Short communication

A novel phospholipase A$_2$ from the venom glands of *Bungarus candidus*: cloning and sequence-comparison

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Abstract

The presence of phospholipase A$_2$ (PLA$_2$) in the venom of Malayan krait (*Bungarus candidus*) and its structure were studied. The PLA$_2$ cDNAs from the venom gland of *B. candidus* (Indonesia origin) were amplified by the polymerase chain reactions (PCR) and cloned. The primers used were based on the cDNA sequences of several homologous *B. multicinctus* venom PLA$_2$s. In addition to the A-chains of β-bungarotoxins, a novel *B. candidus* PLA$_2$ was cloned and its full amino acid sequence deduced. Having totally 125 amino acid residues, the PLA$_2$ contains a pancreatic loop and is 61% identical to the acidic PLA$_2$ of king cobra venom. However, the enzyme was not detected from the venom sample. Its structural relationships to other elapid venom PLA$_2$s were analyzed with a phylogenetic tree and discussed. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Malayan krait (*Bungarus candidus*); Snake venom; cDNA cloning; Phospholipase A$_2$; Phylogenetic analysis

Being a common component in snake venoms, the phospholipases A$_2$ (PLA$_2$s, EC 3.1.1.4) are classified into several structural groups. Amino acid sequences of more than 150 snake venoms PLA$_2$s have been solved; the molecules are 121–125 amino acids long with six or seven disulfide bonds. The group IA and IB PLA$_2$s are found in the venom of elapid and hydrophiid species and in the mammalian pancreases, while group II PLA$_2$s are present in the viperid snake venoms. Interestingly, PLA$_2$s in venom glands may undergo fast adaptive evolution to generate variants of diverse functions (Danse et al., 1997; Francis et al., 1998; Yu et al., 1999).

Snake of the genus *Bungarus*, commonly known as kraits, are distributed from Pakistan eastward through southern Asia and China. The Malayan krait *Bungarus candidus* and the Chinese/Taiwanese krait *B. multicinctus* are medically important, and are responsible for many cases of lethal snakebites in Asian countries. The major symptom of the envenoming is severe neurotoxicity (Warrell, 1993). The venoms of both species show strong lethal potency toward mice and birds (Lee and Tseng, 1969; Tan and Ponnudurai, 1990) and a phylogenetic study on many kraits revealed close relationship between the two (Slowinski, 1994). In both venoms, the most lethal components are β-bungarotoxins, which exist as heterodimers with a PLA$_2$ (A-chain) covalently linked with a Kunitz type inhibitor (B-chain) (Tan et al., 1989). A monomeric PLA$_2$ has also been isolated from the venom of *B. multicinctus* (Kondo et al., 1981), and it has been shown that the PLA$_2$ caused a sharp fall in arterial blood pressure (Lee and Lee, 1979). The purpose of the present study is to identify and clone PLA$_2$s from the venom gland tissue of *B. candidus* and examine its evolutionary relationships with other elapid venom PLA$_2$s.

For cloning and sequencing of the PLA$_2$, mRNA was extracted and purified from the venom glands of *B. candidus* (Bali, Indonesia). The complement DNA was prepared using the cDNA synthesis kits according to the manufacturer instructions (Stratagene, USA). Specific primers were designed based on the highly conserved cDNA sequences encoding the monomeric PLA$_2$s and the A chains of
β-bungarotoxins in the *B. multicinctus* venom glands (Chang et al., 1996). Primers 1 and 2 are 5′-CCA-GACGGCTCTCATAG-3′ and 5′-AAAAAGGAATATRC-CAGG-3′, respectively. Primer 1 was in the sense orientation of the 5′-end from untranslated region to the first two amino acids of signal peptide, whereas primer 2 was in the antisense direction of a conserved region at the 3′-end. The polymerase chain reaction (PCR) (Mullis and Faloona, 1987) was conducted to amplify the PLA2 cDNAs, using venom gland cDNA as templates as well as SuperTaq DNA polymerase (HT Biotech., UK).

DNA fragments of expected size (0.4 kb) were specifically amplified as shown by 1.5% agarose gel electrophoresis. After being treated with polynucleotide kinase, they were inserted into the pGEM-T vector and transformed into *Escherichia coli* strain JM109 (Maniatis et al., 1989). White transformants were picked up and sequenced on the DNA-Sequencing-System (model 373A, PE-Applied Biosystems, USA). About 20 PLA2 clones were sequenced and most of them were found to encode the β-bungarotoxins A-chains. Only two of the cDNA clones encode a novel PLA2 which we designated as Bc-PL. Its amino acid sequence was deduced from the cDNA sequence (Fig. 1A). However, by gel filtration and high performance liquid chromatography (HPLC) fractionation of the *B. candidus* venom, we tried but failed to isolate Bc-PL or any other PLA2 except the β-bungarotoxins (data not shown). It would have been isolated if its content is >0.1% of the crude venom by mass. The highly conserved signal peptide of Bc-PL as compared with other kraits’ PLA2s (Fig. 1B) suggests that mRNA of Bc-PL is originated from venom glands rather than other snake tissues.

Having totally 125 amino acid residues, Bc-PL belongs to group IB in the classification of the PLA2 superfamily (Danse et al., 1997). Its calculated molecular weight is 13982 assuming seven-disulfide bonds between the conserved Cys residues, and the theoretical isoelectric point is 5.1. Bc-PL contains a ‘pancreatic loop’ at 62–66, an acidic N-terminal half but a highly basic C-terminal region. In comparison to other venom PLA2s, Bc-PL has uncommon substitutions including F2, N6, E10, N22, C25, and T31 and contains no Trp residue. Although an intact calcium binding Asp 49 and Gly-rich loop (residues 26–33) as well as other residues presumably essential for the enzymatic action (e.g. 13982 assuming seven-disulfide bonds between the conserved Cys residues, and the theoretical isoelectric point is 5.1. Bc-PL contains a ‘pancreatic loop’ at 62–66, an acidic N-terminal half but a highly basic C-terminal region. In comparison to other venom PLA2s, Bc-PL has uncommon substitutions including F2, N6, E10, N22, C25, and T31 and contains no Trp residue. Although an intact calcium binding Asp 49 and Gly-rich loop (residues 26–33) as well as other residues presumably essential for the enzymatic action (e.g.
His48, Asp99 and Tyr52) are present in Bc-PL, the enzyme activity might be lowered as judged from previous chemical modification and mutagenesis studies at positions 2, 6, 10, 22 and 31 (Kuiipers et al., 1990; Yang and Chang, 1989; Yuan and Tsai, 1999).

According to the Blast search of the sequence databank, Bc-PL is structurally most similar (about 61% identical) to the myotoxic/cardiotoxic PLA2 from Ophiophagus hannah (king cobra) venom from Kunming, China and the haemorrhagic/myotoxic PLA2 (HTe) from Notechis scutatus scutatus venom (Francis et al., 1995). Previous studies showed the possible presence of two acidic PLA2s in king cobra venom (Huang et al., 1997), and geographic variations apparently exist such that the sequences of the king cobra PLA2s from the three Chinese provinces, Kunming, Guangxi, and Fujian are not identical (Fig. 1A). The ‘pancreatic loop’ has been found in the venom PLA2s of king cobra (Huang et al., 1997), several Australian elapids (Francis et al., 1995; Lambeau et al., 1995; Pearson et al., 1991), and New Guinea Micropechis ikaheka (Gao et al., 1999) but not in other krait’s PLA2s. The sequences of the pancreatic loops are rather variable (Fig. 1A).

Highly similar PLA2s from Asian elapid venoms have been purified and characterized in the past three decades. Recently, the cDNAs encoding many PLA2 isoforms in the venoms of Naja sputatrix (Armugam et al., 1997), Naja kaouthia (Chuman et al., 2000), and Naja atra (Pan et al., 1994) were cloned and sequenced. As many as eight PLA2 isoforms have been isolated and sequenced from the pooled venom of golden krait Bungarus fasciatus (Liu et al., 1989; Liu and Lo, 1994), including four catalytically active PLA2s (III, Vb-1, X-1, X-2), three abundant but less-active PLA2s (Va, Vb-2 and VI), and one inactive Ala49 PLA2. A phylogenetic tree has been constructed based on the amino acid sequences of the group IA and group IB PLA2s from the venoms. The bioinformatics tool used was the neighboring methodology using PHYLIP (Felsenstein, 1992). The bovine pancreatic PLA2 sequence was used as the outgroup.

The dendrogram (Fig. 2) has focused on the structural relationships between the cobra and the krait venom PLA2s. It shows linkage of the group IB members, i.e. Bc-PL and the king cobra venom PLA2, independent of other group IA PLA2s. All the B. fasciatus PLA2s are closely related to each other and to the B. multicinctus PLA2. Moreover, cobras appear to be evolved under two phylogeographic groups, i.e. Asian and African, respectively. The topology of the tree (Fig. 2) showed taxonomic affinity between cobras and kraits with the robustness at most of the major nodes supported by bootstrap analysis. We did not consider the PLA2s of hydrophiid and Australian/New Guinea elapid venoms since previous phylogenetic analysis of the group I PLA2s showed that the venom PLA2s of Hydrophiinae and Australian Elapidae evolved independently of those of the Asian and African Elapidae (Tsai, 1997).

This is the first case that a group IB PLA2 is found in krait venom glands although the protein is not expressed in significant amount. The presence of variable surface loop at

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![Fig. 2. Dendrogram showing structural relationships among the venom PLA2s of Asian and African Elapidae. The tree was constructed using the amino acid sequences of the elapid venom PLA2s (Armugam et al., 1997; Chuman et al., 2000; Danse et al., 1997; Liu et al., 1989; Liu and Lo, 1994; Pan et al., 1994), and of bovine pancreatic PLA2 (as the outgroup). Abbreviations are: N., Naja; O., Ophiophagus; and B., Bungarus, and the GenBank accession numbers for B. fasciatus PL-I and PL-II and O. hannah PLA are AAK62362, AAK62361 and AF297034, respectively.](image-url)
residues 62–66 of a PLA₂ supposedly would not affect its over-all structure (White et al., 1990) but may decrease the hydrolytic activity (Kuipers et al., 1989; Thunnissen et al., 1990) and possibly regulates its function (Gao et al., 1999). The phylogenetic analysis (Fig. 2) represents an attempt to study the snake taxonomy based on the sequences of their venom PLA₂K, this has been demonstrated to be rather successful in another case (Tsai et al., 2001). Nevertheless, it is known that B. candidus and B. multicinctus are very similar but not so to king cobra (Słowskii, 1994, and our unpublished result). The expression of distinct monomeric PLA₂S in both krait venoms, as either group IA or group IB, probably resulted from natural selection between the venom PLA₂ paralogs through evolution, and both groups of PLA₂ may be present in their ancestral species.

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