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Toxicon 40 (2002) 1363–1367

TOXICON

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Short communication

A novel phospholipase A_2 from the venom glands of *Bungarus candidus*: cloning and sequence-comparison☆

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Received 26 March 2002; accepted 14 May 2002

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_2s. 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_2s were analyzed with a phylogenetic tree and discussed.

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_2s, EC 3.1.1.4) are classified into several structural groups. Amino acid sequences of more than 150 snake venoms PLA_2 have been solved; the molecules are 121–125 amino acids long with six or seven disulfide bonds. The group IA and IB PLA_2s are found in the venom of elapid and hydrophiid species and in the mammalian pancreases, while group II PLA_2s are present in the viperid snake venoms. Interestingly, PLA_2s 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 from the venom gland tissue of *B. candidus* and examine its evolutionary relationships with other elapid venom PLA_2s.

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_2s and the A chains of

Abbreviations: Bc-PL, phospholipase of *Bungarus candidus*; PLA_2 or PLA, phospholipase A_2.

☆ The novel nucleotide sequences and deduced protein sequences were deposited in GenBank with the accession number AF492561.

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0041-0101/02/$ - see front matter
PII: S0041-0101(02)00150-2

A

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                1           11          21          31          41          51          61
B. candidus Bc-PL  TFLQFNEMIE CTIPGSFPLL DNMDCGCYCG TGGRGTPVDM LDRCCKEHDD CYAQIKENLK CSSLLNVPYV
O. hannah (KM)     NL····Y··Q ·····R·F· ·Y··Y····· ··VA·H···E ·····QT··L ··SKAE·QP· ·····S·LM
O. hannah (GX)     HLV···G··R ·····I·WW ·YS·Y····· S··S·····E ·····QV··N ··T·AQQLTE ·-----S··S
O. hannah OHV-A    HLI··GN··Q ··V··FLSWI KYA·T····· A··S·····K ·····QV··N ··T·AQKLPA ···IMDS···
B. multicinctus PLA NLY··KN··V ·--A·TR·WI GYVNY····· A··S·····E ·····YV··N ··GEAEKIPG ·-----N·KT
B. fasciatus PL-III NLF··KN··Q ·--A·TRSWT ·YVSY····· K··S·····Q ·L···KV··· ··GDAEKIP· ·-----K··Y
N. scutatus HTe    NLY··GN··Q ·ANH·RR·TR HY··Y····· K··S·····E ·····QT··· ··GEAEKLPA ·NYMMSG···

                71          81          91          101         111         121         % identity
B. candidus Bc-PL  KQYSFTCSEG DLTCSDAAGT CARIV-CDCD RTAALCFAEA PYKRRNFKID YKTR--CQ        100
O. hannah (KM)     ·K··Y···G· T···N·DNDE ·GAFI-·N·· ·A·RI···G· ··NKE·KEL· IA··--··        61
O. hannah (GX)     ·R··YD···· T···KADNDE ··AF······ ·V··I···G· ··NKE·IN·· TT··--·-        58
O. hannah OHV-A    ·I··YD···R TV··KADNDE ··AFI-·N·· ·V··H···AS ··NNN·YN·· TT··--·-        53
B. multicinctus PLA ·T··Y··TKP N···T····· ·····-···· ····I···A· ··NIN··M·S SS·H---·        56
B. fasciatus PL-III ·T··YD···· K···KADNDE ··AFI-·N·· ·V··I···G· ··NDN··M·· S···--··        53
N. scutatus HTe    NT··YE·N·· E···K·NNDE ·KAFI-·N·· ····I···R· ··NDA·WN·· T···--··        54
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B

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                    -27                 -1
(1) B. candidus Bc-PL          AVCVSLLGAANIPPQPL
(2) B. multicinctus PLA  MNPAHLLILS·····H·
(3) β-bungarotoxin A1    MNPAHLLVLS·····H··
(4) B. fasciatus PL-I    MNPAHLLVLL·····
(5) O. hannah            MNPAHLLVLS·····SS·····
(6) N. scutatus PLA2-like precursor  MYPAHLLVLLT·····E·SS··AR··
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Fig. 1. (A) Alignment of the deduced amino acid sequences of the venom PLA_2s from *B. candidus* and other related elapids. Numbering follows that of Renetseder et al. (1988). Single letter codes for amino acids were used. Dots denote the amino acid residues identical to those in the first line and gaps are (−). The GenBank accession numbers are: *O. hannah* (KM, i.e. Kunming), AF297034; *O. hannah* (GX, i.e. Guangxi), AF302907; *O. hannah* OHV-A (of the venom from Fujian, China), AF302908; *B. multicinctus*, 350460, respectively; and the Swissprot numbers are: *B. fasciatus* PL-III, P14615; *N. scutatus* HTe, S65624, respectively. (B) Alignment of the signal peptide sequences. Non-conserved substitutions are bolded. References are: (1) the present study (residues used to design the primer at 5′-end are underlined); (2)–(4) GenBank accession numbers CAA34782, CAD24466, and AAK62362, respectively; (5) see the legend of A, the signal sequences of the three *O. hannah* PLA_2s are identical; (6) GenBank accession number X12605.

β-bungarotoxins in the *B. multicinctus* venom glands (Chang et al., 1996). Primers 1 and 2 are 5′ -CCAGACGGCTTCATCATG-3′ and 5′-AAAAGGAATRATCCAGG-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 PLA_2 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 PLA_2 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 PLA_2 which we designated as Bc-PL. Its amino acid sequence was deduced from the cDNA sequence (Fig. 1(A)). 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 PLA_2 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' PLA_2s (Fig. 1(B)) 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 PLA_2 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 PLA_2s, 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 (Kuipers 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 PLA_2 from *Ophiophagus hannah* (king cobra) venom from Kunming, China and the haemorrhagic/myotoxic PLA_2 (HTe) from *Notechis scutatus scutatus* venom (Francis et al., 1995). Previous studies showed the possible presence of two acidic PLA_2s in king cobra venom (Huang et al., 1997), and geographic variations apparently exist such that the sequences of the king cobra PLA_2s from the three Chinese provinces, Kunming, Guangxi, and Fujian are not identical (Fig. 1(A)). The 'pancreatic loop' has been found in the venom PLA_2s 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 kraits' PLA_2s. The sequences of the pancreatic loops are rather variable (Fig. 1(A)).

Highly similar PLA_2s from Asian elapid venoms have been purified and characterized in the past three decades. Recently, the cDNAs encoding many PLA_2 isoforms in the venoms of *Naja sputatix* (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 PLA_2 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 PLA_2s (III, Vb-1, X-1, X-2), three abundant but less-active PLA_2s (Va, Vb-2 and VI), and one inactive Ala49 PLA_2. A phylogenetic tree has been constructed based on the amino acid sequences of the group IA and group IB PLA_2s from the venoms. The bioinformatics tool used was the neighbor-joining methodology using PHYLIP (Felsenstein, 1992). The bovine pancreatic PLA_2 sequence was used as the outgroup.

The dendrogram (Fig. 2) has focused on the structural relationships between the cobra and the krait venom PLA_2s. It shows linkage of the group IB members, i.e. Bc-PL and the king cobra venom PLA_2, independent of other group IA PLA_2s. All the *B. fasciatus* PLA_2s are closely related to each other and to the *B. multicinctus* PLA_2. 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 PLA_2s of hydrophiid and Australian/New Guinea elapid venoms since previous phylogenetic analysis of the group I PLA_2s showed that the venom PLA_2s 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 PLA_2 is found in krait venom glands although the protein is not expressed in significant amount. The presence of variable surface loop at

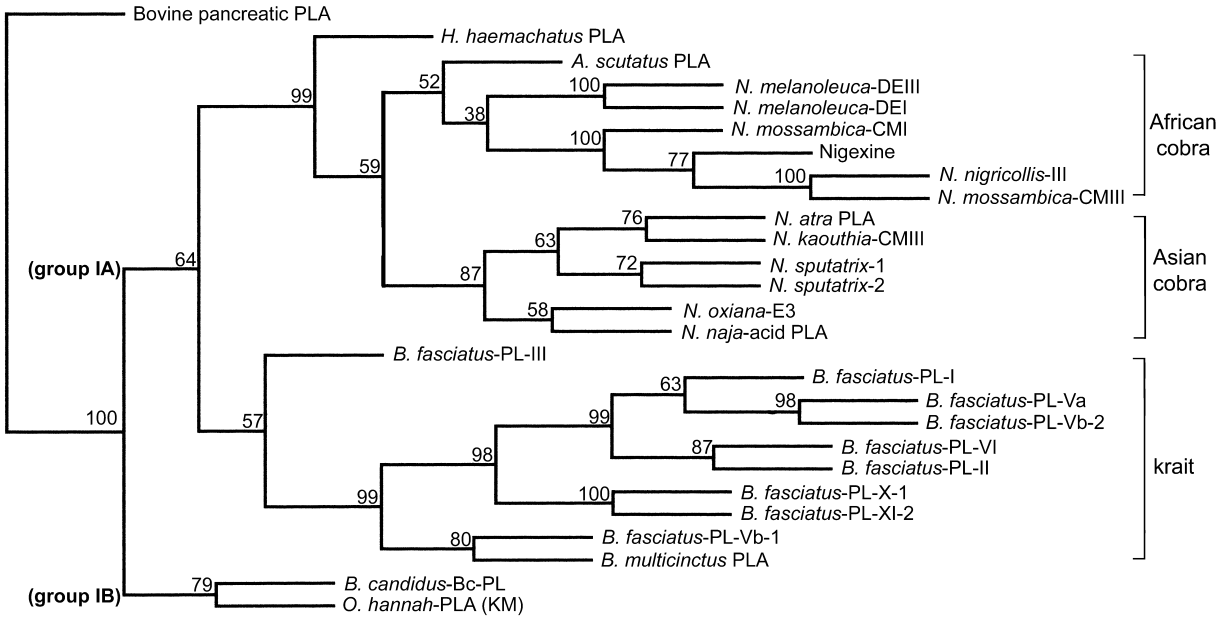

Fig. 2. Dendrogram showing structural relationships among the venom PLA_2s of Asian and African *Elapidae*. The tree was constructed using the amino acid sequences of the elapid venom PLA_2s (Armugam et al., 1997; Chuman et al., 2000; Danse et al., 1997; Liu et al., 1989; Liu and Lo, 1994; Pan et al., 1994), and of bovine pancreatic PLA_2 (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.

residues 62–66 of a PLA_2 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 PLA_2s, 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 (Slowinski, 1994, and our unpublished result). The expression of distinct monomeric PLA_2s in both krait venoms, as either group IA or group IB, probably resulted from natural selection between the venom PLA_2 paralogs through evolution, and both groups of PLA_2 may be present in their ancestrial species.

Acknowledgements

We are grateful for the gift of a live specimen of *B. candidus* from Mr Duncan MacRae. This research was supported by grants from National Science Council, Taiwan.

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