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Research paper

Phosphodiesterase from *Vipera lebetina* venom – Structure and characterization☆

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ABSTRACT

Nucleases and phosphatases are ubiquitous but mostly marginal components of snake venoms. These proteins have been studied quite extensively but up to now no data regarding their amino acid sequences confirmed at protein level have been published. The present study deals with purification, characterization, and structural properties of a phosphodiesterase from *Vipera lebetina* venom (VLPDE). The VLPDE with molecular mass of about 120 kDa hydrolyses ADP but not ATP and 5′-AMP. The aggregation of platelets induced by ADP or collagen is dose-dependently inhibited by VLPDE. The cloning and sequencing of the VLPDE-encoding cDNA resulted in 2772-nt sequence with ORF of 2556 nt. The translated sequence comprises 851 amino acids including the 23-amino acid signal peptide. VLPDE is synthesized as a 828-amino acid single-chain protein but subsequently cleaved to form a two-chain protein held together with disulfide bonds. In reducing conditions the enzyme behaves like a heterodimeric protein but, differently from the real heterodimers, it is synthesized as a single-chain protein. VLPDE is the first snake venom phosphodiesterase with established and confirmed primary structure.

1. Introduction

Vipera lebetina venom as a typical representative of viperid venoms is extremely rich in proteolytic enzymes – serine and metalloproteases, necessary for digestion of the prey [1–3]. The venom contains smaller amounts of phospholipases [4], disintegrins [5], nerve growth factor [6], L-amino acid oxidase [7], C-type lectin-like proteins [8], VEGF-like protein [9], etc. Nucleases and nucleotidases, ubiquitously present in snake venoms, are minor components of *V. lebetina* venom. Although their specificity in venoms has been characterized (reviews [10,11]), their structural properties need thorough investigation. Phosphodiesterases (PDE) (non-specific exonucleases) hydrolysing double- and single-stranded nucleic acids (DNA and RNA), ATP, ADP, NAD have been extensively characterized in 1960s–1970s when they were used for nucleic acid studies [10]. While *V. lebetina* PDE specificity on DNA and RNA has been characterized in several studies [12,13], no individual enzyme has been isolated so far. The main goal of these studies has been separation of PDE and 5′-nucleotidase [14–16]. PDEs hydrolyse phosphodiester bonds giving rise to 5′-mononucleotides. Currently snake venom PDE is not used in nucleic acid studies due to abundance of specific restrictases, thus the interest for PDE has been diminished. Nevertheless, in recent years several papers concerning biological properties of snake venom PDEs have been published [17–19]. In addition some transcripts connected with snake venom gland high-throughput transcriptome studies can be found in gene bank sequence database (JU173674; AB848153; GAAZ01003059, etc) [20–22]. Hereby we present data on the purification and characterization of one PDE isoform from *V. lebetina* venom, as well as cloning and sequencing of the PDE-coding cDNA.

2. Materials and methods

2.1. Materials

Sephadex G-100 superfine – Pharmacia (Sweden), CM-cellulose CM52 – Whatman (UK), DEAE-Toyopearl 650M – Toyo Soda MFG, Co., Ltd (Japan), Na-bis-*p*NPP, thymidine-5′-*p*NPP, 5′-AMP, 3′-AMP, 5′-GMP, cAMP, cGMP, ADP, ATP, 2,5-dihydroxybenzoic acid (DHB), oxidized insulin B chain from bovine pancreas and azocasein were

☆ The sequence of VLPDE is registered in GenBank (Accession No. KF408295; the protein id AHJ80885).

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purchased from Sigma–Aldrich (St. Louis, MO, USA), fluorescamine from Serva (Germany), trypsin from Promega (Madison, WI, USA). Oligonucleotide primers were ordered from DNA Technology (Aarhus, Denmark), Taq DNA Polymerase and InsTAclone PCR Cloning Kit from ThermoScientific (Lithuania). The venom of *V. lebetina* originates from Tashkent Integrated Zoo Plant (Uzbekistan). All other reagents used were of analytical grade.

2.2. Purification of the enzyme

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.

2.2.2. Ion exchange chromatography on CM-52 cellulose

The CM-52 cellulose column (2.3 × 35 cm) was equilibrated with 0.05 M ammonium acetate, pH 5.5. PDE-containing fraction from step I (820–950 ml, Fig. 1A), combined and concentrated by lyophilization (0.5 g in 30 ml of 0.05 M ammonium acetate, pH 5.5), was applied onto the column. Non-adsorbed material was washed out with the equilibration solution. The column was eluted sequentially with 0.1 M, 0.2 M, 0.35 M, 0.5 M and 1 M ammonium acetate, pH 5.5, flow rate 24 ml/h; fractions of 12 ml were collected.

2.2.3. Ion exchange chromatography on DEAE-Toyopearl

The DEAE-Toyopearl column (1.5 × 11 cm) was equilibrated with 0.02 M ammonium acetate, pH 8.5. The respective activities containing CM-52 cellulose fractions (21–30, Fig. 1B; 15 mg) were lyophilized, dissolved in 0.02 M CH_3COONH_4, pH 8.0 and applied onto the column. Non-adsorbed material was washed out with the equilibration solution and the column was eluted sequentially with 0.1 M and 1 M ammonium acetate, pH 8.0, flow rate 14 ml/h; fractions of 3 ml were collected.

2.3. SDS PAGE

SDS-PAGE was performed by the method of Laemmli [23] in 10% gels. The proteins were loaded as follows: venom sample 50 μg per well, other samples 10 μg per well. Molecular mass markers were β-galactosidase (116.0 kDa), bovine serum albumine (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), REase Bsp98I (25.0 kDa) and β-lactoglobulin (18.4 kDa). The proteins were stained with Coomassie BB R250.

2.4. Determination of enzymatic activities

2.4.1. PDE activity with p-nitrophenyl thymidine-5′-phosphate

The assay is essentially that of Razzell and Khorana [24] where the reaction velocity is determined by an increase in absorbance at 400 nm resulting from the hydrolysis of *p*-nitrophenyl thymidine-5′-phosphate.

The activity was measured as follows: 0.1 ml of 5 mM *p*-nitrophenyl thymidine-5′-phosphate was added to 0.9 ml of 0.1 M Tris–HCl buffer, pH 8.8, with 10 mM $MgSO_4$, the cuvette was incubated in spectrophotometer for 3–5 min to reach temperature equilibrium, 10 μl of diluted enzyme was added and the increase in optical density at 400 nm was recorded for 3–5 min.

2.4.2. PDE activity with bis(p-nitrophenyl)phosphate

The activity was measured by slightly modified method of Babkina and Vasilenko [25] as follows: 25 μl of enzyme solution (1 mg/ml) was added to 0.1 ml of 10 mM bis(*p*-nitrophenyl)phosphate in 0.1 M Tris–HCl buffer, pH 8.8, with 10 mM $MgSO_4$ 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 optical density was determined at 400 nm.

2.4.3. 5′-Nucleotidase

5′-Nucleotidase has been estimated by the method of Eibl and Lands [26] with 5′-AMP as substrate, ADPase and ATPase were detected with ADP and ATP, respectively, by the same method. Ten μl of sample solution (1 mg/ml) was added to 250 μl of 5′-AMP (ADP, ATP) solution (2.5 mM 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

The phosphomonoesterase activity was localized by lysis of *p*-nitrophenyl phosphate according to the method of Sulkowski et al. [27].

2.4.4. Proteolytic activity

Proteolytic activity was measured as described by Siigur et al. [28] using azocasein as substrate

2.4.5. Thermostability

The thermostability was studied by incubating the enzyme solution (50 μl; 1 mg/ml in 0.1 M Tris–HCl, 0.01 M $MgCl_2$, pH 8.8) at various temperatures (4, 20, 37, 50, 60, 65, 70, 80, 100 °C) for 15 min; then quickly cooled and PDE activities were determined using bis(*p*-nitrophenyl)phosphate as the substrate at 400 nm.

2.4.6. pH dependence

The dependence of the activity of the PDE on pH was determined in the following buffers: 0.1 M CH_3COONa, 0.01 M $MgCl_2$ at pH 4.0–6.5 and 0.1 M Tris–HCl, 0.01 M $MgCl_2$ at pH 7.0–10.0. The PDE activity was measured using bis(*p*-nitrophenyl)phosphate as substrate at 400 nm.

2.4.7. Effect on platelet aggregation

The effect of VLPDE on platelet aggregation was studied in human platelet-rich plasma in a Chrono-Log aggregometer as described by Siigur et al. [28]. The corresponding permissions for the blood collection are as follows: LO2354 (14.12.2010) and LO2513 (21.07.2011). Aggregation was induced with ADP (10 μM) or collagen (2 μg/ml) after 2-min incubation of VLPDE (0–3.4 μM) with platelet suspension.

2.5. Tryptic digestion and MALDI-TOF mass-spectrometry

After visualization with Coomassie BB the gel-electrophoresis bands of protein of interest (reduced or non-reduced) were excised from SDS-polyacrylamide gels, each gel slice was cut into small pieces (1 mm^2), placed into eppendorf tubes and treated as described earlier [29]. The MALDI-TOF mass spectra of tryptic peptides were measured with a home-built MALDI mass spectrometer (National Institute of Chemical Physics and Biophysics) [6,29,30] and with LTQ-Orbitrap MS. For MALDI-TOF analysis the dried samples were dissolved in 5 μl of 50% ACN, 0.1% TFA. Sample aliquots of 0.5 μl were applied onto the target, allowed to air dry and 0.5 μl of the matrix solution (DHB) was applied to the target