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Purification, characterization, and cDNA cloning of acidic platelet aggregation inhibiting phospholipases A_2 from the snake venom of *Vipera lebetina* (Levantine viper)☆

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ABSTRACT

Two novel acidic phospholipase A_2s (PLA_2) were isolated by size exclusion chromatography and reversed-phase chromatography from the crude *Vipera lebetina* venom. The molecular masses of $VLPLA_2$-1 (13,704 Da) and $VLPLA_2$-2 (13,683 Da) and their internal tryptic peptides were determined by MALDI-TOF mass-spectrometry. When tested in human platelet-rich plasma, both enzymes showed a potent inhibitory effect on aggregation induced by ADP and collagen. Chemical modification with *p*-bromophenacylbromide abolished the enzymatic activity of PLA_2; its anti-platelet activity was fully inhibited in case of collagen as inducer and partially inhibited in case of ADP as inducer. The complete cDNAs encoding PLA_2 were cloned from a single venom gland cDNA library. Complete amino acid sequences of the $VLPLA_2$ were deduced from the cDNA sequences. The full-length cDNA sequences of the $VLPLA_2$ possess 615 bp and encode an open reading frame of 138 amino acids that include signal peptide (16 amino acids) and mature enzyme (122 amino acids). The $VLPLA_2$s have significant sequence similarity to many other phospholipase A_2s from snake venoms. The phylogenetic analysis on the basis of the amino acid sequence homology demonstrates that $VLPLA_2$s grouped with other Asp49 PLA_2s and they appear to share a close evolutionary relationship with the European vipers.

1. Introduction

Snake venoms are complex mixtures of bioactive components, predominantly proteins and polypeptides that play offensive, defensive and digestive roles. A lot of snake venom proteins have enzymatic activities, e.g. proteases, phospholipases, phosphodiesterases, nucleases, nucleotidases, hyaluronidases, L-amino acid oxidases, acetylcholinesterases etc. In addition to their enzymatic activity, these enzymes also induce various pharmacological effects including hemorrhagic, haemolytic, myotoxic, cardiotoxic, neurotoxic, procoagulant and anticoagulant effects (Mebs, 1998; Markland, 1997; Markland, 1998). Among these components are phospholipases A_2 (PLA_2, E.C. 3.1.1.4), which catalyze the hydrolysis of the *sn*-2 fatty acyl ester bond of *sn*-3 phosphoglycerides, liberating free fatty acids and lysophospholipids.

PLA_2s have been found in mammalian tissues, arthropods, in all snake venoms and bee venoms. Based on their source,

Abbreviations: $VLPLA_2$, *Vipera lebetina* phospholipase A_2; TFA, trifluoroacetic acid; *p*-BPB, *p*-bromophenacylbromide; MALDI-TOF MS, matrix assisted laser desorption ionization time-of-flight mass spectrometry; UPLC, ultra performance liquid chromatography; lysoPC, lysophosphatidylcholine.

☆ The nucleotide sequences of $VLPLA_2$s were deposited to GenBank with the accession numbers: FJ905321 and EU421953.

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amino acid sequence, chain length and disulfide bond patterns, PLA_2s are recently divided in 15 groups (Schaloske and Dennis, 2006) and many subgroups and include five distinct types of enzymes, namely the secreted PLA_2s ($sPLA_2$), the cytosolic PLA_2 ($cPLA_2$) (Ohto et al., 2005), the Ca^{2+}-independent PLA_2s ($iPLA_2$) (Jenkins et al., 2004), the platelet-activating factor acetylhydrolases (PAF-AH) (Abe and Shayman, 1998), and the lysosomal PLA_2s (Hiraoka et al., 2002, 2006).

In snake venoms, only secreted PLA_2s belonging to groups I and II (GI and GII) were found. GI includes $sPLA_2$s from Elapinae and Hydrophiinae snakes, which are homologous to mammalian pancreatic GIB $sPLA_2$, while GII includes enzymes from Crotalinae and Viperinae snakes, homologous to mammalian non-pancreatic GIIA $sPLA_2$ (Six and Dennis, 2000). Phospholipase A_2s are the most thoroughly studied snake venom enzymes because of their abundance, small size, and structural stability. Several hundreds of snake venom PLA_2 enzymes have been purified and characterized. Snake venom PLA_2s are multifunctional proteins; in addition to their enzymatic action on membrane phospholipids, they exhibit a wide variety of physiological and pathological effects due to their ability to bind to specific target proteins (reviews Kini, 1997, 2003, 2005; Kini and Evans, 1989; Lambeau and Lazdunski, 1999; Valentin and Lambeau, 2000). Snake venom PLA_2s display several biological effects, including pre- or postsynaptic neurotoxicity (Thouin et al., 1982; Kuruppu et al., 2005; Tsai et al., 1995, 2007), cardiotoxicity (Huang et al., 1997), myotoxicity (review Lomonte et al., 2003; Gutierrez and Ownby, 2003), influence to platelet aggregation (Prasad et al., 1996; Fuly et al., 1997, 2004; Kini and Evans, 1997; Chow et al., 1998; Kemparaiu et al., 1999; Serrano et al., 1999; Roberto et al., 2004; Satish et al., 2004; de Albuquerque Modesto et al., 2006). A variety of $sPLA_2$s possess potent anticoagulant activity (Kini, 2005; Mounier et al., 2001). The $sPLA_2$s were found to play an important role in exocytosis. Both the snake venom crotoxin, which belongs to the GIIA PLA_2s, and synovial GIIA PLA_2 were found to induce the release of catecholamines from pheochromocytoma cells (Wei et al., 2003). Certain $sPLA_2$s may play a role in the killing of bacteria and could be involved in the host defence against bacterial infection (Koduri et al., 2002). Recently bactericidal properties of $sPLA_2$s including snake venom phospholipases A_2 were reviewed (Nevalainen et al., 2008).

To our knowledge this is the first report of the presence of platelet aggregation inhibitor PLA_2s in *Vipera lebetina* venom. Here we report the isolation, cDNA cloning and interactions with platelets of phospholipase A_2s from *V. lebetina* venom. Phylogenetic trees of PLA_2s from Viperidae and Elapidae venoms were constructed.

2. Materials and methods

2.1. Materials

2,5-Dihydroxybenzoic acid (DHB), Substance P and Cytochrome C were from Sigma (St. Louis, MO, USA), trypsin (Promega, USA). The venom batches of *V. lebetina* were commercial preparations from Tashkent Integrated Zoo Plant (Uzbekistan). Sephadex G-100 (superfine) was product of Pharmacia (Uppsala, Sweden). Oligonucleotide primers were ordered from DNA Technology (Aarhus, Denmark). All other reagents used were of analytical grade.

2.2. Purification of the enzyme

V. lebetina venom (1.5 g) was dissolved in 10 ml of 0.2 M ammonium acetate. Insoluble material was removed by centrifugation ($5000 \times g$ for 15 min) and the supernatant was applied to the column of Sephadex G-100 sf (2.2×140 cm) equilibrated with 0.2 M ammonium acetate. The elution was carried out with the same solution at a flow rate of 4 ml/h and 6 ml fractions were collected at 4 °C. The absorbance was continuously monitored at 280 nm.

Combined and concentrated by lyophilization fraction V from size exclusion chromatography was used for phospholipase A_2 purification. About 50 mg of fraction V proteins were dissolved in 1 ml of 0.1% TFA and the proteins were separated using DuPont HPLC system with Waters C4 column (19×150 mm, 5 μm particle size). Elution was performed at 15 ml/min using a linear gradient of acetonitrile (20–70% in 30 min). The protein detection was at 230 nm and fractions were collected manually. Fractions with PLA_2 activity were concentrated and rechromatographed on the same column using a linear gradient of acetonitrile (30–55% in 30 min). UPLC (Acquity™ Ultra Performance LC, Waters) on C18 column (Acquity BEH 300 C18, 2.1×100 mm) was used for characterization of the composition of the fraction V of gel-filtration and for characterization of purified products. Elution was performed at 0.15 ml/min using a linear gradient of acetonitrile (10–50% in 25 min) followed by an isocratic elution with 95% acetonitrile in 0.1% TFA for 5 min.

2.3. PLA_2 assay

Phospholipase A_2 activity was assayed by titrimetric method using egg yolk phosphatidylcholine as a substrate (de Haas et al., 1968). One egg yolk was added to 100 ml of bidistilled water and aqueous emulsion was prepared by homogenisation. Per assay, 1.5 ml of the egg yolk emulsion was diluted with 3 ml Triton X-100 and $CaCl_2$ being 15 mM. The pH was set at 8.0; 10 μl (0.1 mg/ml) of PLA_2 sample was added and the fatty acids released titrated with 10 mM KOH using a pH-stat (TTT80/pHM84/ABU80, Radiometer, Copenhagen) at 25 °C.

2.4. PLA_2 activity inhibition with p-bromophenacylbromide

V. lebetina PLA_2s (0.4 mg) were dissolved in 0.4 ml of 0.1 M ammonium acetate (pH 7.4) containing 0.4 mM of *p*-BPB and incubated for 24 h at room temperature. Excess reagent was removed by ultrafiltration through the microspin filter 5000 MW (Cole-Parmer, USA) and washed with 0.1 M ammonium acetate (pH 7.4), followed by lyophilization.

After that, the samples were assayed for phospholipase A_2 activity and the inhibitory effects on platelet aggregation induced by ADP (10 μM) and collagen (2 μg/ml). Control experiments were performed in parallel with non-treated PLA_2 assayed under the same conditions.