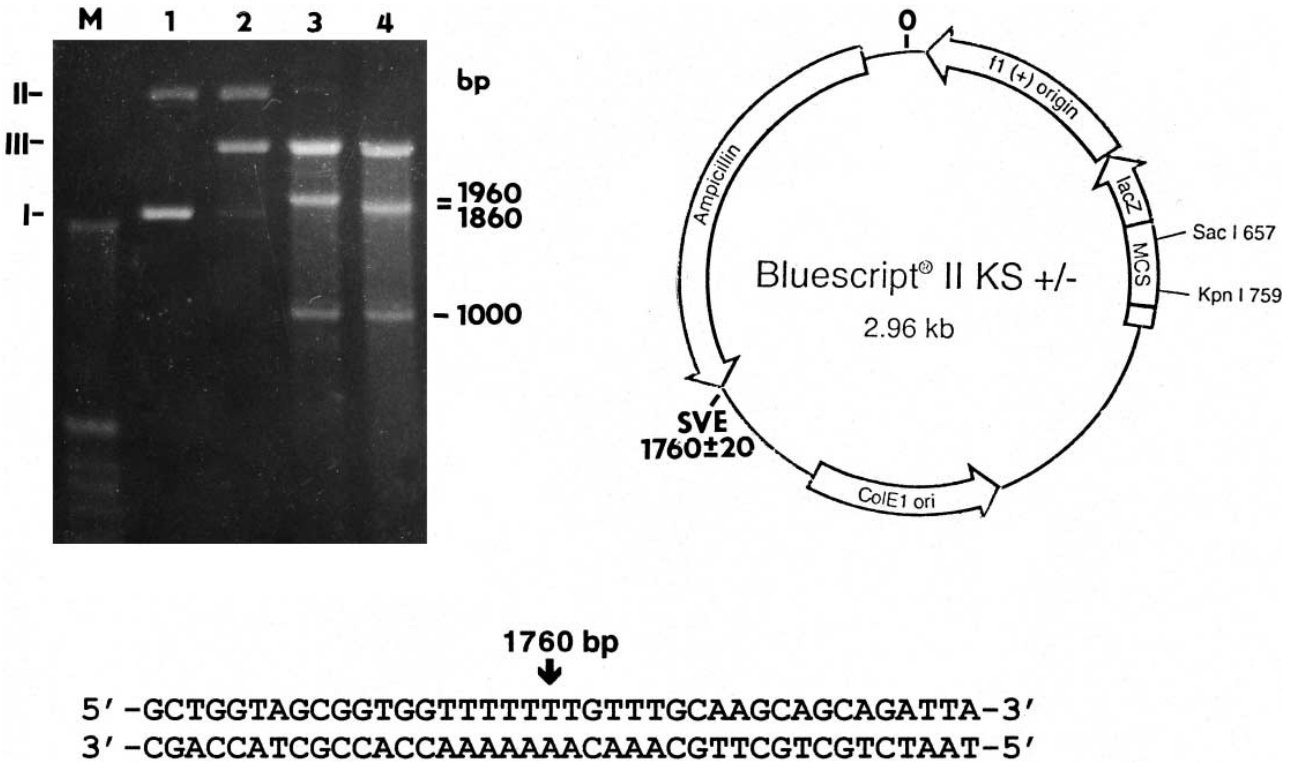

Fig. 2. Single-strand-specific endonucleolytic activity of SVE and its localization. Agarose electrophoresis (1%) of Bluescript II KS$^+$ (lane 1). Lane 2, the plasmid hydrolyzed with SVE. Lanes 3 and 4, the plasmid was hydrolyzed with SVE and consecutively treated with *Kpn*I only (lane 3) and with *Kpn*I and *Sac*I (lane 4). Lane M shows the marker DNA fragments of 1681 and 517 bp. The place for the endonucleolytic attack on Bluescript II KS$^+$ is marked on the scheme (right panel) which corresponds to 40-bp sequence near the Ampicilline gene terminating sequence, shown below.

precipitation [1]. This material contains a number of proteins with different enzymatic activities including the endonuclease. The immunoblotting of the proteins from the acetone precipitate with the obtained anti-SVE antibody (Fig. 1C) reveals only one band corresponding to 140-kDa SVE protein (Fig. 1B).

To follow the single-strand-endonucleolytic activity of SVE, we used the property of negatively supercoiled DNA to unwind at regions where it is thermodynamically unstable. These regions are recognized and cut by the single-strand-specific endonuclease [3].

The electrophoretic pattern before (left panel, lane 1) and after hydrolysis of Bluescript II KS$^+$ with SVE (lane 2) is presented on Fig. 2. As a result of the endonucleolytic attack the supercoiled form of DNA (I) disappears and a linear form (III) becomes visible. The treatment of the SVE hydrolysed plasmid with *Kpn*I (lane 3) led to the appearance of two well-defined DNA bands (1960 and 1000 bp ± 20 bp). This indicates that SVE cuts the superhelical DNA (form I) at one well defined site. To assign the location of the SVE cleavage, the SVE-treated plasmid was cut with *Kpn*I and *Sac*I (lane 4). The observed truncation of the 1960-bp fragment with ca. 100 bp showed that SVE has split the plasmid at the 40-bp AT-rich sequence (the right scheme and the sequence below in Fig. 2) near the Ampicillin gene terminating region at 1760 ± 20 bp.

It was shown earlier [9] that the polyclonal antibodies inhibited both phosphodiesterase and pyrophosphatase activities of SVE. We applied the same strategy to assign the single-strand-specific endonucleolytic activity to the exonuclease protein.

We observed an inhibition of endonucleolytic activity towards supercoiled DNA when pure preparation of SVE was pretreated with the specific antibody in comparison with that of an enzyme preincubated with bovine serum albumin (Fig. 3A) By this way, the single-strand-specific endonucleolytic activity is assigned to the 140-kDa protein of the venom exonuclease.

An important characteristic of any exonuclease, attacking a highly polymerised substrate such as DNA is its processive or non-processive mode of action. If after splitting one or few monomers the enzyme dissociates from the substrate attacking another molecule (non-processive mode of action) the role of the endonucleolytic activity becomes significant. In the opposite case, if it remains bound to the same DNA molecule (processive mode of action) it will release a number of monomers before the enzyme-substrate complex dissociates and the parallel endonucleolytic activity generating new 3′ ends will not interfere.

We used the heparin trapping method [8] to analyse the processivity of the *Crotalus adamanteus* venom exonuclease.

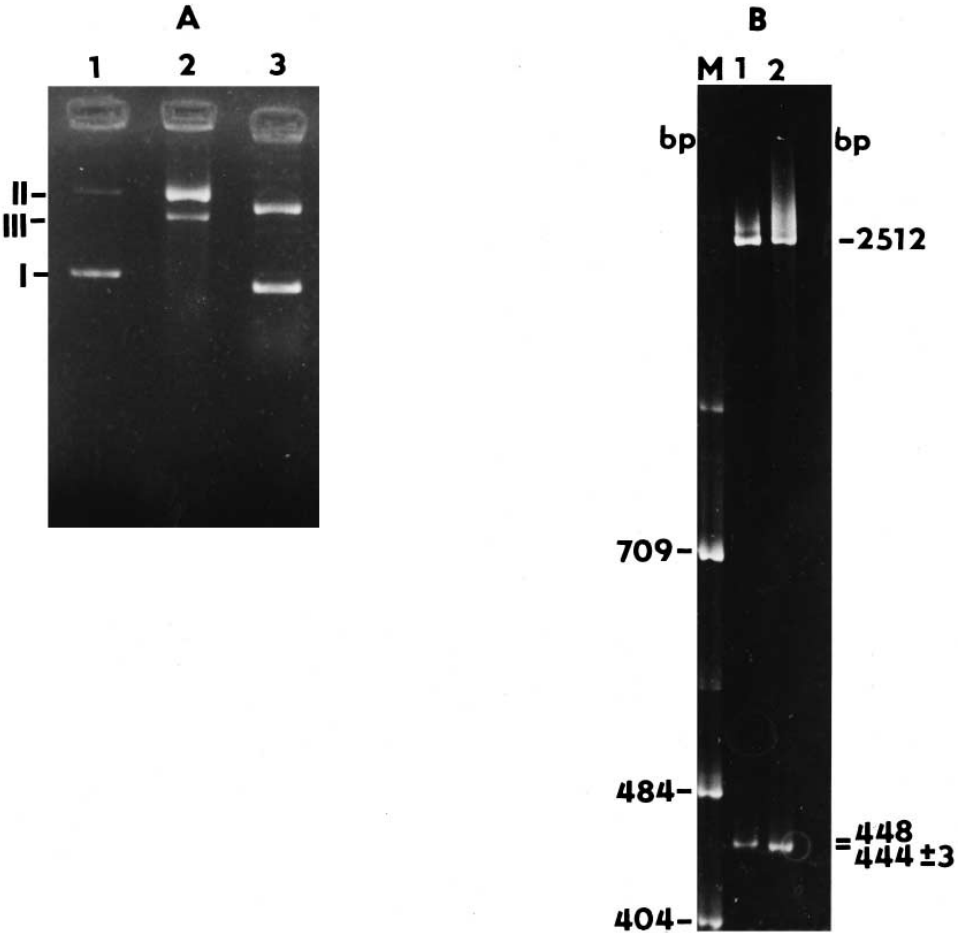

Fig. 3. Single-strand-specific endonucleolytic activity and processivity of the exonucleolytic attack of SVE. (A) Ethidium bromide agarose electrophoresis of Bluescript II KS$^+$ (lane 1) and its hydrolysate with snake venom exonuclease preincubated with BSA as control (lane 2) or with anti-SVE antibody (lane 3). (B) PAGE (5%) of DNA from the heparin trapping experiments. Lane M, DNA markers. Lane 1, blunt end DNA fragments from Bluescript II KS$^+$ with 2512 bp and 448 bp. Lane 2, the same after mixing with SVE and immediate addition of heparin.

It is known that a number of nucleolytic enzymes have high affinity towards heparin. Enzyme molecules, not bound to the DNA, and enzyme molecules which dissociates from the DNA, are trapped by the heparin and therefore become unable to carry out further catalysis.

We first studied the inhibitory effect of heparin on the venom exonuclease and found that while thymus DNA hydrolysis was inhibited above 90% the heparin slowed down the hydrolysis of the low-molecular-mass substrates *bis-p*-NPP and *p*-NPh.pT three and two times respectively. These observations made possible the application of the heparin trapping experiments to venom exonuclease as has been done to a number of other DNA binding enzymes and proteins [8,10].

The immediate (within 2 to 3 s after the enzyme addition) mixing of high excess of heparin to the incubation mixture, if the exonuclease is in a free form or has dissociated from the DNA molecule would stop the hydrolysis. Conversely, if a processive course of the exonucleolytic reaction takes place, a number of mononucleotides would be split before the dissociation of the exonuclease·DNA complex.

Two blunt-end fragments of 2512 bp and 448 bp from Bluescript II KS^+ plasmid were used as substrates for the heparin trapping experiments. As seen on Fig. 3B (lane 2) the shortening of the 448-bp fragment was to 444 ± 3 only. Bearing in mind that the enzyme attacks the double-stranded substrate from both 3′ ends the processivity of the hydrolysis should be not more than 3 mononucleotides.

In conclusion, the single-strand-specific endonucleolytic activity, observed in highly purified preparations of snake venom exonuclease is assigned to the 140-kDa protein of the exonuclease. The exonucleolytic mode of action of the *Crotalus adamanteus* venom exonuclease is non-processive.

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