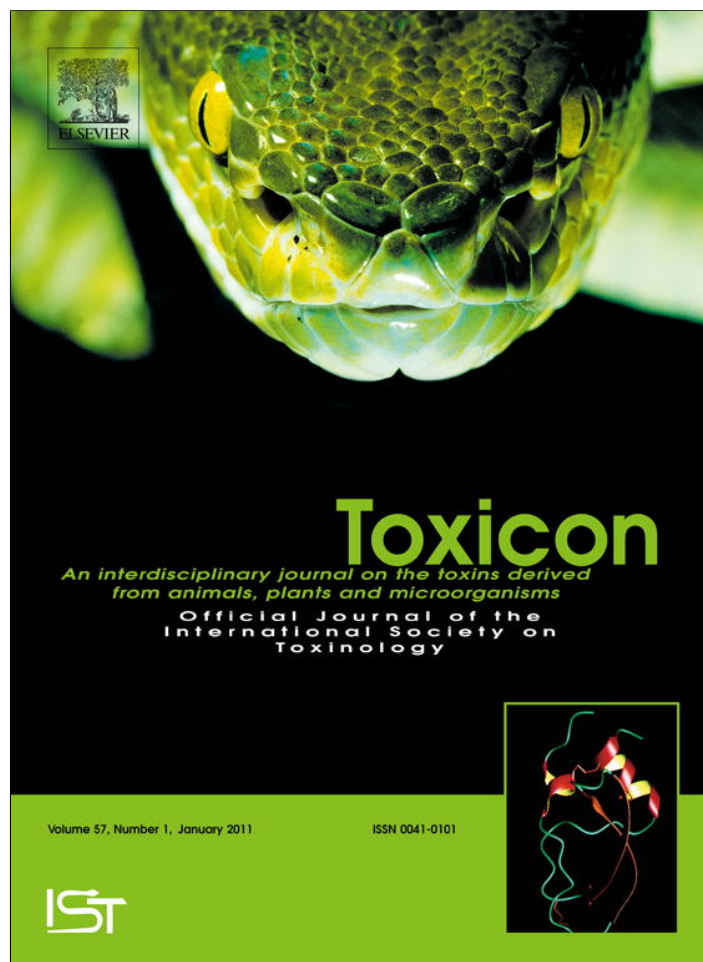


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Short communication

Phospholipase A₂ inhibitors (β PLIs) are encoded in the venom glands of *Lachesis muta* (Crotalinae, Viperidae) snakes

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ABSTRACT

Phospholipase A₂ inhibitors (PLIs) are glycoproteins secreted by snake liver into the circulating blood aiming the self-protection against toxic venom phospholipases A₂. In the present study, we describe the first complete nucleotide sequence of a β PLI from venom glands of a New World snake, *Lachesis muta*. The deduced primary structure was compared to other known β PLIs and recent literature findings of other possible roles of PLIs in snakes are discussed.

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The genus *Lachesis* (Crotalinae, Viperidae) contains the longest pit viper snakes in the world and it currently comprises three species: *L. muta* (the South American bushmaster), *L. melanocephala* and *L. stenophrys* (both in Central America) (Zamudio and Greene, 1997; Fernandes et al., 2004). Among a large variety of toxic components, *Lachesis* spp. venoms display high levels of phospholipase A₂ (PLA₂, E.C. 3.1.1.4) activity (Valiente et al., 1992; Fuly et al., 1993, 1997; Fortes-Dias et al., 1999; Assis et al., 2008). Thus, the presence of endogenous phospholipase A₂ inhibitors (PLIs) in the circulating blood of those snakes would be expected as part of their self-protection mechanism that is known to occur in several venomous snake species. The PLIs described so far are glycoproteins produced by the snake liver from where they are secreted into the bloodstream to act as soluble receptors for toxic

phospholipases A₂ (PLA₂) eventually present there. PLIs have been organized into three structural classes (α , β and γ), the members of which can be concomitantly found in certain snake species (Lizano et al., 2000; Nobuhisa et al., 1998; Ohkura et al., 1997). Each class is characterized by the presence of known protein domains, such as the carbohydrate-recognition domain (CRD) in α PLIs, leucine-rich repeats (LRR) in β PLIs and cysteine-rich three-finger domains in γ PLIs. According to the structural class which they belong to, PLIs may show distinct phospholipase A₂ specificities (Ohkura et al., 1997).

Generally speaking, PLIs have not only been purified as proteins from the blood plasma of snakes but putative α and γ PLI homologues have been shown to be encoded in snake liver. Both α and γ members are more widely distributed over snake species, whereas β PLIs have been solely purified from three snake species: *Gloydus brevicaudus* (a viperid formerly named *Agkistrodon blomhoffii siniticus*) (Okumura et al., 1998) and from the nonvenomous colubrids *Elaphe quadrigata* and *Elaphe*

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climacophora (Okumura et al., 2002; Shirai et al., 2009). β PLIs are trimers of 50 kDa subunits containing nine leucine repeats each of 24 residues, encompassing 2/3 of the molecule. Each subunit binds group II basic PLA₂ molecules from homologous or heterologous venoms with 1:1 stoichiometry (Okumura et al., 1998, 2002; Shirai et al., 2009).

The presence of β PLIs transcripts has been investigated in several *E. quadrivirgata* organs (spleen, liver, lung, pancreas, fat body, small intestine, gallbladder, testis, kidney, esophagus and heart) with negative results except for liver and lung tissues (Okumura et al., 1999). Nevertheless, expressed sequence tags (EST) matching partial β PLI sequences have been found in a cDNA library from *L. muta* venom glands that has been constructed as previously described (Junqueira-de-Azevedo et al., 2006). Sequence data from that library have been deposited with the EMBL/GenBank Data Libraries under the following full-length confirmed cDNA accession numbers DQ396473 to DQ396477. EST clusters in GenBank dbEST are from DY403159 to DY404320. The finding of β PLI sequences in that library has motivated the present study aiming to the isolation and complete nucleotide sequencing of the first putative β PLI from the venom gland of a New World snake species, *L. muta*.

Clones from that cDNA library have then been screened by PCR in the presence of primers designed on the basis of the published sequence for *E. quadrivirgata* β PLI (Okumura et al., 2002): sense signal peptide P1 (3'ATGA AGTCTTCGGTTCATCTC5'), antisense carboxy-terminus P2 (3'TTAGCAGGGACAAATTTGGGAT5') and antisense internal sequence P3 (3'GCACAGACAATTCCTCCTTGA5'). The following conditions were used: 5 min at 94 °C, 35 cycles of 3 min at 94 °C, 30 s at 55 °C and 30 s at 72 °C, followed by an extension period of 7 min at 72 °C (GeneAmp PCR System 9700, Perkin Elmer, USA). Negative control was carried out with no DNA. One long-insert containing clone originated amplicons with sizes compatible with a β PLI. Fresh PCR products were then subcloned into pGEM easy vector (Promega, USA) according to the manufacturer's instructions. After transformation of NM522 *E.coli*, recombinant plasmid DNAs were purified (Pure Yield Plasmid Kit, Promega USA) and submitted to PCR for confirmation of the presence of expected insert before complete nucleotide sequencing (Big Dye Terminator on an automated ABI 3130 Genetic Analyser, Applied Biosystem, USA). Deduced and determined primary structures were aligned using the Mac Vector 11.1.1 software (Mac Vector Inc., USA).

The DNA amplification with P1/P2 and P1/P3 primers has showed that one clone, LmBO4HO2BIPPLA, contained the expected insert sizes (data not shown). After subcloning, three pGEM positive clones were randomly selected and the presence of inserts with the expected sizes (932 bp for P1/P3 and 999 bp for P1/P2 primers) was confirmed (Fig. 1). Nucleotide sequencing (data not shown) was performed in both senses and proved to be almost identical in the three clones, except for two nucleotide substitutions. In the first case, there was a third position substitution in nt286 to 288 (AGT in clones 1 and 3, AGC in clone 2) with conservation of the Ser⁹⁶ residue. The second substitution occurred in the

first nt of the 817th–819th codon (CTC in clone 1 and TTC in clones 2 and 3) leading to Leu²⁷³ and Phe²⁷³ codification, respectively. The primary structures of those clones were deduced and aligned against the primary structures of known β PLI subunits from other snake species (Fig. 2). The putative β inhibitor from *L. muta* venom glands displayed average identity scores of 63.4%, 63.7%, 64.1% and 63.7% with β PLIs from *G. brevicaudus*, *E. quadrivirgata* A, *E. quadrivirgata* B and *E. climacophora*, respectively. The mean molecular mass for the *L. muta* β PLI – as calculated from the deduced amino acid composition – was 36,556.33 \pm 19.63. The main general characteristics of β PLIs were present in the putative *L. muta* β PLI: nine tandem LRRs with 24 residues each, an amino-terminal proline-rich flanking region, ten cysteinyl residues (seven of which, including the carboxy-terminus one, were strictly conserved at the same position in every sequence). The two free thiol cysteine residues – assigned as Cys170 and Cys213 in *G. brevicaudus* β PLI – are conserved in the Old World snake species, but replaced by isoleucine and serine, respectively, in *L. muta* β PLI (Fig. 2). Furthermore, a two-residue deletion was noted exclusively in *L. muta* β PLI at 12th and 13th positions downstream the strictly conserved amino-terminal valine (Fig. 2). The leucine-rich repeats (LRRs) are also marked in Fig. 2. Those repeats were firstly discovered in leucine-rich α_2 -glycoprotein (LRG), a protein with unknown function in human serum. Since their discovery, they have been found in more than 6000 proteins in different organisms, ranging from viruses to eukaryotes (Kobe and Deisenhofer, 1995; Wei et al., 2008). These repeats are thought to play an important role in protein–ligand interactions (Wei et al., 2008).

Until not long ago, β PLIs have been solely identified in two snake species: *G. brevicaudus* (Ohkura et al., 1997) and *E. quadrivirgata* (Okumura et al., 1999). The former is a venomous species containing acidic, neutral and basic PLA₂ in its venom composition (Ohkura et al., 1993), whereas the latter is a nonvenomous species that is supposed to prey upon venomous snakes, including *G. brevicaudus* (Shirai et al., 2009). In both cases, the role played by β PLIs is in accordance with the known self-protection mechanism against an eventual presence of toxic venom PLA₂ in the snake circulating blood. That role has been recently reinforced by the first demonstration of up-regulation of α , β and γ PLI expression in *G. brevicaudus* liver by intramuscular injection of the homologous venom (Kinkawa et al., 2010). Nevertheless, the recent identification of a β PLI in the blood plasma of *Elaphe climaphora* (Shirai et al., 2009), a nonvenomous and non-ophiophagous snake, has again called attention to other possible roles for the PLIs in snakes which are unrelated to venom PLA₂ inactivation. A first step in that direction was the latest demonstration that *G. brevicaudus* β PLI binds more tightly to snake cytochrome c than to basic, toxic PLA₂ from its own venom (Shirai et al., 2010). These authors suggested that β PLIs may be originally involved in the clearance of Cyt c released from dead cells in the circulatory system. If so, the role played by β PLIs in snakes could be extended to self-protection against Cyt c release, besides the leaking of toxic PLA₂ from venom glands.

Although clusters coding for α PLIs had been previously found in the transcriptome of *Bothrops jararaca* venom

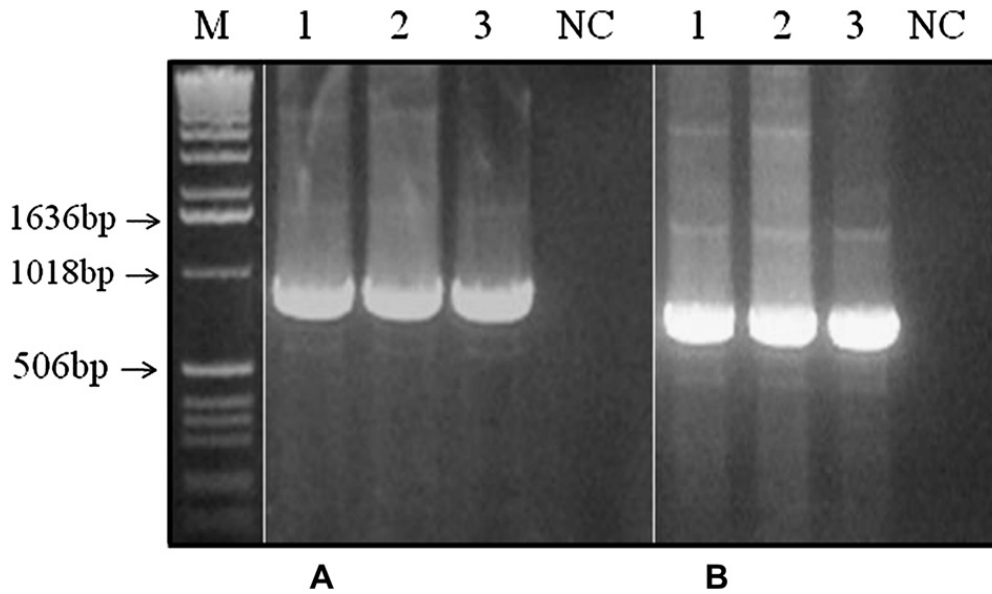


Fig. 1. Electrophoresis on 1% agarose gel of recombinant pGEM clones after amplification in the presence of P1/P3 (A) and P1/P2 (B) oligonucleotides for β -PLIs. Well samples: M. Size marker (1 Kb DNA ladder, Gibco BRL). 1–3. pGEM clones (Lm1 to 3); 4 – Negative control (no DNA).

gland with 0.9% of representation (Cidade et al., 2006) and partial β PLI sequences identified in *L. muta* venom gland library (Junqueira-de-Azevedo et al., 2006), to our knowledge, the present report is the first one comprising a complete β PLI sequence in venom glands of a New World snake. Nevertheless, the exact function of β PLIs as possible components of *L. muta* venom remains to be clarified.

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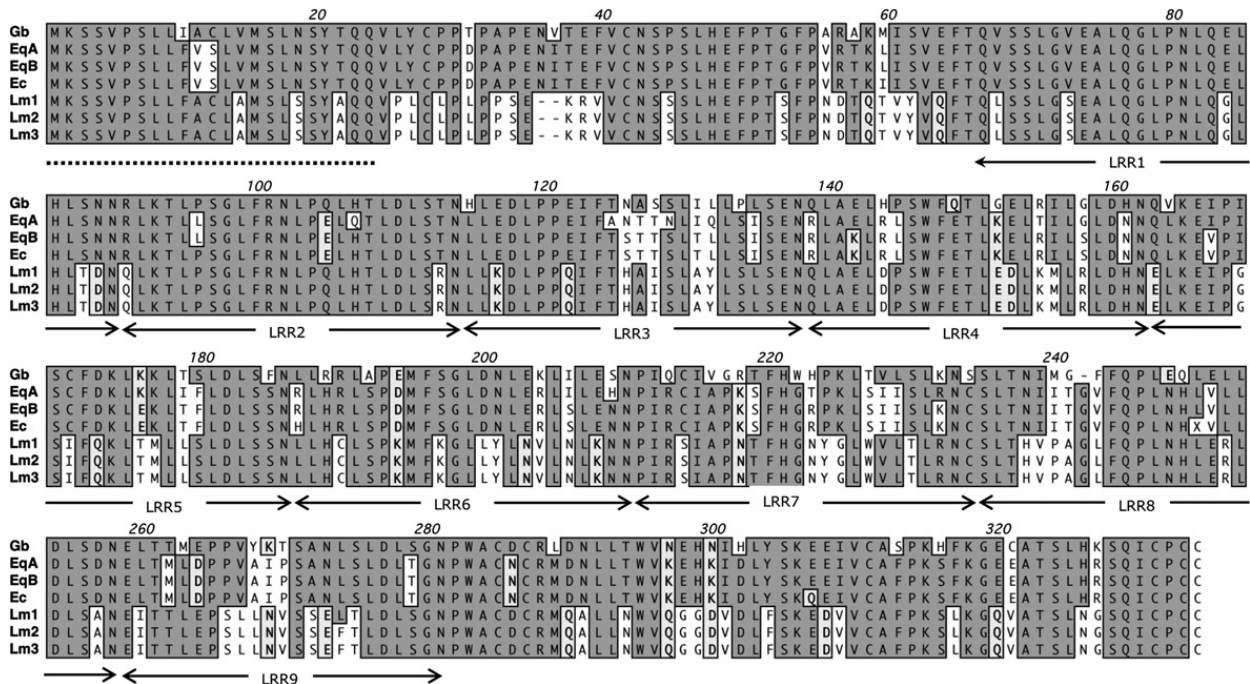


Fig. 2. Multiple alignment of primary sequences of β -PLIs from snakes and their respective source. Gc – *G. brevicaudus* blood [gi3358088]; Ec – *Elaphe climacophora* blood [gi226235136]; EqA – *E. quadrivirgata* blood subunit A [gi19911057]; EqB – *E. quadrivirgata* blood subunit B [gi19911059]; Lm (1–3) – *L. muta* venom glands (present study). Signal sequences are marked by a dotted line and leucine-rich repeats are indicated between arrows (based on Shirai et al., 2009). Amino acid identities are boxed and shadowed in dark gray. Similarities are boxed and shadowed in light gray.

Conflict of interest statement

On behalf of authors and co-authors I declare that there is no conflict of interest related to the short manuscript “Phospholipase A₂ inhibitors (βPLIs) are encoded in the venom glands of *Lachesis muta* (Crotalinae, Viperidae) snakes” submitted to *Toxicon*.

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