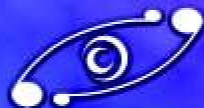

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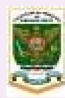

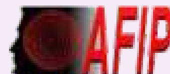

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# cDNA and deduced primary structure of basic phospholipase $A_2$ with neurotoxic activity from the venom secretion of the *Crotalus durissus collilineatus* rattlesnake

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## Abstract

To illustrate the construction of precursor complementary DNAs, we isolated mRNAs from whole venom samples. After reverse transcription polymerase chain reaction (RT-PCR), we amplified the cDNA coding for a neurotoxic protein, phospholipase $A_2$ D49 ($PLA_2$ D49), from the venom of *Crotalus durissus collilineatus* (Cdc $PLA_2$). The cDNA encoding Cdc $PLA_2$ from whole venom was sequenced. The deduced amino acid sequence of this cDNA has high overall sequence identity with the group II $PLA_2$ protein family. Cdc $PLA_2$ has 14 cysteine residues capable of forming seven disulfide bonds that characterize this group of $PLA_2$ enzymes. Cdc $PLA_2$ was isolated using conventional Sephadex G75 column chromatography and reverse-phase high performance liquid chromatography (RP-HPLC). The molecular mass was estimated using matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry. We tested the neuromuscular blocking activities on chick biventer cervicis neuromuscular tissue. Phylogenetic analysis of Cdc $PLA_2$ showed the existence of two lines of N6-$PLA_2$, denominated F24 and S24. Apparently, the sequences of the New World's N6-F24-$PLA_2$ are similar to those of the agkistrodotoxin from the Asian genus *Gloydius.* The sequences of N6-S24-$PLA_2$ are similar to the sequence of trimucrotoxin from the genus *Protobothrops*, found in the Old World.

Key words: Cdc $PLA_2$; cDNA; Chick biventer cervicis; *Crotalus durissus collilineatus*; Mass spectrometry MALDI-TOF; Neurotoxic agents

## Introduction

Phospholipase $A_2$ ($PLA_2$; EC 3.1.1.4) enzymes are small proteins widespread in nature and frequently the major toxic component of snake venoms (1). Despite their similar structures and enzymatic mechanisms for the catalysis of the hydrolysis of sn-2 bonds in phosphoglycerides in a $Ca^{2+}$-dependent manner, they have evolved to acquire a wide range of activities, including neurotoxic, myotoxic, cardiotoxic, edema-forming, platelet-aggregating or -inhibiting, anticoagulant, convulsive, and hypotensive effects (2). These activities are attributed primarily to crotoxin and crotoxin B ($PLA_2$), the principal toxin of *Crotalus durissus terrificus* (South American rattlesnake) venom, which is also present in other subspecies such as *C. durissus collilineatus*, *C. durissus ruruima*, and *C. durissus cumanensis* (3-5).

The cDNAs and genes encoding $PLA_2$ have been cloned; however, comparison of their nucleotide sequences led to a novel discovery that Darwinian-type accelerated evolution has occurred and diverse physiological activities have been acquired (6-8). Therefore, the study of the neurotoxic protein $PLA_2$ D49, derived from the venom of a rattlesnake subspecies, *C. durissus collilineatus* (Cdc $PLA_2$), is of particular interest. When comparing the chromatography profiles of other crotalic venoms with the venom of *C. durissus collilineatus*, the absence of crotamine in *C. durissus collilineatus* venom is particularly interesting since this toxin has myotoxin and neurotoxin activities. These findings suggest that the crotoxin and $PLA_2$ F6 from *C. durissus collilineatus* probably play an important role in this

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venom's action (3), suggesting a molecular microevolution. However, acquiring information on the molecular genetics of precursor structures derived from cDNAs involves sacrificing the specimens for subsequent dissection of the venom gland (9,10).

We describe here a simple and rapid technique for the synthesis of venom gland protein cDNAs from lyophilized venom samples that does not require the sacrifice and dissection of the specimen. The venom from Cdc was chosen because, although it is a clinically important species of rattlesnake found in central Brazil, few studies have been carried out on its venom and its main toxin, a crotoxin homolog (11).

## Material and Methods

### Venom, chemicals and reagents

Lyophilized Cdc venom was obtained from "Centro de Extração de Toxina Animal" (CETA) snakes farmed in Morungaba, SP, Brazil. Crotoxin and Cdc $PLA_2$ were purified from this venom as described by Ponce-Soto et al. (3). All chemicals and reagents used were of analytical, sequencing or molecular grade.

### Animals

Male HY-LINE W36 chickens (*Gallus gallus*), 4-8 days old were supplied by Granja Ito S/A (Sumaré, SP, Brazil). Animals had free access to food and water.

### mRNA isolation and cDNA construction

Lyophilized venoms (10 mg) were reconstituted with 0.25 mL Trizol LS (Gibco-BRL, USA), 50 µL chloroform (-20°C) was added and the preparation was incubated at 4°C for 5 min. Samples were centrifuged at 16,000 *g* for 5 min at 4°C. Following centrifugation, the upper aqueous phase, containing all RNAs, was transferred to a fresh tube. The RNA was precipitated with 300 µL cold isopropyl alcohol and collected by centrifugation at 16,000 *g* for 5 min at 4°C. The supernatant was removed and the RNA pellet was washed once with 400 µL 75% ethanol and collected by centrifugation at 10,000 *g* for 2 min at 4°C. The supernatant was discarded and the pellet was resuspended in 8 µL diethyl pyrocarbonate water for cDNA synthesis.

Reverse transcription polymerase chain reaction (RT-PCR) was conducted using RETROTOOLS cDNA/DNA polymerase (BIOTools, USA), according to manufacturer protocols. RT-PCR was carried out using oligo (dT) as primer. DNAs were amplified by PCR using total RT-PCR products as template and two oligonucleotide primers. The two primers were designed according to the reported nucleotide sequences of venom $PLA_2$ (12). The forward primer was designed around the start codon: primer 1 (5' tCT GGA TTG AGG AGG ATG 3'); the reverse primer was designed around the stop codon: primer 2 (5' cAT GCC TGC AGA GAC TTA 3'). Both primers corresponded to the conserved non-coding regions of venom $PLA_2$. PCR conditions were: 95°C/3 min (1 cycle), 94°C/30 s, 52°C/30 s, and 72°C/30 s (25 cycles), and 72°C/10 min (1 cycle). The amplified DNA fragment obtained was analyzed on 1.5% (w/v) agarose gel stained with ethidium bromide and visualized under UV light.

### cDNA sequencing

PCR products were sequenced directly using a big dye terminator cycle sequencing kit (Applied Biosystems, USA) and an ABIPrism 377 Sequencer automated DNA sequencer (Perkin Elmer, USA). The structure of the product was compared to that of the GenBank nucleotide and protein data bases using the Blastn and Blastx programs available at the National Center for Biology Information (NCBI) for identification (www.ncbi.nlm.nih.gov).

### Phylogenetic tree of the N6-$PLA_2$ and G6-$PLA_2$

The amino acid sequences of the $PLA_2$ Cdcoll cDNAs were aligned to $PLA_2$ isoform sequences from related viperid species: Crotoxin B1 and B2 (P07517, P24027) *C. durissus terrificus*, Sistruxin B (AAR14164) *Sistrurus catenatus tergeminus*, Agkistrotoxin (P14421) *Gloydius halys*, Smstoxin (AAR14160) *S. miliarius streckeri*, Cvvtoxin (AAQ13337) *C. viridis viridis*, Trimucrotoxin (Q90W39) *Protobothrops mucrosquamatus*, Cgtoxin (AAR14161) *Cerrophidion godmani*, Bstoxin (AAR14162) *Bothriechis schlegelii*, TfPLA-N (BAC56893) *Trimeresurus flavoviridis*, Batoxin (S09314) *Bothrops asper*, Bjtoxin (AAO27454) *B. jararacussu*, using the Lasergene software (DNASTAR, USA) and a phylogenetic tree constructed for the latter sequences using the CLUSTAL W program (13). The W6 $PLA_2$ D49 of *Calloselasma rhodostoma* venom (14) was used as out-group. Bootstrap values higher than 50 at each node support the robustness of the cladogram.

The predicted isoelectric point of $PLA_2$ examined here was determined using the Lasergene software (Protean, DNASTAR).

### Measurement of $PLA_2$ activity

$PLA_2$ activity was measured using the assay described by Holzer and Mackessy (15) in the use of a chromogenic substratum, modified for 96-well plates (3). The standard assay mixture contained 200 µL buffer (10 mM Tris-HCl, 10 mM $CaCl_2$, 100 mM NaCl, pH 8.0), 20 µL chromogenic lipid substrate 4-nitro-3-octanoyloxy-benzoic acid, 20 µL water, and 20 µL $PLA_2$ in a final volume of 260 µL. After the addition of $PLA_2$ (20 µg), the mixture was incubated for up to 40 min at 37°C, and the chromophore, 4-nitro-3-hydroxy-benzoic acid, detected by absorbance measurements at 425 nm, at 10-min intervals. Enzyme activity, reported as the initial reaction velocity, was calculated on the basis of the increase of absorbance at 425 nm after 20 min (3).

### MALDI-TOF mass spectrometric analysis

The molecular mass of Cdc $PLA_2$ was determined by

matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry using a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems). One microliter of sample (Cdc $PLA_2$) in 0.1% TFA was mixed with 2 µL of the matrix α-cyano-4-hydroxycinnamic acid, 50% acetonitrile, and 0.1% TFA (v/v). The matrix was prepared with 30% acetonitrile and 0.1% (v/v) TFA. Ion masses were determined under the following conditions: accelerate voltage 25 kV, the laser operated at 2890 µJ/com2, delay 300 ns and in the linear analysis mode (16).

### N-terminal amino acid sequence

One milligram of purified Cdc $PLA_2$ was dissolved in 6 M guanidine hydrochloride containing 0.4 M Tris-HCl and 2 mM EDTA at a final pH of 8.15. After reduction with DTT the products were carboxymethylated with $^{14}C$-iodoacetic acid (16). The mixture was desalted on a Sephadex G25 column equilibrated with 1 M acetic acid at 25°C and the reduced and carboxymethylated proteins were lyophilized. The N-terminal amino acid sequence of Cdc $PLA_2$ protein was determined by automated Edman degradation in a model Procise automatic sequencer (Applied Biosystems). PTH amino acids were identified with a model 120A PTH amino acid analyzer (Applied Biosystems) based on their retention times. Cdc $PLA_2$ was sequenced to double check the N-terminal sequence of up to 42 residues in order to confirm and compare it with the $PLA_2$ sequence deduced from nucleotide sequencing.

### Effect on the chick biventer cervicis preparation

Animals were anesthetized with halothane and sacrificed by exsanguination. The biventer cervicis muscles were removed and mounted under a tension of 0.5 g, in a 5-mL organ bath (Automatic organ multiple-bath LE01 Letica Scientific Instruments, Spain) at 37°C containing aerated (95% $O_2$-5% $CO_2$) Krebs solution, pH 7.5, of the following composition: 118.7 mM NaCl, 4.7 mM KCl, 1.88 mM $CaCl_2$, 1.17 mM $KH_2PO_4$, 1.17 mM $MgSO_4$, 25.0 mM $NaHCO_3$ and 11.65 mM glucose. Contraction to exogenously applied acetylcholine (ACh; 55 and 110 µM for 60 s) and KCl (20.1 mM for 130 s) was obtained in the absence of field stimulation, prior to the addition of a single dose of venom or crotoxin. A bipolar platinum ring electrode was placed around the tendon, which runs along the nerve trunk supplying the muscle. Indirect stimulation was performed with a MAIN BOX LE 12404 Panlab s.l. stimulator (Powerlab AD Instruments, Spain; 0.1 Hz, 0.2 ms, 3-4 V). Muscle contractions and contractures were isometrically recorded by force-displacement transducers (Model MLT0201 Force transducer 5 mg-25 g Panlab s.l. AD Instruments Pty Ltd.) connected to a PowerLab/4SP (OUAD Bridge AD Instruments, Spain).

### Statistical analysis

Data are reported as the mean ± SEM of *n* experiments. The significance of differences between means was assessed by analysis of variance followed by the Dunnett test when several experimental groups were compared with the control group. The confidence limit for significance was 5%.

## Results

### mRNA isolation and cDNA construction

The single PCR product generated was within the expected size of approximately 450 bp (Figure 1) and nucleotide sequencing, as shown by trawling unedited data on the database, which unequivocally identified the product, demonstrating that the polyadenylated mRNAs constituted representative products of the venom secretion of a neurotoxic venomous snake.

### cDNA sequencing

The cDNA of $PLA_2$ D49 from Cdc has 451 bp, which includes the start codon ATG and the termination codon TAA (Figure 2). By comparing this with other $PLA_2$ cDNAs, we deduced that this cDNA encodes a putative signal peptide of 16 amino acids and a mature protein of 122 residues. There are 14 cysteines in the mature peptide, its isoelectric point was estimated to be 8.5 and its molecular mass to be about 14 kDa.

### Purification of $PLA_2$ and measurement of $PLA_2$ activity

Purification of $PLA_2$ from Cdc venom was performed according to the procedure described by Ponce-Soto et al.

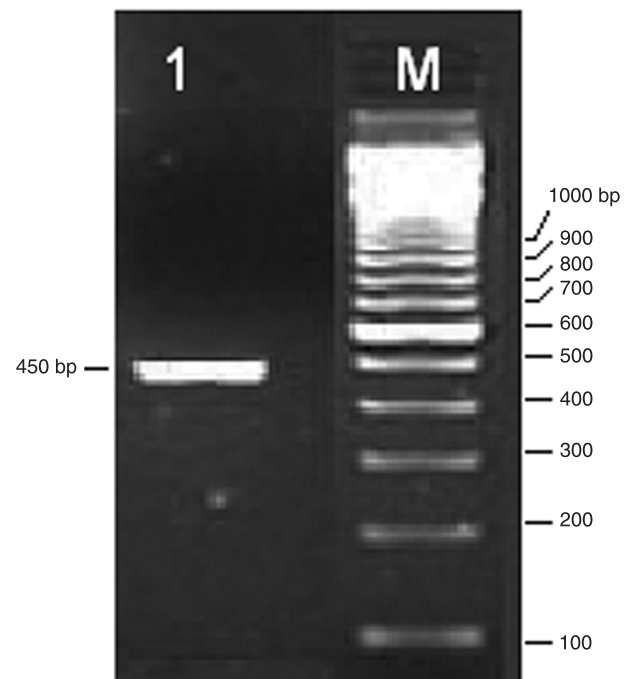


**Figure 1.** Reverse transcription polymerase chain reaction amplification of mRNA encoding protein transcripts from lyophilized venom. *Lane 1*, RT-PCR products from cDNA phospholipase $A_2$ primers, around 450 bp. *Lane M* contains a standard DNA ladder, each band representing 100-bp increments.

(3). Reverse-phase-HPLC of the crotoxin of the Cdc venom resulted in one $PLA_2$ peak (Cdc $PLA_2$) that eluted at 35 min, and any other isoforms of $PLA_2$ were absent. The specific activity of purified $PLA_2$ was 9.87 $nmol \cdot min^{-1} \cdot mg^{-1}$.

### N-terminal amino acid sequence of the expressed protein

The complete amino acid sequence deduced was confirmed up to the 42nd residue by direct protein N-terminal sequencing (HLLQF NKMIK FETRR NAIPF YAFYG CYCGW GGRGR PKDAT DR). Amino acid analysis was deduced as the sequence of cDNA of the protein, using the Lasergene software (Protean, DNASTAR, USA), and revealed the following composition of Cdc $PLA_2$: A/6, C/14, D/6, E/5, F/6, G/11, H/2, I/5, K/7, L/6, M/2, N/3, P/4, Q/3, R/13, S/6, T/7, V/2, W/3, and Y/11.

### MALDI-TOF mass spectrometric analysis

MALDI-TOF results showed that Cdc $PLA_2$ has a molecular mass of 14,276.90 Da (Figure 3), confirming the results obtained by analysis of the amino acids deduced from the cDNA (14,340.54 Da). The molecular mass of Cdc $PLA_2$ was confirmed by MALDI-TOF mass spectrometry, indicating the protonated protein species of homodimer with a molecular mass of 28,395.68 Da ($2M+2H^+$), monomers of 14,185.48 Da ($M+H^+$) and half of the mass of 7,105.18 Da ($MH_2^{2+}$) showed in the mass spectrum (Figure 3).

### Effects on the chick biventer cervicis preparation

The neuromuscular effect of Cdc $PLA_2$ was studied using chick biventer cervicis nerve-muscle preparations. Concentrations of 10 and 20 µg/mL Cdc $PLA_2$ affected neuromuscular transmission, and the times required to reach a 50% blockade were 42.61 min for 10 µg/mL and 36.14 min for 20 µg/mL (Figure 4). In both cases, the blockade was irreversible. $PLA_2$ did not block ACh and KCl-induced contractions significantly compared to control values. In control preparations, the contractions induced by ACh and KCl remained stable after 120 min of indirect stimulation compared to control values (data not shown).

```
ggtctggattgaggagg atg agg gct ctc tgg ata gtg gcc gtg ttg ctg gtg ggc gtc gag ggg
                   M   R   A   L   W   I   V   A   V   L   L   V   G   V   E   G
cac ctg ctg caa ttc aac aag atg atc aag ttt gag aca agg aga aac gct att ccc ttc
 H   L   L   Q   F   N   K   M   I   K   F   E   T   R   R   N   A   I   P   F
 1   2   3   4   5   6   7   8   9   10  11  12  13  14  15  16  17  18  19  20
tat gcc ttt tac ggc tgc tac tgt ggc tgg ggg ggc cga ggc cgg cca aag gac gcc act
 Y   A   F   Y   G   C   Y   C   G   W   G   G   R   G   R   P   K   D   A   T
 21  22  23  24  25  26  27  28  29  30  31  32  33  34  35  36  37  38  39  40
gac cgc tgc tgc att gtg cat gac tgc tgt tac gga aaa ctg gcc aag tgc aac acc aaa
 D   R   C   C   I   V   H   D   C   C   Y   G   K   L   A   K   C   N   T   K
 41  42  43  44  45  46  47  48  49  50  51  52  53  54  55  56  57  58  59  60
tgg gac ttc tat cgc tac agc ttg agg agt ggg tat ttc acc tgc gga aag ggc acc tgg
 W   D   F   Y   R   Y   S   L   R   S   G   Y   F   T   C   G   K   G   T   W
 61  62  63  64  65  66  67  68  69  70  71  72  73  74  75  76  77  78  79  80
tgc gag caa cag att tgt gag tgc gac agg gtc gcg gca gaa tgc ctc aga agg agt ctg
 C   E   Q   Q   I   C   E   C   D   R   V   A   A   E   C   L   R   R   S   L
 81  82  83  84  85  86  87  88  89  90  91  92  93  94  95  96  97  98  99  100
agc acg tac agg tat gga tat atg att tac ccg gac tct cgt tgc agg ggg cct tca gag
 S   T   Y   R   Y   G   Y   M   I   Y   P   D   S   R   C   R   G   P   S   E
 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120
aca tgc taa gtctctgcaggcatgca
 T   C  END
 121 122
```

**Figure 2.** The cDNA sequence determined by sequencing and the deduced amino acid sequence of Cdc $PLA_2$. One-letter codes of amino acids are used and the numbering is shown below the sequences. PCR primers are in bold and the signal peptide is underlined. The sequence confirmed by direct n-terminal sequence is double underlined.

### Phylogenetic tree of the N6-$PLA_2$ and G6-$PLA_2$

We constructed the phylogenetic tree (Figure 5) based on the protein sequences of neurotoxic N6-$PLA_2$ and G6 or A6-myotoxic $PLA_2$ (17-20) from pit viper venom (Figure 5). Molecular phylogeny of basic crotalid $PLA_2$ D49 includes all sequences listed in Figure 6. The comparison of Cdc $PLA_2$ sequences revealed a high level of conservation within the $PLA_2$ protein family and showed that there are extremely

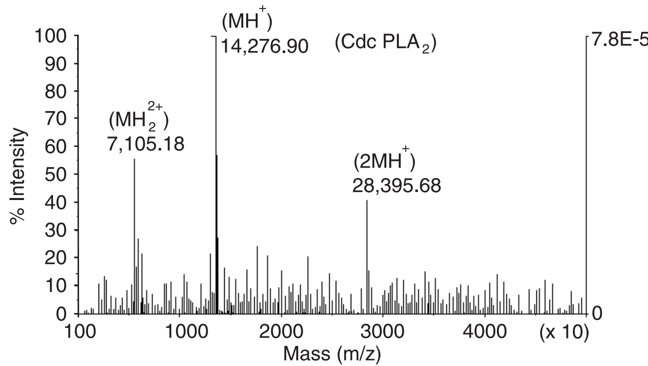


**Figure 3.** Determination of the molecular mass of Cdc $PLA_2$ by MALDI-TOF mass spectrometry, using a Voyager DE PRO MALDI-TOF mass spectrometry (Applied Biosystems, USA). The ionic $MH^+$ corresponds to the real mass of the Cdc $PLA_2$ and $MH_2^{2+}$ and $2MH^+$ species are shown in the mass spectrum. The value of the real mass of Cdc $PLA_2$ corresponds to the average of several spectrum ($\pm 7.8 \times 10^{-5}$).

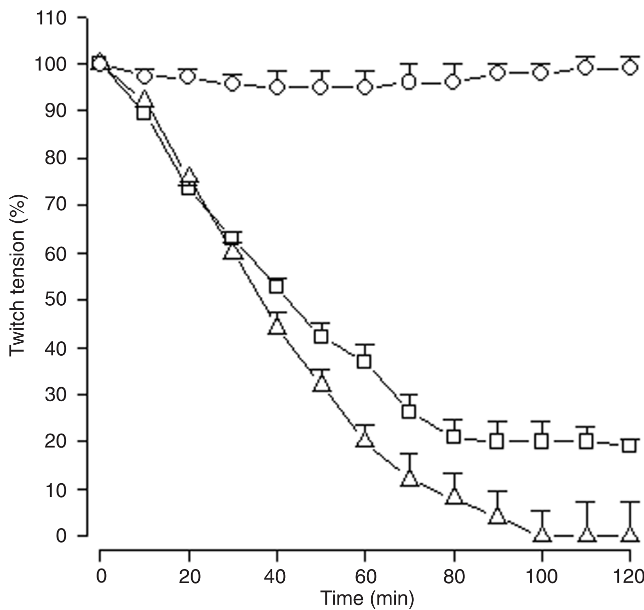


**Figure 4.** Neurotoxic activity of Cdc $PLA_2$ on the chick biventer cervicis preparation. Control (circles) without $PLA_2$. Doses of 10 (squares) and 20 µg/mL (triangles) of Cdc $PLA_2$. Each point represents the average of five experiments ± SEM. $P < 0.05$ compared to control (Dunnett test).

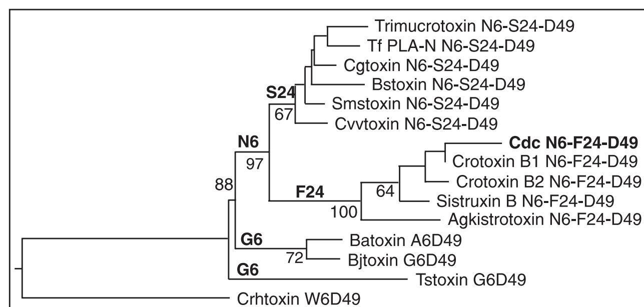


**Figure 5.** Molecular phylogeny of basic crotalid $PLA_2$ D49. The data used include all sequences listed in Figure 6. Clustal X (DNA Star program) was employed for the comparison of Cdc $PLA_2$ sequence with other Crotoxin B1 and B2 (P07517, P24027) *Crotalus durissus terrificus*, Sistruxin B (AAR14164) *Sistrurus catenatus tergeminus*, Agkistrotoxin (P14421) *Gloydius halys*, Smstoxin (AAR14160) *Sistrurus miliarius streckeri*, Cvvtoxin (AAQ13337) *Crotalus viridis viridis*, Trimucrotoxin (Q90W39) *Protobothrops mucrosquamatus*, Cgtoxin (AAR14161) *Cerrophidion godmani*, Bstoxin (AAR14162) *Bothriechis schlegelii*, TfPLA-N (BAC56893) *Trimeresurus flavoviridis*, Batoxin (S09314) *Bothrops asper*, Bjtoxin (AAO27454) *Bothrops jararacussu*. The phylogeny relationship of Cdc $PLA_2$ to other $PLA_2$ isoforms was statistically evaluated by the Bootstrap method.

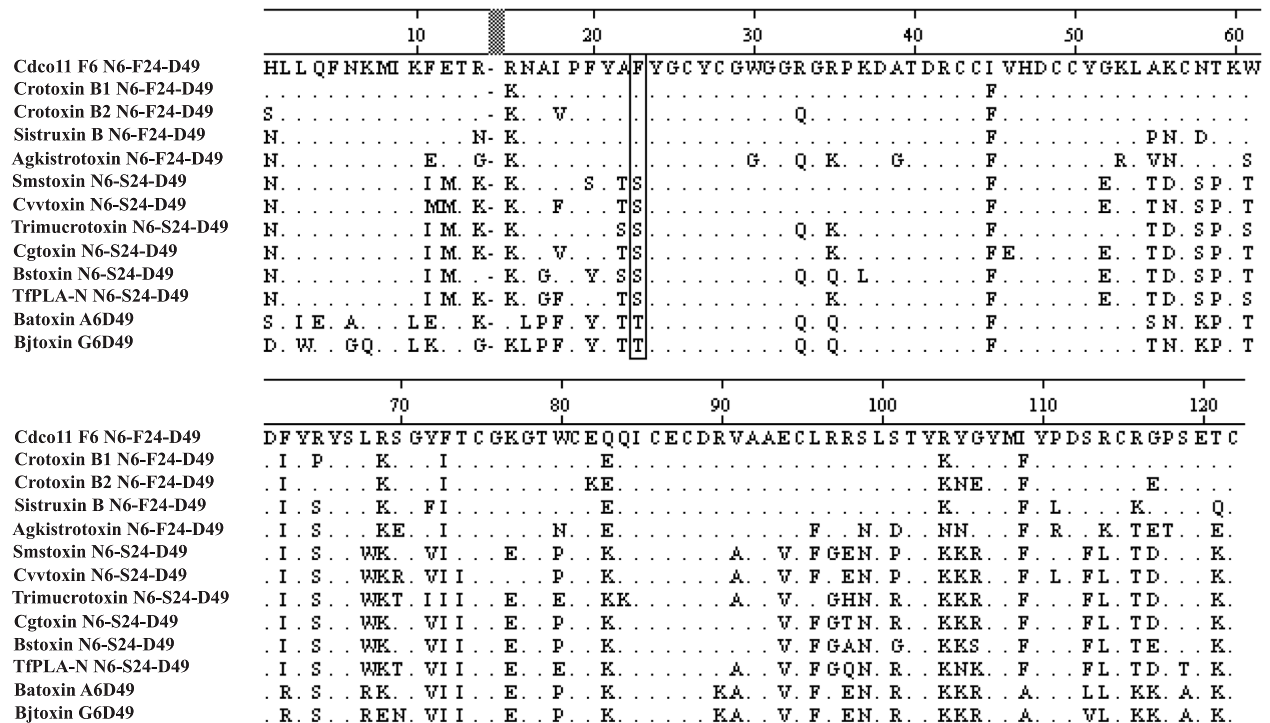


**Figure 6.** Amino acid sequence and homology of Cdc $PLA_2$ with other basic $PLA_2$ D49. The $PLA_2$, with GenBank accession numbers and the venom species are: Crotoxin B1 and B2 (P07517, P24027) *Crotalus durissus terrificus*, Sistruxin B (AAR14164) *Sistrurus catenatus tergeminus*, Agkistrotoxin (P14421) *Gloydius halys*, Smstoxin (AAR14160) *Sistrurus miliarius streckeri*, Cvvtoxin (AAQ13337) *Crotalus viridis viridis*, Trimucrotoxin (Q90W39) *Protobothrops mucrosquamatus*, Cgtoxin (AAR14161) *Cerrophidion godmani*, Bstoxin (AAR14162) *Bothriechis schlegelii*, TfPLA-N (BAC56893) *Trimeresurus flavoviridis*, Batoxin (S09314) *Bothrops asper*, Bjtoxin (AAO27454) *Bothrops jararacussu*.

conserved positions in the $PLA_2$ (Figure 6).

## Discussion

Venoms from many invertebrates and vertebrates are rich in amphipathic/cationic peptides that have been shown to possess nucleic acid binding properties and indeed to afford protection against degradation (21,22). The presence of these peptides permits the retrieval of these RNAs in a RT-PCR-compatible form.

Chen et al. (23) reported that it was impossible to recover high quality mRNAs from reptile venom for RT-PCR using the precipitation method with organic solvents. We succeeded in recovering these mRNAs by conventional techniques of precipitation with organic solvents. For the construction of the $PLA_2$ cDNA encoder from Cdc, we then used samples of total RNA extracted from the venom. These findings show that it is unnecessary to separate the mRNA from other RNAs to construct the cDNA.

The single PCR product generated was within the expected size, approximately 450 bp (Figure 1), and nucleotide sequencing unequivocally identified the product, demonstrating that the polyadenylated mRNAs constituted representative products of the venom gland transcriptome of a neurotoxic venomous snake.

The cDNA of $PLA_2$ D49 from Cdc has 451 bp, include the start codon ATG and the termination codon TAA (Figure 2). By comparing this with other $PLA_2$ cDNAs, we deduced that this cDNA encodes a putative signal peptide of 16 amino acids and a mature protein of 122 residues. The deduced signal peptide agrees with the characteristics of a general signal peptide: i) the central core is rich in hydrophobic amino acid residues (Leu), which can form a transmembrane α-helix; ii) the signal peptide cleavage site is present after an apolar residue (Gly) at position 16 (Figure 2). There are 14 cysteines in the mature peptide, which can form 7 disulfide bridges, its isoelectric point is estimated to be 8.5 and molecular mass is about 14 kDa. Since position 49 in the mature peptide is Asp, we conclude that it is a form of $PLA_2$ D49.

A pattern of biochemical and genetic variation exists within any species with respect to the protein fractions expressed in their venom (24). Therefore, we opted to isolate the mRNA and $PLA_2$ from the same extracted venom

sample to avoid differences among samples due to mixing the venom of more than one individual animal.

Purification of $PLA_2$ from Cdc venom was performed in accordance with the procedure described by Ponce-Soto et al. (3) for the comparison of its deduced molecular structure with its primary structure and with its neurotoxic activity.

In comparison with the chromatographic profiles of other crotalid venoms, the venom of Cdc lacks the fraction corresponding to crotamine. The absence of crotamine in Cdc venoms is interesting because this toxin is typically found in *C. durissus terrificus* venom. This toxin is responsible for myotoxic and neurotoxic activities and in this rattlesnake species (*C. durissus terrificus*) it accounts for 20% of the dry venom. These findings suggest that the crotoxin homolog in Cdc probably plays an important role in the neurotoxic action of this venom (3).

The effect of Cdc $PLA_2$ on neuromuscular preparations was studied using chick biventer cervicis nerve-muscle preparations. The blockade was irreversible (Figure 4). $PLA_2$ did not block Ach and KCl-induced contractions significantly compared to control values. In control preparations, contractions induced by ACh and KCl remained stable after 120 min of indirect stimulation compared to control values (data not shown). These results confirm that isolated Cdc $PLA_2$ may be considered a presynaptic neurotoxin that presents different times of blockade. Furthermore, these results suggest that its pharmacological activity is dose-dependent.

The side chain of F24 has proven to be important for the toxicity of ammodytoxins A and C (25), and modification of Y22 by tetranitromethane greatly reduces the specific binding of crotoxin to synaptosomal vesicles (26). In addition, residues 6-8 have been shown to be essential for the neurotoxicity of trimucrotoxin by site-directed mutagenesis (27). Therefore, the neurotoxic effects induced by Cdc $PLA_2$ may be related to the presence of residue F at position 24, making it a member of the group of F24-N6-$PLA_2$. Moreover, the importance of residues F24, Y22 and residues 6-8 is to preserve the neurotoxic activity of Cdc $PLA_2$ (Figure 5).

Recently, an R34Q mutation in basic $PLA_2$ from Chinese pit viper venom reduced its hemolytic activity *in vitro* (28), suggesting that R34 in the group II $PLA_2$ is favorable for membrane binding while Q34 may be more favorable for binding to specific neuronal proteins or receptors. In Figure 5, the presence of an arginine residue can be clearly seen at position 34. $PLA_2$ Cdc may exert some hemolytic activity in addition to its neurotoxic activity.

Rattlesnakes (genus *Crotalus*) and other pit vipers of the New World are probably the descendants of some species of Asian pit vipers (29). The group II $PLA_2$ in pit viper venom have been classified into different pharmacological subtypes that play different roles, e.g., as platelet aggregation inhibitors, anticoagulants, neurotoxins, or myotoxins (30,31). One of these subtypes is the myotoxic/neurotoxic $PLA_2$ D49 with an Asn 6 substitution (hereafter designated as N6-$PLA_2$). The known members of this venom $PLA_2$ subtype include the basic subunits of crotoxin and Mojave toxin, agkistrodotoxin from *Gloydius halys brevicaudus* (32), trimucrotoxin from *Protobothrops mucrosquamatus* (33), Tf-PLA-N from *flavoviridis* (34), and the myotoxin from *C. viridis viridis* (designated as Cvv-N6) (14,35).

The phylogenetic tree based on protein sequences of neurotoxic N6-$PLA_2$ and G6 or A6-miotoxic $PLA_2$ (18-20) from pit viper venom classifies the group of N6-$PLA_2$, separately from G6-$PLA_2$ D49 and the existence of two lineages of N6-$PLA_2$, denominated F24 and S24, according to the presence of residue at position 24 of $PLA_2$. In addition to the residue at position 24 that can be distinguished in the N-terminal, the amino acids N1, I11, K14, and T/S23, conserved in the S24-subtype, and the amino acids, H/S1, F11, R14, and A23, conserved in the F24-subtype, can be found (Figure 5).

Apparently, the sequences of the New World's N6-F24-$PLA_2$ are similar to those of agkistrodotoxin from the Asian genus *Gloydius.* The sequences of N6-S24-$PLA_2$, also from the New World, are similar to the sequence of trimucrotoxin from *Protobothrops*, found in the Old World (Figure 6). Therefore, ancient pit vipers found in the Old World and related to the present day *Gloydius* and *Protobothrops*, possibly migrated to the New World and evolved into various species of rattlesnakes.

While proteome analysis has shown the secretion to be non-lethal, transcriptome analysis has until now required sacrificing the specimens to dissect the venom gland for cDNA library construction. Here, we have shown that polyadenylated mRNAs encoding proteins from venom are present in the secreted venom. Therefore, secretory proteome and transcriptome analyses can be performed simultaneously without sacrificing the specimen.

The simple protocol described here should circumvent the need to sacrifice snakes hitherto killed for their venom glands in the pursuit of what are essentially molecular biological approaches to the structural characterization of toxic venom proteins. In addition, the way has been paved to facilitate accelerated acquisition of this information for modern biotechnological applications.

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