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ORIGINAL ARTICLE

Crotacetin, a Novel Snake Venom C-Type Lectin Homolog of Convulxin, Exhibits an Unpredictable Antimicrobial Activity

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Abstract

Snake venom (sv) C-type lectins encompass a group of hemorrhagic toxins that are capable of interfering with blood stasis. A very well-studied svC-type lectin is the heterodimeric toxin, convulxin (CVX), from the venom of South American rattlesnake *Crotalus durissus terrificus.* CVX is able to activate platelets and induce their aggregation by acting via p62/GPVI collagen receptor. By using polymerase chain reaction homology screening, we have cloned several cDNA precursors of CVX subunit homologs. One of them, named crotacetin *(CTC)* β-subunit, predicts a polypeptide with a topology very similar to the tridimensional conformations of other subunits of CVX-like snake toxins, as determined by computational analysis. Using gel permeation and reverse-phase high-performance liquid chromatography, CTC was purified from *C. durissus* venoms. CTC can be isolated from the venom of several *C. durissus* subspecies, but its quantitative predominance is in the venom of *C. durissus cascavella.* Functional analysis indicates that CTC induces platelet aggregation, and, importantly, exhibits an antimicrobial activity against Gram-positive and -negative bacteria, comparable with CVX.

Index Entries: *Crotalus durisssu*s venom; snake venom C-type lectin; crotacetin; convulxin; platelet aggregation; antimicrobial activity.

INTRODUCTION

By molecular cloning and polymerase chain reaction (PCR) homology screening, several snake venom C-type lectin homologs can be isolated that share nucleotide (nt) and predicted amino acid sequences with one another. The conformational topologies of these toxin homologs must present common features, but not necessarily common biological or pharmacological functions. Based on this premise, we used reverse biology to isolate and characterize a novel convulxin (CVX)-like toxin with the ability to aggregate platelets and to inhibit bacterial growth.

Snake venoms are complex mixtures of polypeptides and nonprotein components able to disturb the homeostasis of prey organisms. The venom composition is diverse, differing among species (interspecific venom variations) and individuals (intraspecific venom variations) *(1).* Hereditary and epigenetic factors (e.g., geographical distribution, diet, and snake maturity) contribute to the venom variability *(2–6).* Based on a victim's symptoms of snakebite, snake venom can basically be classified as neurotoxic or hemorrhagic. Neurotoxins act on ion channels and neural receptors *(7).* Hemorrhagic toxins interfere with blood stasis, and major classes of

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these toxins encompass metalloproteases, phospholipases, disintegrins, and C-type lectins (reviewed in ref. *8*). C-type lectins are animal proteins of approx 130 amino acids, containing at least a carbohydrate recognition domain (CRD) capable of mediating sugar and calcium binding. The carbohydrate recognition is directly related to some biological activities, such as cell–cell adhesion, serum glycoprotein turnover, and innate immune responses against potential pathogens *(9).* Snake venom C-type lectins also contain the conserved CRD and share significant primary structure similarities with the animal C-type lectins, but they neither necessarily bind carbohydrate molecules nor require calcium ions for their activity. More than 80 C-type lectin-like proteins that affect thrombosis and hemostasis by inhibiting or activating specific platelet receptors or blood coagulation factors have been characterized from the venom of various snake species *(10).*

A very well-studied snake venom C-type lectin is the heterodimeric toxin CVX, from the venom of South American rattlesnake *Crotalus durissus terrificus (11).* This protein consists of two subunits, α (CVXα, 13.9 kDa) and β (CVXβ, 12.6 kDa), joined by inter- and intrachain disulfide bounds, arranged in a tetrameric $\alpha_4\beta_4$ conformation *(12,13).* CVX activates platelets and induces their aggregation via p62/GPVI collagen receptor *(14).* Some other snake toxins with similar properties of activating and aggregating platelets are also known. They are, for example, bitiscetin, from the venoms of *Bitis arietans (15)*; botrocetin, from *Bothrops jararaca (16)*; flavocetin A *(17)*; ophioluxin, a protein from *Ophiophagus hannah (18),* and mucrocetin, a platelet-agglutinin from *Trimerusurus mucrosquamatus (19).*

Recombinant convulxin (rCVX) has been cloned and expressed in *Drosophila* cells. Purified rCVX from cell culture supernatants binds strongly to human platelet GPVI in Western blot assay when using whole platelet proteins or recombinant human GPVI as target. Importantly, rCVX induces the aggregation of platelets in platelet-rich-plasma, indicating that the recombinant CVX subunits can assemble into a functionally competent complex *(20).*

By PCR homology screening, we have cloned several complementary DNA (cDNA) precursors that are homologous of CVX subunits. In this work, we have characterized one of them, named crotacetin *(CTC),* a new member of snake venom C-type lectin family, with the property of aggregating platelets and with an unexpected antimicrobial activity.

MATERIALS AND METHODS

Snake and Snake Venom

For the construction of venom gland cDNA library, a pair of glands was excised from an adult specimen of *C. durissus terrificus,* captured in São Paulo state and provided by the Laboratory of Herpetology, Instituto Butantan (São Paulo, Brazil). The snake was milked to collect the venom and to induce the maximum level of RNA synthesis in venom gland *(21).* The crude venom was vacuum-dried and kept at –20°C until protein purification. The venom of *C. durissus cascavella* was a gift from the Regional Snake Laboratory of Fortaleza (LAROF), Ceará, Brazil. The venom of *C. durissus collilineatus* was purchased from the Bio-Agents Serpentarium (Batatais, São Paulo, Brazil).

C. durissus terrificus *Venom Gland cDNA Library Construction*

The cDNA library was constructed as described previously *(22).* Briefly, poly(A^+) RNAs were purified from a pair of venom glands excised from a single specimen of South American rattlesnake, *C. durissus terrificus* (*Cdt 9706*). The cDNAs were synthesized, selected by size, and cloned into a phagemide vector—a λ phage derivative (λ ZapII; Strategene, La Jolla, CA). Recombinant phagemids were packed into viable phage particles and used to infect *Escherichia coli* cells XL1 Blue MRF' (Stratagene). The venom gland cDNA library was titrated, amplified, and stored at –80°C, in 7% dimethyl sulfoxide, for posterior utilization.

PCR Homology Cloning

Based on nt sequences of subunit α (or A) and β (or B) of CVX from *C. durissus terrificus* (GenBank accession no. AF541882 and Y16348, CVX subunit α, AF541881 and Y16349, CVX subunit β) one oligonucleotide primer forward CVXA/B-FW1 (5'-TCTCTCTGCAGGGAAG-GAAG-3'), and two reverse primers, CVXA-RV1A (5'-TCCTTGCTTCTCCAGACTTCA-3') and CVXB-RV2B (5'-ACTTCACACAGCCGGATCTT- 3') were synthesized (Invitrogen, São Paulo, Brazil), which correspond to the 5'-untranslated region (UTR) of CVX subunit α and β genes (forward primer), and to 3'-UTRs of CVX subunit α (reverse primer RV1A) and of CVX subunit β (reverse primer RV2B), respectively. The gene of one subunit of CTC (β-subunit) was amplified with the same primer pair used to amplify CVX subunit α, in the following way: phage particles (10^7–10^8 plaque-forming units) of *C. durissus terrificus* venom gland cDNA library, 10 pmol of each primer and 25 μL of ExLONGase enzyme mix (Invitrogen, Carlsbad, CA), were mixed and the PCR was performed according to the manufacturer's instructions. The long-distance–PCR products were purified from the gel slice and cloned into pCR2.1-TOPO (Invitrogen).

The gene was sequenced with ABI Prism Big Dye Terminator (Applied Biosystems, Foster City, CA) in an automated sequencer (ABI Prism 373 or 377; Applied Biosystems) by using synthetic oligonucleotides designed for CVXA/B sequences (this work). The cloned *CTC* mRNA (accession no. AF541884) was compared

against nt sequences in the GenBank at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), using the BLAST algorithm and the Biocomputing software Lasergene (DNAStar, Madison, WI).

Similarity Search and Homology Modeling

The predicted amino acid sequence from the respective *C. durissus terrificus* precursor cDNA was compared against the Protein Data Bank (PDB) (http://www.rcsb.org/pdb/) by using BLASTP, a tool for protein search and alignment *(23).* From PDB, several sequences were chosen as the best score after alignment and used to identify conserved residues in homologous sequences. Snake venom C-type lectin sequences from the venoms of *Trimeresurus flavoviridis* (PDB entry code 1C3A; chain B) *(17), T. mucrosquamatus* (1V4L; chain B) *(19),* and *C. durissus terrificus* (1UOS, chain B; and 1UMR, chain B) *(12,13)* were aligned with ClustalW *(24),* by using default parameters.

To build a tridimensional structure of the CTC β-subunit C-type lectin, 1000 models were generated with PARMODEL, a Web server at http://www.biocristalografia.df.ibilce.unesp.br/tools/parmodel *(25),* which paralyzes the MODELLER *(26)* software in a Beowulf cluster (16 nodes). The best model was selected according to the Modeller objective function and evaluated by PROCHECK *(27),* WHATCHECK *(28),* and 3DANALYSIS *(29).* The permissible angles of amino acid residues in the spatial structure of CTC β-subunit were also evaluated by RAMACHANDRAN plot *(30).*

CTC Purification

Whole venom (35 mg) was dissolved in a 0.2 *M* ammonium bicarbonate (pH 8.0) buffer and then clarified by high-speed centrifugation (4500*g* for 1 min). The supernatant was injected onto a molecular exclusion high-performance liquid chromatography (HPLC) column (Superdex 75; 1 × 60 cm; GE Healthcare, Little Chalfont, Buckinghamshire, UK), previously equilibrated with the same buffer that was used for solubilization of the whole venom. Chromatography process was performed with a flow rate of 0.2 mL/min and monitored at 280 nm. Fractions corresponding to CTC were pooled and lyophilized. After this first step of purification, CTC was repurified by reverse-phase HPLC. Approximately 1 mg of the purified protein was dissolved in the same buffer (buffer A, 0.1% trifluoroacetic acid [TFA] in aqueous solution) for equilibration of the analytical μ-Bondapack C18 column (0.39 × 30 cm). The elution of highly purified CTC was carried out using a linear and discontinuous buffer B gradient (66% acetonitrile in buffer A). The chromatographic run was conducted at constant flow rate of 1.0 mL/min and monitored at 214 nm.

Reduction, S-Carboxymethylation and Determination of N-Terminal Sequence

Two milligrams of purified CTC was dissolved in 200 mL of a 6.0 *M* guanidine chloride solution (Merck, Darmstadt, Germany), containing 0.4 *M* of Tris-HCl and 2 m*M* EDTA, pH 8.15. Nitrogen was blown over the top of the protein solution for 15 min, followed by molecular reduction with 200 mL of a 6.0 *M* dithiothreitol and further carboxymethylated with [^{14} C]iodoacetic acid and cold iodoacetic acid. Nitrogen was again blown over the surface of the solution and the reaction tube was sealed. This solution was incubated in the dark at 37°C for 1 h and desalted in a Sephadex G25 column (0.7 × 12 cm), equilibrated with 1.0 *M* acetic acid buffer. The eluted reduced CTC was then applied on the reverse-phase HPLC μ-Bondapack C18 column (0.39 × 30 cm), previously equilibrated with buffer A (0.1% TFA in aqueous solution). The subunits of CTC were eluted with a nonlinear gradient concentration of buffer B (66% acetonitrile in buffer A), and the fraction corresponding to each CTC subunit was recovered, lyophilized, and stored at –80°C. CTC subunits were then sequenced by automatic Edman degradation, using a gas–liquid protein sequencer (model Precise; Applied Biosystems). The amino acid phenylthiohydantoin (PTH) derivatives were identified using a PTH-analyser (model 450 microgradient; Applied Biosystems).

Platelet Aggregation

Platelet-rich plasma (PRP) was prepared as described by Chudzinski-Tavassi et al. *(31),* and the evaluation of platelet aggregation was performed with a Chrono-Log Lumi aggregometer (Chrono-Log, Havertown, PA). PRP samples were incubated at 37°C for 2 min with different concentrations of purified CTC. Platelet agglutination was monitored by turbidimetry and expressed as an increase of light transmittance. Collagen was used as control of platelet aggregation.

Antimicrobial Activity and Electron Microscopy

Xanthomonas axonopodis pv. *passiflorae* (Gram-negative) or *Clavibacter michiganensis michigansis* cells were harvested from fresh agar plates and suspended in sterile distilled water (A_{650nm} = 0.3/cc 10^3 CFU/mL). Aliquots of bacterial suspension were diluted to a 10^{-5} colony-forming units [CFU]/mL and incubated, during 20 min at 37°C, with the same concentration (150 (g/mL) of purified CTC or CVX. Then, the capacity of microbial cell survival was assayed on nutrient medium (Difco, Detroit, MI) plates (n = 5). In these antibacterial assays, morphological alterations were visualized by

electron microscopy, in absence (control) or presence of CTC or CVX (A_{650nm} = 0.3/cc 10^3 CFU/mL). For this purpose, bacterial samples were fixed with 1% osmium tetroxide (Agar Scientific), for 2 h, at 25°C. Sections were washed three times, dehydrated in increasing concentrations of ethanol and propylene oxide, and embedded in Epon resin (Agar Scientific, Stansted, UK). Polymerization was performed at 60°C for 48 h, and ultra-thin sections were prepared with a Sorvall MT2 ultramicrotome. Sections were placed on 5% collo-dion-coated 100-mesh grids and stained with 4% uranyl acetate (Agar Scientific) for 15 min, followed by 2.6% lead citrate (Agar Scientific) for 15 min. Samples were observed with a Hitachi 1100 transmission electron microscope (Hitachi Scientific Instruments, Tokyo, Japan), operating at 100 kV.

RESULTS

PCR Homology Cloning of CTC β*-Subunit*

The gene coding for *CTC*β (GenBank accession no. AF541884) was isolated from *C. durissus terrificus* venom gland cDNA library by PCR homology screening with specific primers for the 5′-UTR and for 3′-UTR of both *CVX*α and *CVX*β, as described in Materials and Methods.

The cloned *CTCβ* gene is 513-bp long with a 5′-UTR strictly conserved in all CVX-like and antithrombin-like precursors (ATLs) isolated so far (Fig. 1). In *CTCβ* gene, the start codon (ATG) is located 28-bp downstream, and the stop codon (TGA) is located 496-bp downstream. A sequence corresponding to the signal peptide lies between nt 28 and 96 (Fig. 1, underlined).

At the nt level, the similarity between *CTC*β and *CVX*β is higher than between *CTC*β and *CVX*α or *CTC*β or *ATLs*. In contrast, the similarity of *CVX*α and *ATLs* is proportionally high. The *CTC*β gene precursor predicts a polypeptide of 148 residues of amino acids, 23 corresponding to the leader sequence, and a molecular mass of mature subunit of 14.3 kDa.

Sequence Alignment and Tridimensional Modeling

Comparison of the predicted amino acid sequence of *CTC*β gene against protein data bank reveals high similarity of CTCβ protein with C-type lectin subunits from the venom of *T. flavoviridis* (PDB entry code 1C3A; chain B), *T. mucrosquamatus* (1V4L; chain B), and *C. durissus terrificus* (1UOS, chain B; and 1UMR, chain B) (Fig. 2A). The similarity of CTCβ and these other snake venom-aggregating toxins is approx 50%. If conserved substitutions are considered, then the similarity exceeds 70%. In primary structure of CTCβ, the eight residues of cysteine are located in the same position of all aligned sequences. These positions include six residues that are involved in the intrachain disulfide bridges and two other "extra" cysteines in the carboxy (Cys3) and amino (Cys77) terminals. The computer-generated model of CTCβ shows an overall topology of five principal β-strands and two α-helices, forming a globular structure with a lateral loop similar to the other CVX-like C-type lectins (Fig. 2B). The lateral loop contributes to the formation of heterodimers that are stabilized by the extra cysteine residue. Ramachandran diagram of CTCβ model indicates that psi (ψ) and phi (φ) angles of the majority of amino acid residues are located in thermodinamically favorable region (Fig. 2C).

Isolation, Reduction, and N-Terminal Sequence of CTC From C. durissus

By exclusion chromatography, CTC heterodimer seems to be a minor fraction peak in the venom of at least three *C. durissus* subspecies, namely, *C. durissus cascavella, C. durissus terrificus,* and *C. durissus collilineatus* (Fig. 3A). In the venom of *C. durissus cascavella,* the amount of CTC is significantly abundant (representing ~0.8% of the proteins in the crude venom). Thus, CTC was isolated and characterized from this venom. Purified CTC from *C. durissus cascavella* shows an apparent molecular mass of 70 kDa, by gel permeation chromatography, and it appears as two subunits of different size by reverse phase-HPLC (Fig. 3B). Furthermore, by molecular exclusion chromatography, it seems to have a high molecular weight, indicating the presence of oligomeric forms. The partial N-terminal amino acid sequence of purified CTCβ corresponds to that predicted from the gene sequence (Fig. 3C).

Platelet Aggregation Assay

CTC is capable of aggregating human platelets in PRP in a dose-dependent manner: 22% at concentration of 32.8 μg/mL (47 μ*M*), 36% at 49.3 μg/mL (70 μ*M*), and 84% at 65.5 μg/mL (94 μ*M*) (data not shown).

Antimicrobial Activity of Naive CTC, CVX, and Their Isolated α*- and* β*-Subunits*

The oligomeric CTC protein decreases the bacterial growth of two plant pathogens: *X. axonopodis* pv. *passiflorae,* a Gram-negative bacterium and *C. michiganensis michigansis* , a Gram-positive bacterium. The inhibition rates were in the range of 87.8 and 96.4%, respectively. The isolated reduced chains did not show any significant antimicrobial activity (Fig. 4A,B) against both species of tested bacteria. In *Xanthomonas axonopodis* pv. *passiflorae,* CTC induced vacuolation of cell cytoplasm, and, in some cases, ruptured the cell membrane (Fig. 4C,D). Similarly to CTC, reduced CVX subunits failed in

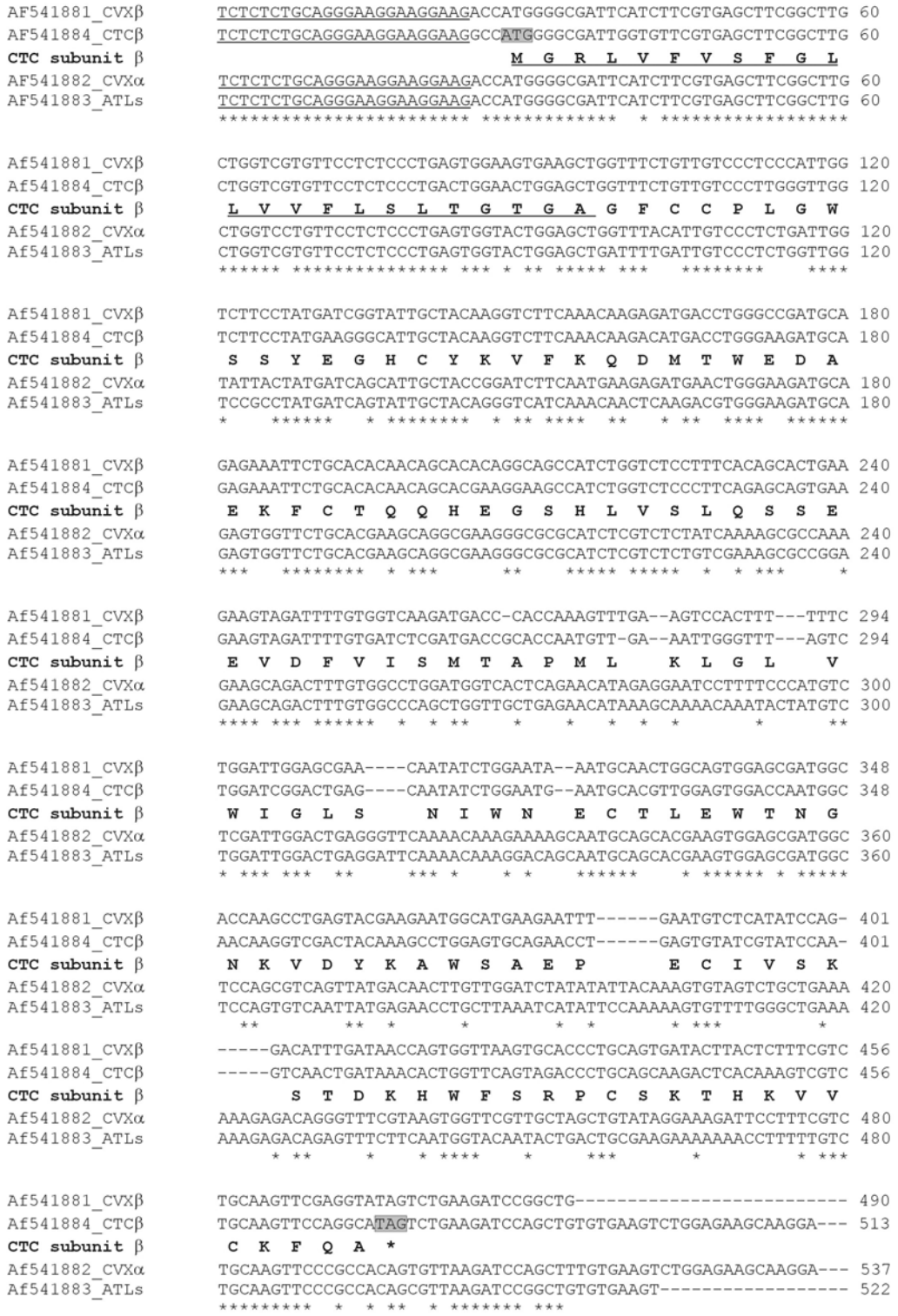

Fig. 1. Comparison of nucleotide sequence of *CTC*β, *CVX*α and β subunits, and antithrombin-like subunit cDNA precursors. All genes were isolated from *C. durissus terrificus* venom gland cDNA phage library by PCR homology screening. Sequences were aligned using ClustalW accessible at http://www.ebi.ac.uk/clustalw/index.html with parameters in default. The numbers in the left side of each precursor correspond to the GenBank accession nos. AF541881, *CVX*β mRNA; AF541882, *CVX*α subunit mRNA; AF541883, antithrombin-like *(ATL)* mRNA; and AF541884, *CTC*β mRNA, all from *C. durissus terrificus.* Conserved 5′-UTRs, where the primer CVXA/B FW exactly anneals, are underlined. Dots represent conserved nucleotides in homologous sequences.

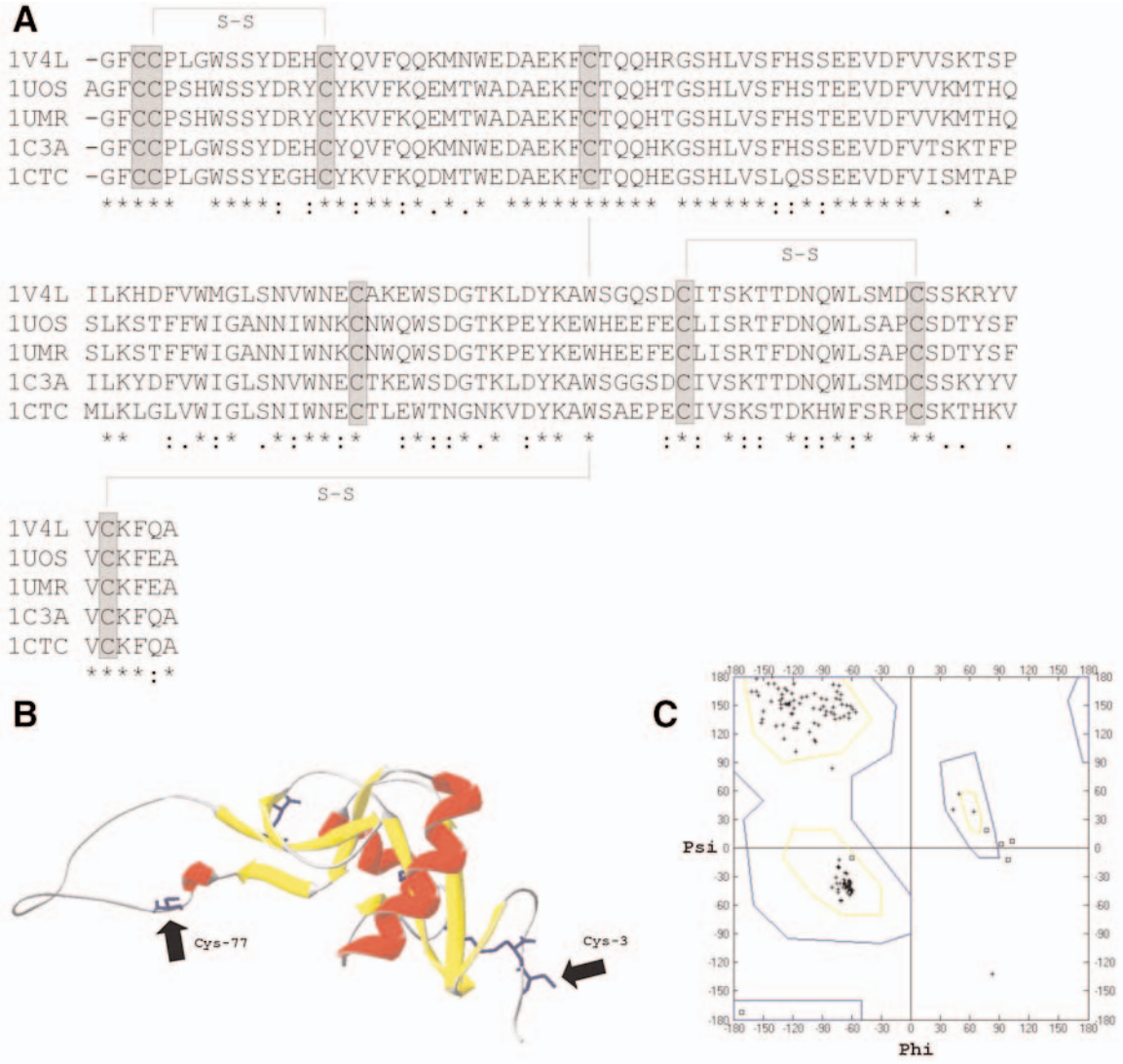

Fig. 2. Structural analysis of CTCβ. **(A)** Comparison of deduced CTCβ amino acid sequence from *C. durissus terrificus* (this work) with other snake venom C-type lectin subunit sequences. The alignment was performed by ClustalW. Amino acid sequences are from *T. flavoviridis* (PDB entry 1C3A; chain B), *T. mucrosquamatus* (1V4L; chain B), and *C. durissus terrificus* (1UOS, chain B; and 1UMR, chain B). Cysteine residues are represented in gray and disulfide bridges by dotted lines. **(B)** Computer generated model of CTCβ. The 3-D structure was generated with PARMODEL, a Web server at http://www.biocristalografia.df.ibilce.unesp.br/tools/parmodel. Nonpairing intrachain, or extra cysteine residues at positions 3 and 77, are indicated by arrows. **(C)** Ramachandran plot of CTC.

inhibiting the bacterial growth of both bacteria, whereas the naïve CVX induced more extensive vesiculation of preferential Gram-positive *C. michiganensis michigansis* (Fig. 5A–D).

DISCUSSION

Using a specific forward primer for the 5′-UTR and reverse for 3′-UTR for both subunits of *CVX*, *CVX*α, and *CVX*β, we have isolated several cDNA precursors homologs of *CVX* from *C. durissus terrificus* venom gland cDNA library. The nt sequence of two of these precursors, specifically of their subunits; both *CVX*α and *CVX*β subunit sequences; and the deduced amino acid sequence of *CTC*β are shown in Fig. 1. These cDNA sequences present conserved nt starting at –27 bp, from the ATG initiation codon, and extending several bases along the precursor. All of the isolated genes, coding for CVX-like subunits, encompass open reading frames of approx 500 nt (*CVX*β, 490 nt; *CTC*β, 513 nt; *CVX*α, 537 nt; and *ATLs*, 522 nt). *CTC*β and *CVX*β genes are almost identical (82% of similarity; 439/537 nt), whereas *CVX*α and *ATLs* genes are more related to each other (~83%; 409/490 nt). When *CTC*β mRNA is compared with other

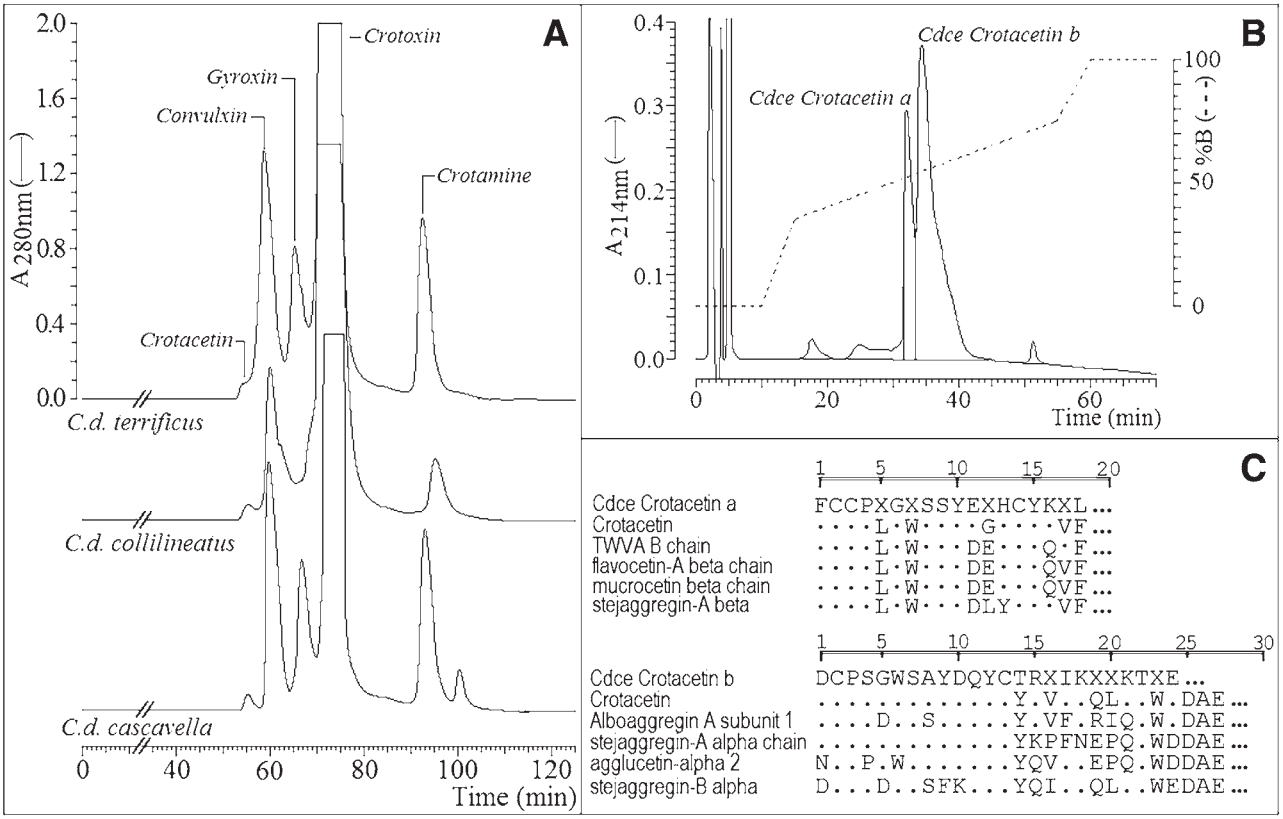

Fig. 3. Isolation, reduction, and N-terminal sequence of CTC from *C. durissus* spp. CTC was purified from the most abundant source, the crude venom of *C. durissus cascavella,* by a combination of gel permeation HPLC (Superdex 75, 1 × 60 cm) and sequential reverse phase HPLC, as described in Materials and Methods. **(A)** Gel permeation chromatography of the crude venom of three different subspecies of *C. durissus* (*C. durissus terrificus, C. durissus collilineatus,* and *C. durissus cascavella*). The peaks corresponding to CTC and other toxins are indicated. **(B)** Reverse phase HPLC (μ-Bond pack C18 column) profile of reduced CTC, where the peaks of the α- and β-subunit are separated. **(C)** N-terminal sequencing of CTCα and β and comparison with predicted sequences (this work) and the alignment with the N-terminal of other snake venom C-type lectins.

sequences in the gene databank, identities of more than 86% are found. For example, mucrocetin β chain mRNA (AY390534), from *Protobhotrops mucrosquamatus,* shares 87% identity (439/537 nt). Almost 200 mRNA sequences of snake venom C-type lectin share some similarity with *CTC*β and each other.

The high homology of *C. durissus* C-type lectin genes, and some other aggregating toxin genes from different snakes, points out their own evolutionary history. These genes seem to have arisen by more than one single event of gene duplication, which probably occurred after the division of Viperidae and Colubridae. The α- and β-subunits of the C-type lectins are restricted to viper and pit viper snakes; therefore, they compose a monophyletic gene clade *(32).* Indeed, phylogenetic analysis of nt sequences of coding region of C-type lectins and C-type lectin-like proteins from snake venom shows that these proteins diverged into three groups: C-type lectins, α-subunits, and β-subunits. Furthermore, α- and β-subunits are branched into three functional groups based on their protein targets, which include coagulation factor, platelet receptor, and α2β1 integrin *(9).*

However, duplication is not the only event for gene evolution. Nucleotide substitution, transversion and transition, and deletion also play some role. It is now evident that genes coding for toxins present extremely conserved noncoding regions, whereas the coding regions are hyperdivergent *(33,34).* In general, these coding regions of toxin genes lie in exons separated from the exon that encompasses the leader sequence of the toxin and its 5′-UTR *(35–40).* This structural gene organization influences not only its own evolution, such as gene duplication and recombination, but also affects protein diversity. Thus, structural gene organization allows accelerated evolution of toxins and polypeptides to operate, and the functional hypervariation of these genes provides an evolutionary advantage for the snakes (*9,41,42*).

Comparison of deduced amino acid sequence of CTCβ and members of snake venom hemorrhagic toxins

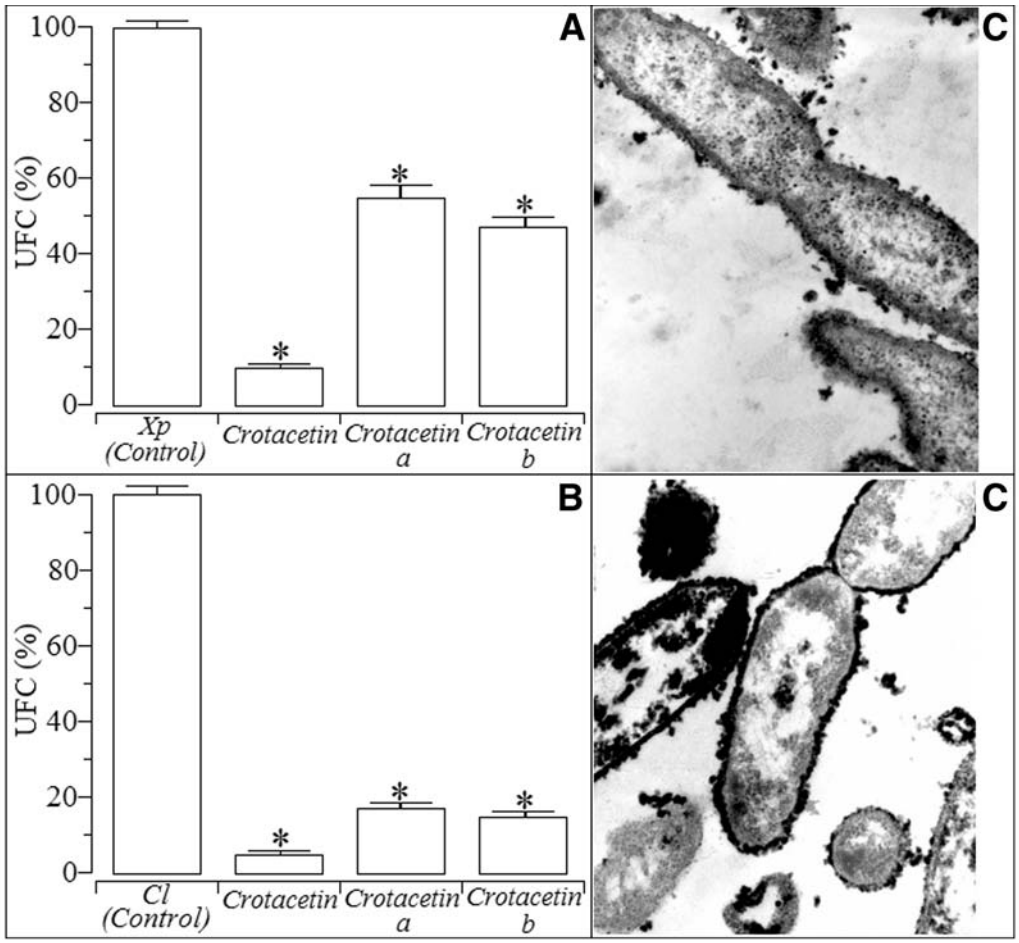

Fig. 4. Antimicrobial activity of native CTC and reduced α- and β-subunits. *Xanthomonas* a. pv. *passiflorae* **(A)** and *C. michiganensis michiganensis* **(B)** were incubated with native crotacetin or with reduced α- and β-subunits for 20 min at 37°C. The reduction of antibacterial activity is compared. Transmission electron microscopy of untreated *X. axonopodis* pv. *passiflorae* **(C)** and treated with crotacetin **(D)** is observed. Vesiculation of bacterial cell is evident.

corroborates a consensual structural characteristic of CVX-like proteins: the eight conserved residues of cysteine. The six cysteine residues are involved in intrachain disulfide bridges, linking at 4–15, 32–121, and 98–113 residues, and the other two cysteines are recruited in the formation of the heterodimer, in a head-to-head manner, resulting in the α_4 β_4 quaternary structure *(12,19).* To explore the structural conformation adopted by CTCβ and to build a tridimensional (3D) model of CTCβ, we used amino acid sequences and coordinate data of other crystalized CVX-like snake venom C-type lectins. It is well known that the two most critical problems in homology modeling are the degree of similarity among target sequences and templates, and evidently the fidelity of the alignment *(26).* In CTCβ, its predicted 3D structural model is practically identical to the experimentally resolved model.

Because CTCβ seems to belong to the class of CVX-like proteins, with the consensual motif of C-type lectin domains, including the CRD, elucidative biological assays with purified CTC were conducted. CTCβ was initially cloned from *C. durissus terrificus* venom gland, but CTC was further found in the venom of various *C. durissus* subspecies, occurring as minor fraction peak in the chromatograms of the venom of *C. durissus cascavella, C. durissus terrificus,* and *C. durissus collilineatus.* In the venom of *C. durissus cascavella,* the amount of CTC is significantly abundant (representing ~0.8% of all proteins in the crude venom). The observation that CTC is expressed in different amounts in the venom of several subspecies of South American rattlesnakes, inhabiting distinct geographical locations, confirms the phenomena of hypervariability widely seen in snake venom composition *(3,4).* Interestingly, even if snake venom C-type lectins are diverse, they are restricted to the venom of

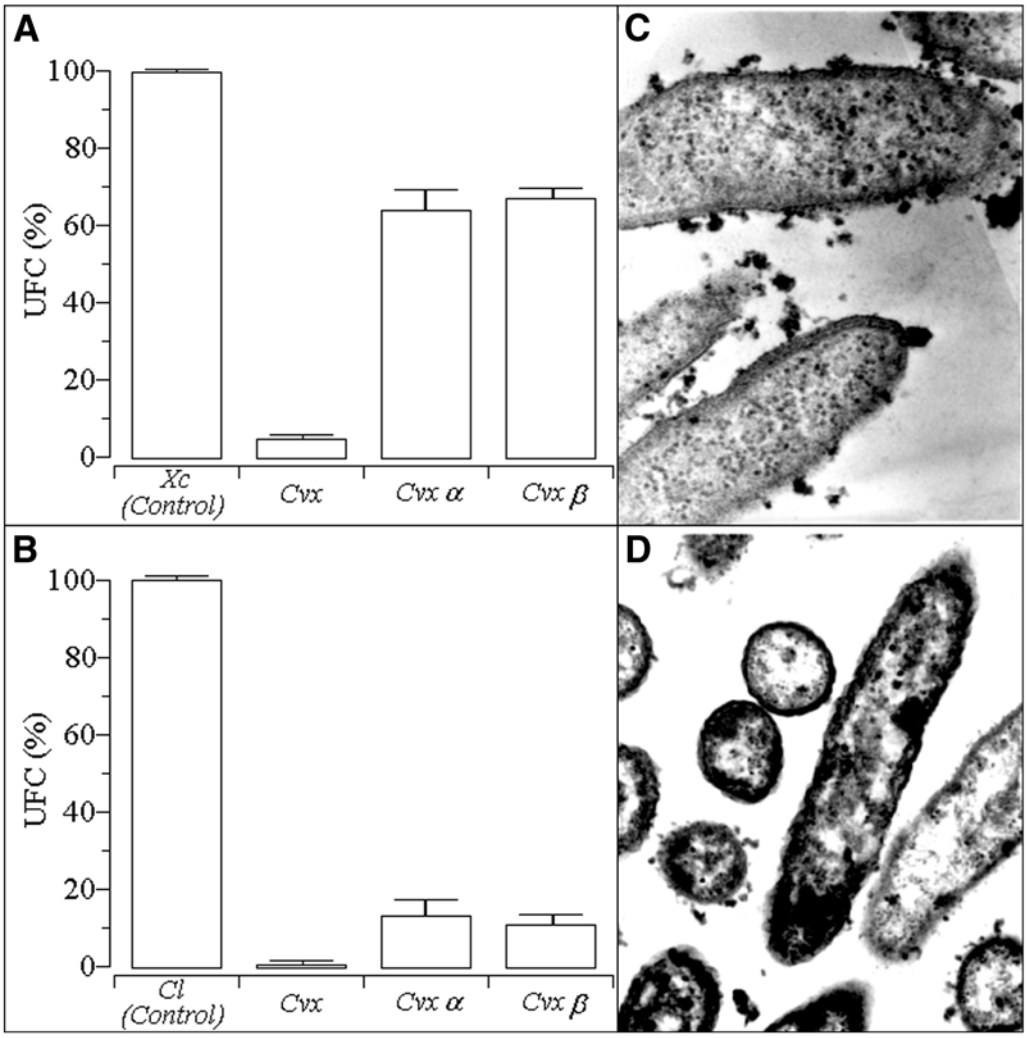

Fig. 5. Antimicrobial activity of native CVX and reduced α- and β-subunits. *X. axonopodis* pv. *passiflorae* **(A)** and *C. michiganensis michiganensis* **(B)** were incubated with native CVX or with isolated α- and β-subunits, as for CTC in Fig. 4. The reduction of bacterial viability is compared in the graphics of **A** and **B.** Transmission electron microscopy of untreated *X. axonopodis* pv. *passiflorae* **(C)** and treated with whole CVX **(D).** In bacteria treated with CVX, high vesiculation of cytoplasm and of membrane are observed. Dark regions in the cytoplasmic membrane are indicative of CVX adhesion.

Viperidae, the family that *C. durissus* species belong to, as discussed above and in more detailed studies *(9,32).*

Thus far, CTC was isolated and characterized from the venom of *C. durissus cascavella.* Purified CTC occurs as two subunits of different size, and by gel permeation a high-molecular-weight oligomeric form protein is evidenced (apparent molecular mass of 70 kDa). By using human PRP, it was observed that CTC is capable of aggregating platelets in a dose-dependent manner, and, in higher doses, the platelet aggregation is more sustainable than collagen. Actually, platelet aggregation is a common phenomenon of snake venom C-type lectins, usually mediated by glycoprotein VI receptor on platelet membranes. However, Kaniji et al. *(43)* demonstrated that in addition to glycoprotein VI collagen receptor, CVX mediates platelet aggregation by binding to native human GPIbα, exhibiting dual specificity to both platelet receptors. Thus, it remains to be verified with what receptors CTC can interact, i.e., whether it has the ability to bind and activate platelet via GPVI, GPIbα, or even other collagen receptors, such as GPIa-IIa ($\alpha_2\beta_1$ integrin). In addition, glycoprotein receptors on erythrocyte membrane might seem like specific molecular targets for CVX-like snake toxins, as we demonstrated in a recent study with recombinant BJcuL, a C-type lectin of *Bhotrops jararacussu* venom *(44).*

Significantly, CTC showed antimicrobial activity against two different bacterial strains, *X. axonopodis* pv. *passiflorae* and *C. michiganensis michiganesis.* The oligomeric form of CTC decreased the overall growth of

both bacteria. However, the separation of intact oligomeric protein into isolated and reduced subunits significantly abolishes the antimicrobial activity of CTC. As mentioned, tetrameric conformation is a typical structure adopted by several snake venom CVX-like C-type lectins, and this quaternary arrangement also seems essential for antimicrobial activity. The microscopic effect of CTC on *X. axonopodis* pv. *passiflorae* involved the induction of cytoplasmic vacuolation and membrane rupture. As surprising as the antimicrobial activity of CTC is, the fact is that CVX, another snake venom C-type lectin and potent agonist of platelet aggregation from the venom of *C. durissus terrificus,* was able to abrogate the bacterial viability of both strains. In this respect, CVX was more effective in decreasing the growth of *C. michiganensis michiganesis,* a Gram-positive bacterium. Similarly to CTC, and in contrast to the corresponding natural oligomeric polypeptide, the reduced CVX subunits failed to restrain the bacterial growth.

From our point of view, these latter results are of particular relevance, because they concern the description of antimicrobial activities of snake venom C-type lectins. The most studied C-type lectin domain-containing proteins with the property of binding to microorganisms are the mannose binding lectins (MBLs) and the lectins receptors (CLRs) located on antigen-presenting cell membranes. MBLs are involved in vertebrate first-line defense by binding to bacteria, viruses, protozoa, and helminths and then initiating a range of host response *(45).* Also, C-type lectin receptors, CLRs, on dendritic cells are type II transmembrane proteins implicated in the pattern recognition of pathogens and in the distinction of self- and no-self antigen recognition in mammals *(46).* Thus, the antimicrobial property of C-type lectins should be further investigated.

Together, it is evident that snake venom C-type lectins have their structural domains derived from a common ancestral precursor. Snake venom C-type lectins comprise not only a multigene family but also homologous polypeptides with diverse biological functions, including the ability to interact with glycoproteins on blood cell membranes, and possibly on microbial cell.

In summary, we report (1) the cloning of several genes for C-type lectin subunits from *C. durissus terrificus,* (2) the isolation and characterization of the gene product of CTC from the venom of three *C. durissus* subspecies, (3) an indication of its predominant presence in the venom of *C. durissus cascavela,* (4) prediction of its 3D structure, and (5) comparable, unexpected antimicrobial activity of CTC and CVX. To our knowledge, this work reports for the first time the antimicrobial activities of snake venom C-type lectin homologs.

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