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Vipera lebetina venom nucleases

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ABSTRACT

Nucleases, in particular ribo- and deoxyribonucleases, are among the least-studied snake venom enzymes. In the present study we have partially purified different nucleases from *Vipera lebetina* venom. The DNase activity has been proved by DNA degradation both in solution as well as in-gel (zymogram-method). In DNA-containing SDS-PAGE *V. lebetina* venom exhibits DNA-degrading activity in bands with molecular masses of ~120, 30–35 and 22–25 kDa. The 120 kDa band corresponds to phosphodiesterase, a 3′, 5′-exonuclease. The endonucleolytic activity of the lower-molecular-mass protein has been confirmed by plasmid degradation and the visualization of the results in agarose gel (with ethidium bromide) electrophoresis. A partial DNA sequence of putative RNase H1 has been determined from the *V. lebetina* venom gland cDNA library. The translated sequence is similar to the assumed RNase H1 from *Crotalus adamanteus* (AFJ51163). The RNA/DNA hybrid is hydrolysed by *V. lebetina* venom and venom fractions. The masses of tryptic peptides from the SDS-PAGE 30–35 kDa band are in concordance with the theoretical peptide masses from the respective translated sequence. For the first time RNase H1-like enzyme activity has been ascertained in snake venom, and sequencing a relevant partial transcript confirmed the identification of this enzyme.

1. Introduction

Snake venom is a rich source of diverse bioactive peptides and proteins; among them enzymes that are divided into proteases (metallo- and serine proteases), phospholipases, L-amino acid oxidases, hyaluronidases, nucleases and nucleotidases. Snake venom nucleases belong to the least studied enzymes due to the low quantity in many venoms. We have isolated and characterized phosphodiesterase (VLPDE) (Trummal et al., 2014) and 5′-nucleotidase (Trummal et al., 2015) from *Vipera lebetina* venom. VLPDE is classified as an exonuclease degrading DNA in 3′,5′-direction. There are many nucleases in numerous animal species including different deoxyribonucleases (DNases) and ribonucleases (RNases). In the case of snakes, RNase has been isolated from *Naja naja oxiana* (cobra) venom by Vassilenko's group. RNase with molecular mass in the range of 14–16 kDa from cobra venom (RNase V_1) is a non-specific endoribonuclease hydrolysing double-stranded RNA (Vassilenko and Babkina, 1965; Vassilenko and Ryte, 1975; Mackessy, 1998; Dhananjaya and D'Souza, 2010; Dhananjaya et al., 2010). The aforementioned enzyme has been widely used for RNA "structure-mapping" experiments (Lockard and Kumar, 1981; Lowman and Draper, 1986). RNase H1, an endoribonuclease, related to DNA replication, repair and transcription, has been found and described in almost all living organisms but not yet in snake venoms. The characteristic feature of RNase H1 is hydrolysis of RNA strand in RNA/DNA hybrids (Cerritelli and Crouch, 2009).

Very limited information has been published concerning venom DNases. An acidic endonuclease with pH-optimum of 5.0 has been isolated from *Bothrops atrox* (Georgatsos and Laskowski, 1962) and *Bothrops alternatus* venom (Nascimento et al., 2007) but there are no published data regarding individual DNases with alkaline pH-optimum. DNase activity has been detected in Brazilian snake venoms (Sales and Santoro, 2008) as well as in Costa Rican crotaline venoms (Sittenfeld et al., 1991); however, the activity could result from venom phosphodiesterase as no fractionation of the venom proteins according to their molecular masses has been performed. de Roodt et al. have fractionated 17 different snake venoms on SDS-PAGE and demonstrated the presence of ~30-kDa components with DNase activity by zymogram-method. The endonucleolytic effect has been confirmed by degradation of pBluescript plasmid (de Roodt et al., 2003). DNase I that is known to be involved in

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apoptosis is a non-specific endonuclease found mostly in pancreas and parotid gland, but also in other tissues (Chen and Liao, 2006). In the present study we attempted to find and describe new nucleases from *Vipera lebetina* venom.

2. Materials and methods

2.1. Materials

The venom of *Vipera lebetina* was collected in Central Asia and purchased from Tashkent Integrated Zoo Plant (Uzbekistan). Sephadex G-100 superfine and CM-Sephadex C25 were from Pharmacia (Sweden) [now Life Technologies], and DNA-cellulose was synthesized at the NICPB; azocasein, deoxyribonucleic acid sodium salt from salmon testes, ribonucleic acid from baker's yeast (*S. cerevisiae*), polyadenylic acid potassium salt and polythymidylic acid sodium salt were from Sigma-Aldrich (USA), and plasmid pTZ57R (2886 bp) was from Thermo Scientific/Fermentas (Lithuania). Ni-NTA was from Qiagen (USA). Oligonucleotide primers were ordered from DNA Technology (Aarhus, Denmark). All other reagents used were of analytical grade.

2.2. Purification of the enzymes

2.2.1. Gel filtration of V. lebetina venom

Crude venom (5 g) was dissolved in 25 ml of 0.2 M ammonium acetate, pH 6.7. Insoluble material was removed by centrifugation (5000×*g* for 15 min) and the supernatant was applied to the column (2.2 × 140 cm) of Sephadex G-100 superfine equilibrated with 0.2 M ammonium acetate. The elution was carried out with the same solution at a flow rate of 5 ml/h and 7.5 ml fractions were collected at 4 °C. The absorbance was continuously monitored at 280 nm. Fractions of peaks I–VIII were pooled and lyophilized.

2.2.2. Ion exchange chromatography

The CM-Sephadex C25 column (1.5 × 13 cm) was equilibrated with 0.6 M ammonium acetate, pH 6.7. Combined and concentrated by lyophilization, the partially purified protein from previous steps (ca 50 mg in 2 ml 0.6 M ammonium acetate, pH 6.7) (Fig. 1) was applied onto the column. Non-adsorbed material was washed out with the equilibration solution. The column was eluted sequentially with 0.7 M, 0.8 M and 1 M ammonium acetate, pH 6.7, flow rate was 12 ml/h; fractions of 3 ml were collected.

2.2.3. Affinity chromatography on DNA-cellulose

The DNA-cellulose column (1.0 × 5 cm) was equilibrated with 0.5 M ammonium bicarbonate, pH 8.9. The active fraction (30 mg) purified by gel filtration on Sephadex G-100 and G-75 (Fig. 1) and lyophilized, was dissolved in 0.5 M ammonium bicarbonate, pH 8.9 and applied onto the column. Non-adsorbed material was washed out with the equilibration solution and the column was eluted sequentially with 1 M ammonium bicarbonate, pH 8.9, and 1 M ammonium acetate, pH 5.1 at a flow rate of 2 ml/h; fractions of 3 ml were collected.

2.3. SDS-PAGE

SDS-PAGE was performed by the method of Laemmli (1970) in 10% or 12.5% gel. Molecular mass markers were β-galactosidase (116.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), REase Bsp98I (25.0 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa). The proteins were detected with Coomassie BB R250.

2.4. Activity determination

2.4.1. RNase

50 μl of enzyme solution (1 mg/ml) was incubated for 2 h at 37 °C with 1 ml of RNA solution (40 μg/ml) in 0.05 M Tris, 7.5 mM $MgSO_4$, pH 7.5 and the increase of absorbance was determined at 260 nm.

2.4.2. RNase H1

200 μg of poly(A) and 200 μg of poly(dT) were dissolved in 1 ml of incubation buffer (20 mM Hepes-KOH, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 8.0) and the mixture was heated for 5 min at 95 °C and then allowed to cool slowly for 2 h to generate a RNA/DNA hybrid. 50 μl of enzyme solution (1 mg/ml) was incubated with 60 μl of RNA/DNA hybrid solution and 840 μl of incubation buffer for 1 h at 37 °C and the increase of absorbance determined at 264 nm (www.roche-applied-science.com).

2.4.3. DNase

50 μl of enzyme solution (1 mg/ml) was incubated for 2 h at 37 °C with 1 ml of DNA solution (40 μg/ml) in 0.05 M Tris, 7.5 mM $MgSO_4$, pH 7.5 and the increase of absorbance was determined at 260 nm.

2.4.4. PDE

The activity was measured by a slightly modified method of Babkina and Vasilenko (1964) as follows: 25 μl of enzyme solution (1 mg/ml) was added to 0.1 ml of 10 mM bis(p-nitrophenyl) phosphate sodium in 0.1 M Tris-HCl buffer, 10 mM $MgSO_4$, pH 8.8 and incubated for 30 min at room temperature. Then 1.4 ml of 0.1 N NaOH was added to stop the reaction and the increase of absorbance was determined at 400 nm.

2.4.5. 5′-nucleotidase

5′-nucleotidase activity was estimated by the method of Eibl and Lands (1969) with 5′-AMP as substrate. Ten μl of enzyme solution (1 mg/ml) was added to 250 μl of 5′-AMP solution (1 mg/ml in 0.05 M Tris-HCl buffer, pH 7.5, with 20 mM $MgSO_4$) and the reaction was incubated at room temperature for 20 min. Then 150 μl of 2.5% ammonium molybdate solution in 6 N H_2SO_4 and 15 μl of 1% Triton X-100 were added, the solution was mixed, 1.1 ml of water was added and the mixture was allowed to stand for 20 min at room temperature. Absorbance was measured at 660 nm.

2.4.6. Azocaseinolytic activity

Azocaseinolytic activity was measured as described by Siigur et al. (1998).

2.5. DNA zymogram

DNAse activity was also determined according to de Roodt et al. (2003) by electrophoresis in DNA-containing SDS-PAGE 10% and 12.5% gels under non-reducing conditions. Twelve micrograms of different samples with DNAse activity were electrophoresed in SDS gels containing 0.15 mg/ml salmon testes DNA. To remove SDS, gels were washed 8 × 15 min in 30 ml of incubation buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM $CaCl_2$, pH 8.0) at room temperature. Then the gels were incubated in the aforementioned buffer for approximately 16 h at 37 °C, stained with ethidium bromide (5 μg/ml) and UV illuminated. Bands of activity were visualized as dark spots against a light background. After that the gels were stained with Coomassie blue.