Toxicon 57 (2011) 332–341

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

cDNA cloning, structural, and functional analyses of venom phospholipases A_2 and a Kunitz-type protease inhibitor from steppe viper *Vipera ursinii renardi*☆

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ARTICLE INFO

Article history:
Received 21 August 2010
Received in revised form 25 November 2010
Accepted 15 December 2010
Available online 23 December 2010

Keywords:
Venom phospholipases A_2
Kunitz inhibitor
Full sequences
Properties
Steppe viper *Vipera ursinii renardi*

ABSTRACT

Snake venom phospholipases A_2 (PLA_2s) display a wide array of biological activities and are each characteristic to the venom. Here, we report on the cDNA cloning and characterization of PLA_2s from the steppe viper *Vipera ursinii renardi* venom glands. Among the five distinct PLA_2 cDNAs cloned and sequenced, the most common were the clones encoding a basic Ser-49 containing PLA_2 (Vur-S49). Other clones encoded either ammodytin analogs I1, I2d and I2a (designated as Vur-PL1, Vur-PL2 and Vur-PL3, respectively) or an ammodytoxin-like PLA_2 (Vurtoxin). Additionally, a novel Kunitz-type trypsin inhibitor for this venom species was cloned and sequenced. Comparison of these PLA_2 and Kunitz inhibitor sequences with those in the sequence data banks suggests that the viper *V. u. renardi* is closely related to *Vipera ammodytes* and *Vipera aspis*. Separation of *V. u. renardi* venom components by gel-filtration and ion-exchange chromatography showed the presence of many PLA_2 isoforms. Remarkably, the most abundant PLA_2 isolated was Vur-PL2 while Vur-S49 analog was in very low yield. There are great differences between the proportion of cDNA clones and that of the proteins isolated. Two Vur-PL2 isoforms (designated as Vur-PL2A and Vur-PL2B) indistinguishable by masses, peptide mass fingerprinting, N-terminal sequences and CD spectroscopy were purified from the pooled venom. However, when rechromatographed on cation-exchanger, Vur-PL2A showed only one peak corresponding to Vur-PL2B, suggesting the existence of conformers for Vur-PL2. Vur-PL2B was weakly cytotoxic to rat pheochromocytoma PC12 cells and showed both strong anticoagulant and anti-platelet activities. This is the first case of a strong anticoagulating ammodytin I analog in *Vipera* venom.

1. Introduction

Phospholipases A_2 (PLA_2s; EC 3.1.1.4) are among the most widely studied snake venom proteins. Several PLA_2 isoforms could exist in the same venom, and they may display a wide array of biological activity ranging from unspecific cell lysis to neurotoxicity (Gubensek et al., 1997; Kini, 1997; Chijiwa et al., 2003). All the venom PLA_2s belong to either groups I or II secretory PLA_2 superfamily (Six and Dennis, 2000). Venoms of Viperinae and Crotalinae subfamilies express group II PLA_2s which can be further classified into various subtypes

Abbreviations: PLA_2, Phospholipase A_2; KIn, Kunitz-type protease Inhibitor; HPLC, High Performance Liquid Chromatography; CD, Circular Dichroism; PRP, Platelet-Rich Plasma; Vur, *Vipera ursinii renardi* or its venom.

☆ The nucleotide sequences for the PLA_2s and the Kunitz inhibitor have been submitted to EMBL Databank and are available under accession numbers: GQ304904 to GQ304908 for Vur-S49, Vur-PL3, Vur-PL1, Vur-PL2 and Vurtoxin, and HM462022 for Vur-KIn, respectively.

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doi:10.1016/j.toxicon.2010.12.012

(Tsai, 1997; Chijiwa et al., 2003). Functionally, PLA_2s have evolved into neurotoxins (Tsai and Hseu, 2009), anticoagulants (Kini, 2005), platelet aggregation inhibitors (Ouyang and Huang, 1984), and more. Kunitz-type protease inhibitors (KIns) are also common components of Viperinae venoms (Ritonja et al., 1983; Siddigi et al., 1991; Zupunski et al., 2003). Both PLA_2 and KIn families show great biodiversity and thus serve as excellent venom markers for Viperinae identification and comparison.

The genus *Vipera* is composed of true vipers distributed mainly from Europe through Mideast to western Asia; the venom proteins of several *Vipera* species, e.g. *Vipera aspis aspis* (Guillemin et al., 2003; Jan et al., 2007) and *Vipera ammodytes ammodytes* (Gubensek et al., 1997), have been extensively studied. The venom gland PLA_2 transcripts from 21 European vipers of central Europe have been cloned and sequenced, including one *Vipera ursinii* (Meadow Viper) specimen from France. Although various Viperinae PLA_2 isoforms have been identified and analyzed, it has been shown that the PLA_2 evolution and their selective expressions are complicated and warrant further study (Jan et al., 2007).

V. ursinii is found not only in regions from France to west Turkey, but also distributes further east in southern Russia, central Asia and west Xinjiang (China). In this study, we investigated venom-gland transcripts of two *V. ursinii* specimens captured in southern Russia (Krasnodar region). Some authors consider the vipers inhabiting this region as part of the *Vipera renardi* species (Nilson and Andren, 2001), while others (Ananjeva et al., 1998) classify them as *V. ursinii* subspecies. As the systematics of *V. ursinii* complex are not yet fully established we shall refer to this species as *Vipera ursinii renardi*.

There are prominent geographic or individual variations in the venom constituents of some Viperinae. This study aims to unravel the subvenomics and full sequences of the PLA_2s and KIns in *V. u. renardi* venom, which has not been studied at molecular levels. The cDNA cloning and sequencing for the venom proteins based on the Russia specimens are presented. For both the venom PLA_2 and KIn families, the transcriptomic data was compared with results of the proteomic analyses. The polymorphism of the PLA_2s was also analyzed and compared to those from European Viperinae venoms. These findings provide clues to the mechanisms leading to venom PLA_2 diversification and geographic variation at the intra-species and interpopulational levels.

2. Material and methods

2.1. Venom and snakes

The snakes used for the *V. u. renardi* venom production were captured in Krasnodar region of southern Russia. The venom glands were dissected from two snakes whose venoms were also collected for the venom protein analyses.

2.2. Cloning and sequence deduction

Two days after venom extraction, the venom glands of the *V. u. renardi* specimens were promptly removed after the snakes were sacrificed. The glands were immediately preserved in RNAlater solution (Ambion, U.S.A.) before being used for RNA extraction. The mRNA was isolated and the cDNA prepared according to the manufacturer's instructions using kits purchased from Stratagene and modification and restriction enzymes from Promega (Maniatis et al., 1989). In order to amplify and clone the PLA_2s, PCR (Mullis and Faloona, 1987) was conducted using SuperTaq DNA polymerase with a pair of mixed-base oligonucleotide primers which were designed according to the highly conserved cDNA regions of the group II PLA_2s from other snake venoms (primer 1 in sense-direction: TCTGGATTSAGGAGGATGAGG; and primer 2 in antisense-direction: GCCTGCAGAGACTTAGCA) (Tsai et al., 2003). In order to amplify and clone the venom KIns, PCR primers 3 and 4 were synthesized based on the conserved UTR and/or signal peptide regions of the cDNA sequences previously published for venom KIn (Cheng et al., 2005). Primer 3 (in sense-direction) is 5′-CCAGACGGCTTCATCATG-3′; and primer 4 (in antisense-direction) is 5′-AAAAGGAA-TRATCCAGG-3′.

For PLA_2 cloning, 0.4 kb fragments were specifically amplified by PCR as shown by electrophoresis of products on a 1% agarose gel, and harvested. Likewise, Kunitz inhibitor cDNAs of 0.2 kb were specifically amplified by PCR. After treatment with polynucleotide kinase, the amplified DNAs were inserted into the pGEM-T easy vector (Promega Corp., U.S.A.) and then transformed into *Escherichia coli* strain JM109. White transformants were picked-up and cDNA clones were selected. The DNA Sequencing System (model 373A) and the Taq-Dye-Deoxy terminator-cycle sequencing kit (PE Applied Biosystems, U.S.A) were used to determine the nucleotide sequences. The protein sequences were deduced from the nucleotide sequences and compared.

2.3. Protein purification

Dried *V. u. renardi* venom (400 mg) was dissolved in 1 ml of 0.1 M ammonium acetate buffer (pH 6.2) containing 0.01% sodium azide. It was applied onto a Sephadex G-50 sf (GE Healthcare, U.S.A.) column (4.5 × 150 cm) that had been equilibrated with the same buffer and was then eluted at a flow rate of 60 ml/h. The eluting proteins were detected by absorbance at 280 nm.

The fraction containing PLA_2s was separated on a HEMA BIO 1000 CM column (8 × 250 mm, Tessek, Czech Republic) with a gradient of 5–600 mM ammonium acetate (pH 7.5) in 120 min, at a flow rate of 1.0 ml/min. Fractions obtained were further separated on a Phenomenex C_{18} column (4.6 × 250 mm) in a gradient of 15%–45% acetonitrile in 30 min in the presence of 0.1% trifluoroacetic acid, at a flow rate of 1.0 ml/min.

For cation-exchange re-chromatography of *V. u. renardi* PLA_2 a small HEMA BIO 1000 CM column (3 × 30 mm, Tessek, Czech Republic) was used. The elution of the proteins was performed using a gradient of 5–600 mM ammonium acetate (pH 7.5) in 120 min, at a flow rate of 0.4 ml/min.

A PLA_2 designated as CM2 (Swiss-Prot access no. P00596) was also purified from *Naja kaouthia* venom, as described earlier (Makarova et al., 2006).

To isolate KIns from the gel-filtration fraction right after the PLA_2 fraction, a Jupiter C18 column (300A, 250 × 4.6 mm, Phenomenex, U.S.A.) was used and eluted with an