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# Cloning of serine protease cDNAs from *Crotalus durissus terrificus* venom gland and expression of a functional Gyroxin homologue in COS-7 cells

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ABSTRACT

Gyroxin is one of main serine proteases of *Crotalus durissus terrificus* venom, representing about 2% of the protein content in the crude venom. It is a 33 kDa glycoprotein with 3.8% by weight of sugar moiety. This toxin induces hemotoxicity in mice and a neurological condition called barrel rotation syndrome. In the present work, we report the molecular cloning of five new nucleotide sequences from a cDNA library of the venom glands of a single specimen of *C. d. terrificus*. These sequences have been analyzed in silico with respect to their cDNA organization and similarity with other snake venom serine proteases (SVSPs). We also describe a rapid and efficient method for screening vectors for mammalian cell expression, based on the fact that SVSPs are difficult-to-express toxins due to the presence of several disulfide bonds and glycosylation in their structures. Thus, one of the Gyroxin cDNAs was subcloned into pSectag2 HygroA and pED vectors and used to transfect COS-7 cells. Expression of the functional recombinant Gyroxin isoform was achieved with this cell line with esterase activity in the conditioned culture medium, as revealed by immunoblot of secreted protein and standard anti-crotalic serum from Butantan Institute.



## 1. Introduction

Venoms from snake specimens belonging to the *Crotalinae* subfamily (pit vipers) are very rich in various types of proteases, like metalloproteases and serine proteases. Such enzymes constitute components of the venom that have been preserved throughout the snake evolution, since they are found in species inhabiting all continents. This fact demonstrates the importance of the recruitment of these enzymes for the survival of snakes, like self defense and feeding, as well capture and digestion of preys.

The members of serine proteases family are characterized by a common catalytic mechanism that includes a highly reactive serine residue, which plays a key role in the formation of a transient acylenzyme complex, stabilized by the presence of histidine and aspartic acid residues within the active site (Serrano and Maroun, 2005).

The amino acid sequence identities of these enzymes range from 50 to 80% and the intramolecular disulfide bonds are precisely conserved at the corresponding positions. This similarity is also reflected in the 3D structure, that is folded into two six-stranded β barrels connected by trans segments and two short α-helices (Parry et al., 1998). Despite such high similarities, every venom serine protease shows an exquisite specificity in its biological activity, which has motivated many studies on cloning, sequencing

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and analysis of the structure–function relationships of snake venom serine proteases (SVSPs).

In this work we report the molecular cloning, sequencing and analysis of five new serine proteases cDNA sequences from the venom glands of one specimen of *Crotalus durissus terrificus*.

Until now, only two serine proteases had been isolated from the venom of this snake: Gyroxin (Barrabin et al., 1978) and Thrombin-like enzyme (Raw et al., 1986). In addition to this, Gyroxin had only its N-terminal sequence partially determined (Da Silva et al., 1989) and the nucleotide sequence has not yet been reported. Both enzymes affect hemostasis; besides, Gyroxin has also a characteristic neurotoxic activity – the barrel rotation syndrome (Camillo et al., 2001). The structure–activity relationship as well as the physio or pathological processes involved in this behavior are still unknown. For this reason, the preparation of recombinant Gyroxin constitutes an important approach to obtain homogeneous toxin samples with better batch reproducibility for careful biological assay.

There are few articles in the literature reporting the expression of toxins in eukaryotic cells. The recombinant Batroxobin from *Bothrops atrox moojeni* venom – expressed in *Pichia pastoris*, was able to coagulate plasma in a dose dependent manner. However, the molecular weight was higher than the native protein, so it was supposed that the recombinant Batroxobin might be containing the yeast-type carbohydrate structures (You et al., 2004). Another example of such scarce toxin expressed in mammalian cells is a C-type lectin from *Bothrops jararaca*. Bothrojaracin from *B. jararaca* venom was expressed transiently in COS cells and the secreted toxin was able to bind to and inhibit thrombin (Arocas et al., 1997). Up to now, none SVSPs has been expressed successfully in mammalian cell systems.

In this work, we present data concerning to molecular cloning of five novel cDNA precursor homologues of *C. d. terrificus* Gyroxin. In addition, we report the effective transfection of COS-7 cells with mammalian expression vectors containing the cDNA sequence coding for one of the Gyroxin-like homologues. The transient expression of such Gyroxin homologue in COS-7 cells proved to be a rapid and efficient method for screening plasmid constructs that drive high level expression of SVSPs in mammalian cells. Thus far, COS-7 cells transfected with either pSecTag2-Gyro or pED-Gyro vectors produced recombinant Gyroxin which was secreted into the medium with the expected molecular mass and preserved antigenic properties. Moreover, Gyroxin was a fully active product as demonstrated by esterase activity.

## 2. Material and methods

### 2.1. Materials

The pED vector, kindly provided by Dr. R.J. Kauffman (Howard Hughes Medical Institute, University of Michigan). UniZAP XR expression vector was from Stratagene (LaJolla, CA, USA). pCR2.1 TOPO vector and *Escherichia coli* TOP 10 competent cells; pSecTag2 HygroA vector; DMEM High Glucose; Lipofectamine 2000 were from Invitrogen Life Sciences (CA-USA) and Penicillin, Streptomycin, and L-glutamine were from Gibco. Fetal bovine serum was from Cultilab (Campinas, SP, Brazil). T4 DNA ligase 6.2 U/mL, XhoI 14 U/μL, Chemiluminescence chemistry kit – ECL, Marker is Low-Range Rainbow Molecular Weight Marker (45 K to 2.5 KDa), Nitrocellulose membrane (Hybond-ECL) and Benzamidine Sepharose 6B resin were from GE Healthcare (Fairfield, CT USA). Toluil-arginine methyl ester (TAME) and Porcine pancreas Trypsin were from Sigma

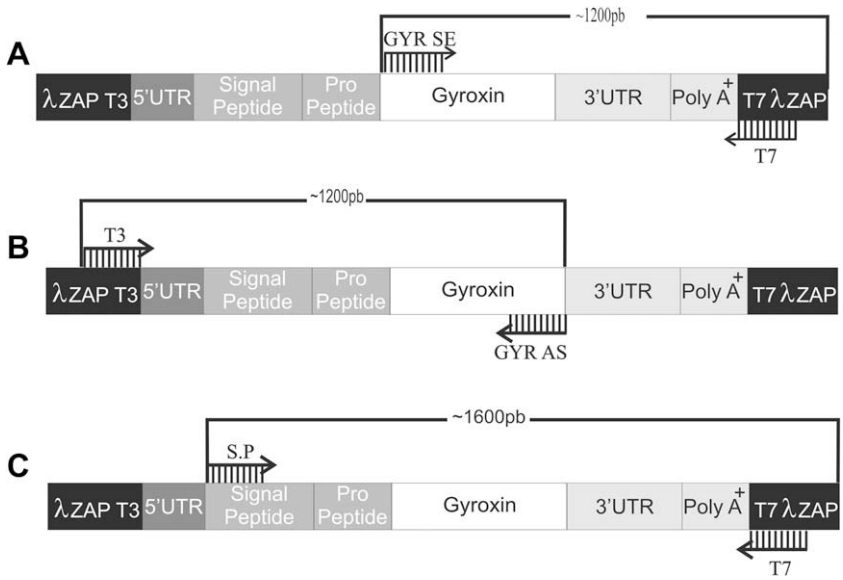



**Fig. 1.** Cloning strategy of Gyroxin-like sequences. PCR amplification of cDNAs clones with three steps (A, B and C) using the following primers: A – GYR SE sense and T7 antisense, obtaining B2.1 clone, B – T3 sense and GYR AS antisense, to sequence the region of cDNA coding for signal peptide, C – SP signal peptide sense and T7 antisense, obtaining B1.3, B1.4, B1.5 and B1.7 clones.

(St Louis, MO, USA). Other restriction endonucleases and modifying enzymes were: Klenow DNA polymerase I 10 U/μl and SmaI 10 U/μL (Fermentas); Nhe I 10 U/μl and Xba I 14 U/μL (USB); Not I 10 U/μL (Jena Bioscience); Spe I 10 U/μL (Q.Bio gene); Taq DNA polymerase Platinum 5 U/μL (Invitrogen Life Sciences, CA, USA). *C. d. terrificus* venom and standard anti-crotalic serum were kindly provided by Butantan Institute (State Secretary of Health, Sao Paulo, Brazil). All the other reagents were of analytical or molecular biology grade.

### 2.2. Method

#### 2.2.1. Molecular cloning of serine proteases and Gyroxin-like from cDNA library

The cDNA library of the venomous glands of a single specimen of *C. d. terrificus* was constructed by using the UniZAP XR expression vector (Rádis-Baptista et al., 1999). The Gyroxin-like cDNAs were retrieved from this cDNA library by (high fidelity) PCR with the primers GYR SE and T7 according to Fig. 1A. The primer GYR SE (5′- GTCATTG-GAGGTG ATGAATGTAAC -3′) is a sense primer that was synthesized based on the N-terminal sequence of Gyroxin from *C. d. terrificus* venom (Da Silva et al., 1989), and the primer T7 is a universal primer with antisense orientation downstream to multiple cloning site (MCS) of UNIZAP XR vector.

In order to sequence the region of cDNA coding for the signal peptide sequence of Gyroxin-like, primers T3 sense and GYR AS were used (Fig. 1B). T3 primer is a universal primer with sense orientation that is complementary to upstream to (MCS) UNIZAP XR vector. Primer GYR AS (5′- CGAGGGGCAATTCACAGT-3′) is an antisense primer complementary to the C-terminal region of Gyroxin-like B2.1 clone.

For the purpose of cloning the full length cDNA of Gyroxin, the cDNA library served as a template for the amplification of Gyroxin sequences with the primers SP signal peptide sense and T7 antisense (Fig. 1C). The signal peptide-coding sequences of Gyroxin-like and other SVSPs were aligned and the primer SP coding for a region of signal peptide was synthesized based on a conserved region of the 6 first amino acids codons of signal peptide (5′- ATG GTGCTGATCAGAGTG -3′). The PCR product was cloned into pCR2.1 TOPO and the recombinant clones were sequenced using DyEnamic ET Dye Terminator chemistry for Mega-BACE 1000, according to dideoxy chain termination method (Sanger et al., 1977). The analyzed DNA sequence traces of all clones had base calling PHRED quality score higher than 30.

#### 2.2.2. Homology search and sequence comparison

Similarity of cDNAs sequences was performed using the program BLAST (Basic Local Alignment Search Tool). These cDNAs sequences and mature toxins of the snake venom serine proteases were aligned by using MACAW program, with the matrix Blosum 62 (Schuler et al., 1991). The coding region for signal peptide, propetide and mature toxins was analyzed by SignalP 3.0 program (Bendtsen et al., 2004) and checked by alignment with precursors and mature toxins.

The N-glycosylation sites of cDNAs sequences were predicted by NetNGlyc Server, which examine the sequence context of Asn-Xaa-Ser/Thr sequons (Johansen et al., 2006).

#### 2.2.3. Data analysis based on sequences and biological functions

The alignment of cDNAs sequences was used to calculate a distance matrix with DNAdist from PHYLIP 3.6 program (Felsenstein, 1989), using the category model of substitution F84. A dendrogram was drawn by a neighbor-joining method (Saitou and Nei, 1987) shown in Fig. 2.

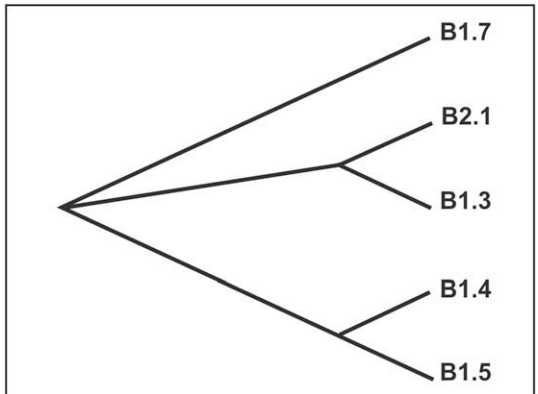
The alignment of 34 snake venom serine proteases corresponding to the mature form of toxins was used to construct a protein dendrogram by Neighbor-Joining method with bootstrap test (Felsenstein, 1985) conducted in MEGA4 (Tamura et al., 2007) shown in Fig. 5.

#### 2.2.4. Construction of expression vectors

The mature toxin Gyroxin-like B2.1 (pCR2.1 Gyro) was subcloned into the vector pSecTag2 HygroA containing the sequences of murine Igκ-chain V-J2-C signal peptide. Purified pSecTag2 HygroA vector was digested with the restriction enzyme XhoI, by treatment with Klenow fragment of DNA polymerase I to create blunt end followed by digestion with the enzyme NotI in order to obtain a cohesive end.

Purified pCR2.1 Gyro was digested with the restriction enzyme SpeI, followed by treatment with Klenow fragment of DNA polymerase I to create downstream blunt end and by digestion with the enzyme NotI. The DNA fragment (0.9 kb) containing the Gyroxin cDNA with upstream cohesive end and a downstream blunt end, was cloned in pSecTag2 HygroA vector with the enzyme T4 DNA ligase.

Removal of nucleotides located between the leader sequence and the codon for the first amino acid of Gyroxin




| | B2.1 | B1.3 | B1.4 | B1.5 | B1.7 |
|---|---|---|---|---|---|
| B2.1 | 100.00 | 99.55 | 94.40 | 94.86 | 84.04 |
| B1.3 | 99.55 | 100.00 | 96.14 | 96.67 | 88.45 |
| B1.4 | 94.40 | 96.14 | 100.00 | 99.76 | 88.57 |
| B1.5 | 94.86 | 96.67 | 99.76 | 100.00 | 89.57 |
| B1.7 | 84.04 | 88.45 | 88.57 | 89.57 | 100.00 |

**Fig. 2.** Similarity of cDNAs sequences of clones B2.1, B1.3, B1.4, B1.5 and B1.7. The table shows the similarity of percentage among the clones amplified from *Crotalus durissus terrificus* venom gland. Dendrogram was made from the alignment of cDNA sequences using PHYLYP 3.6 program.

was performed using site-specific mutagenesis by the method of Kunkel (Kunkel, 1985). The 5′-phosphorylated oligonucleotide used in the mutagenesis was 5′-CATCACCTCCAATGACGTCACCAGTGGAACC-3′. The undigested plasmids, thus lacking the sequences for the restriction enzymes NotI and EcoRV, located in the mutated sequence, were sequenced and this vector was denominated pSecTag2-Gyro.

This vector pSecTag2-Gyro was used as a cassete donor to generate a fragment Igκ signal peptide plus Gyroxin-like cDNA to be subcloned in pED expression vector. To achieve that, the purified pSecTag2-Gyro vector was digested with the restriction enzyme XhoI, followed by treatment with Klenow fragment of DNA polymerase I and by digestion with the enzyme NheI, to obtain a DNA fragment (0.9 kb) containing the Igκ-Gyroxin cDNA with upstream cohesive end and downstream blunt end to be subcloned into pED vector.

The purified pED vector was digested with the restriction enzyme SmaI (that generates blunt end) and the XbaI (that generates cohesive end) which was compatible with cohesive end of the cassete Igκ-Gyroxin was obtained previously. The pED vector and the cassete Igκ-Gyroxin were ligated using the T4 DNA ligase enzyme.

The pED-Gyro construct containing the murine Igκ signal peptide was transformed into *E. coli* TOP 10 and the plasmid construction was confirmed by restriction analysis.

#### 2.2.5. Transfection of COS-7 cells and transient expression of B2.1 Gyroxin

The COS-7 cells were maintained in DMEM culture medium, supplemented with 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamine and 10% fetal bovine serum. Transfections were carried out with 20 μg of the purified pSecTag-Gyro and pED-Gyro vectors, in 50 $cm^2$ culture plates, containing $2 \times 10^6$ cells (85% of confluence), using the Lipofectamine 2000 reagent, according to the manufacturer.

Transfected COS-7 cells were propagated and the conditioned culture medium was collected after 24 h, harvested and stored for further analysis. After 24 h of transfection, the cells were trypsinized and stored in 10% DMSO, under liquid nitrogen. As negative controls, the conditioned culture medium of untransfected COS-7 cells was used. All samples were stored at −80 °C.

Data of our laboratory shows that the native Gyroxin at a concentration of 120 μg/mL was shown to be not cytotoxic for CHO-K1 cells in a cytotoxicity assay. The absence of cytotoxicity enabled COS-7 cells to express recombinant Gyroxin.

#### 2.2.6. Gel electrophoresis and immunoblot analysis

The recombinant Gyroxin expressed by pED-Gyro and pSecTag2-Gyro vectors in extract of COS-7 cells and untransfected cells (negative control) was resolved by SDS-polyacrylamide gel electrophoresis (15% T/2.6% C), under reducing conditions (Laemmli, 1970). Native Gyroxin (0.5 μg and 5 μg) purified from *C. d. terrificus* venom was used as a positive control.

Concomitantly, recombinant Gyroxin previously purified from conditioned culture medium of COS-7 cells transfected with pED-Gyro by affinity resin Benzamidine Sepharose 6B was also run on electrophoresis and native Gyroxin (0.05 μg and 0.5 μg) purified from *C. d. terrificus* venom were used as a positive control.

The proteins in the gel were either stained with Coomassie brilliant blue R-250 or transferred to nitrocellulose membranes for immunoblotting. Thereafter, the membranes were incubated for 12 h, at room temperature, with an aliquot of horse anti-crotalic serum from Butantan Institute diluted 1:1000 in PBS containing 10% of defatted milk. The Gyroxin immunocomplexes were detected with anti-horse conjugated to horseradish peroxidase (1:2000 in PBS/10% defatted milk), as secondary antibody, and with enhanced chemiluminescence chemistry kit – ECL.

#### 2.2.7. Purification of recombinant Gyroxin B2.1 and esterase activity assay

Recombinant B2.1 Gyroxin was purified by Benzamidine Sepharose 6B affinity chromatography as previously described (Camillo et al., 2001). Benzamidine Sepharose 6B resin was activated with 100 mM Tris–HCl, pH 8.5, 400 mM NaCl, and 100 mM Sodium Acetate, pH 4.5, 500 mM NaCl.

Basically, conditioned culture media over 24 h of COS-7 cells transfected with pED-Gyro, supernatant of COS-7 cells lysate transfected with the same vector, conditioned culture medium of untransfected COS-7 cells and supernatant of untransfected COS-7 cells lysate (negative controls), were run on Benzamidine Sepharose 6B affinity previously activated. The incubation proceeded at 4 °C, overnight, followed by centrifugation (700 × g, 4 °C, 5 min) of resin batches which were washed three times with 50 mM Tris–HCl, pH 9.0, 400 mM NaCl. Gyroxin-like B2.1 was eluted with small volumes of 100 mM Ammonium Acetate, pH 5.0 and the purified recombinant toxin was dried under vacuum.

The enzymatic activity of recombinant B2.1 Gyroxin was determined by the method described by Nolan et al. (1976). Samples of 2 μg of purified toxin, as described previously, were solubilized in 80 mM Tris–HCl, pH 8.1, 20 mM $CaCl_2$, and incubated with 1 mL of TAME substrate solution (2.5 mM). Porcine pancreas Trypsin and native *C. d. terrificus* Gyroxin (2 μg) were used as control. The enzymatic reaction proceeded for 5 min, at room temperature, and the absorbance was measured at 247 nm. One unit of esterase activity was defined as 1 μmol of product formed per minute, at room temperature.

## 3. Results and discussion

### 3.1. Cloning of serine proteases and Gyroxin-like from cDNA library

Five different cDNA precursors of *C. d. terrificus* Gyroxin-like serine proteases were cloned, sequenced and analyzed – clones B1.3, B1.4, B1.5 and B1.7 (GenBank accession numbers EU360951; EU360952; EU360953; and EU360954, respectively). In case of clone B2.1, only a partial sequence was obtained (GenBank accession number AY954040). All clones were alignment as shown in Appendix.

The comparison of nucleotide sequences (Fig. 2) showed that the highest similarity 99.55% is shared between B2.1

and B1.3 clones. B1.4 and B1.5 clones, grouped in other branch of dendrogram, showed respectively 94.4% and 94.86% of similarity with B2.1 clone. The B1.7 clone is more divergent than the others, reaching 84.04% of similarity with B2.1 clone. In spite of B1.5 clone showing 99.76% of similarity with B1.4, it represents a truncated cDNA precursor, with a deletion of 146 bases (corresponding to residues 157–205 of Gyroxin-like B1.3) and this deleted region corresponds to exon 4 of the Batroxobin gene (Itoh et al., 1988). The end of exon 3 was probably joined to the beginning of exon 5. Similar observation of the presence of truncated or alternatively spliced cDNA species has also been reported (Wang et al., 2001). In snake venom gland, this mRNA probably would not be translated in a functional protein and, accordingly, it was not analyzed in the functional dendrogram.

The alignment with other SVSPs (Fig. 3) demonstrated that the deduced amino acid sequences of clones B2.1, B1.3, B1.4, and B1.7 share the conserved catalytic triad formed by the amino acids His, Asp and Ser and six disulfide bonds, except clone B1.5 because this clone is truncated by the insertion of a stop codon in translated sequence at the position 472 bp due to the joining of the exon 3 and exon 5.

In Fig. 4, the alignment of nucleotide sequences from B1.3 and B2.1 clones revealed 3 mutations in nucleotides resulting 3 amino acids substitutions in putative toxins. However, at the position 12 bp of B2.1 sequence a silent mutation (A → T) was introduced by the primer GYR SE sense used in PCR amplification.

Clone B1.3 contains two consensus motifs for hypothetical poly($A^+$) signals (5′- AATAAA -3′) at positions 929 and 1380 bp, whereas the B2.1 sequence contains only the

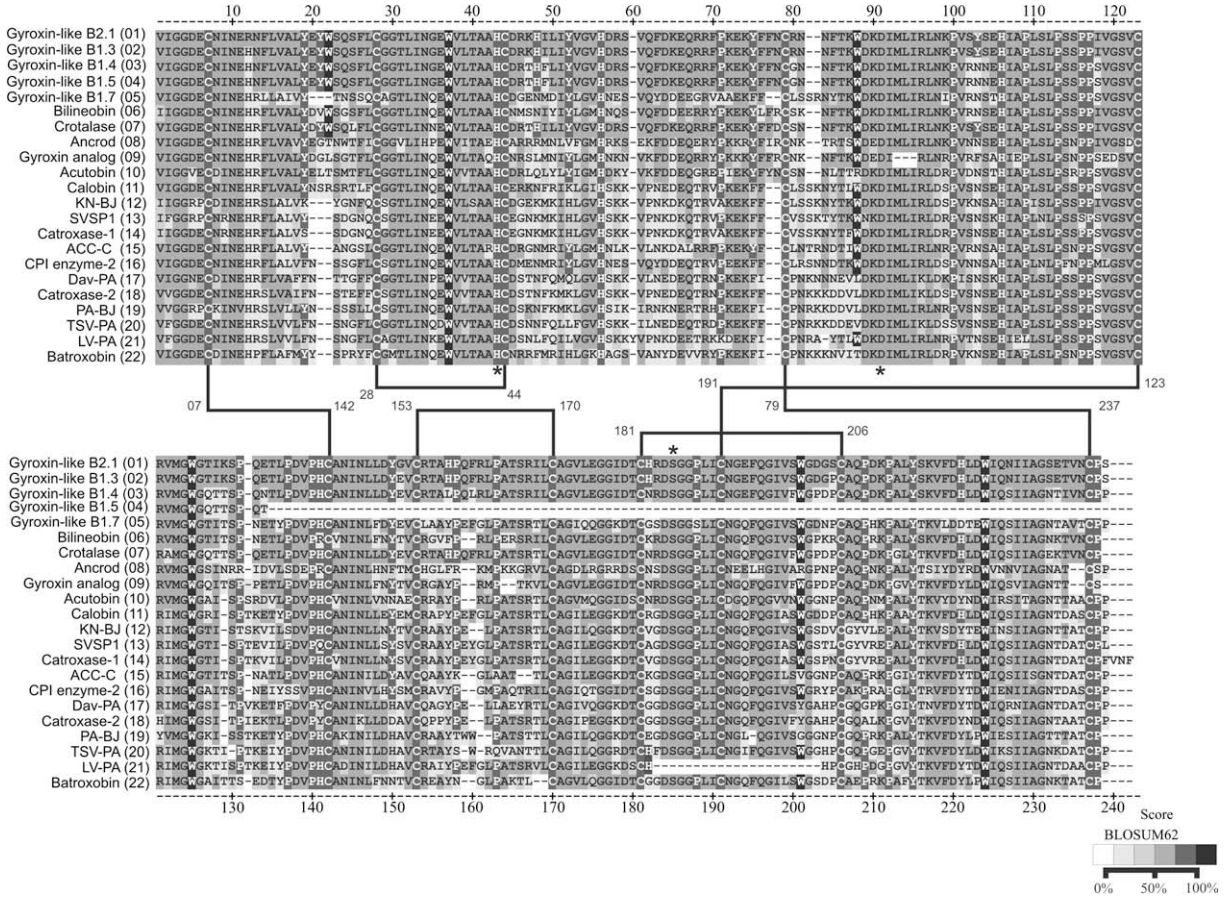



**Fig. 3.** Alignment of snake venom serine proteases. 1) Gyroxin-like B2.1 from *Crotalus durissus terrificus* (Q58G94), 2) Gyroxin-like B1.3 from *Crotalus durissus terrificus* (B0FXM1), 3) Gyroxin-like B1.4 de *Crotalus durissus terrificus* (B0FXM2), 4) Gyroxin-like B1.5 de *Crotalus durissus terrificus* (EU360953) 5) Gyroxin-like B1.7 from *Crotalus durissus terrificus* (B0FXM3), 6) Bilineobin from *Agkistrodon bilineatus* (Q9PSN3) (Nikai et al., 1995) 7) Crotalase from *Crotalus adamanteus* (Henschen-Edman et al., 1999), 8) Ancrod from *Agkistrodon rhodostoma* (P26324) (Burkhart et al., 1992) 9) Gyroxin analog from *Lachesis muta muta* (P33589) (Magalhaes et al., 1993), 10) Acutobin from *Agkistrodon acutus* (Q9I8X2) (Wang et al., 2001); 11) Calobin from *Agkistrodon caliginosus* (Q91053) (Hahn et al., 1996); 12) KN-BJ from *Bothrops jararaca* (O13069) (Serrano et al., 1998), 13) SVSP-1 Venom serine proteinase from *Crotalus adamanteus* (Q8UUK2), 14) Catroxase-1 from *Crotalus atrox* (Q8QHK3), 15) ACC-C Protein C activator from *Agkistrodon contortrix contortrix* (P09872) (Mcmullen et al., 1989), 16) CPI enzyme from *Agkistrodon caliginosus* (O42207) (Hahn et al., 1998); 17) Dav-PA from *Agkistrodon acutus* (Q9I8X1) (Wang et al., 2001), 18) Catroxase-2 from *Crotalus atrox* (Q8QHK2) (Bjarnason et al., 1983), 19) PA-BJ from *Bothrops jararaca* (P81824) (Serrano et al., 1995), 20) TSV-PA from *Trimeresurus stejnegeri* (Q91516) (Zhang et al., 1995), 21) LV-PA from *Lachesis muta muta* (P84036) (Sanchez et al., 2000), 22) Batroxobin from *Bothrops atrox* (P04971) (Itoh et al., 1987). Indicated accession numbers are from Swissprot. The lines indicate the disulfide bonds and the catalytic triad (His, Asp and Ser) is represented by *.

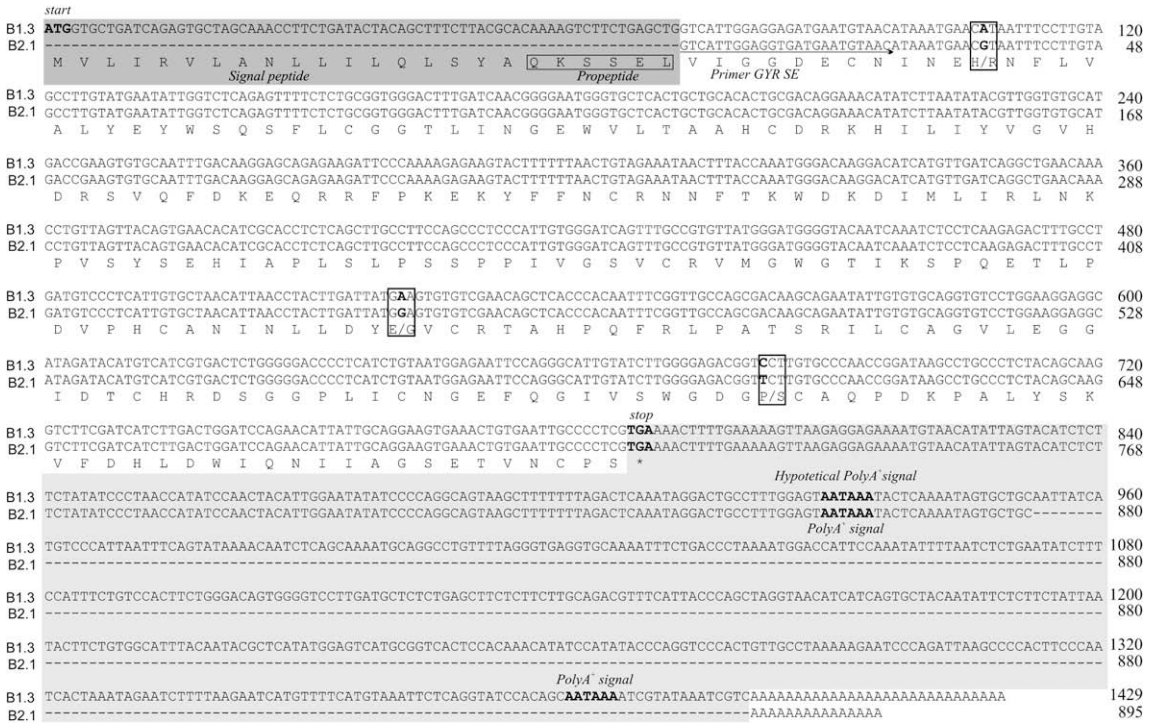



**Fig. 4.** Alignment of nucleotide sequences from clones B1.3 (EU360951) and B2.1 (AY954040). Coding region for signal peptide and propeptide is indicated in dark grey. Start codon and stop codons are in bold. The mature coding region is indicated in white. The mutations between B1.3 and B2.1 sequences are indicated by a box line and differences in nucleotides are in bold. Light grey encompasses the 3′UTR. B1.3 hypothetical poly A$^+$ signal (929–934 bp), B1.3 poly A$^+$ signal (1380–1385 bp) and B2.1 poly A$^+$ signal (857–862 bp) are in bold. Dashes represent gaps introduced for optimal sequence alignment. The sequence of the primer GYR SE is underlined by an arrow.

first poly(A$^+$) signal at the position 857 bp and has a shorter 3′UTR and poly(A$^+$) tail.

The presence of short and long 3′UTRs was also described for myogenin, XmyogU1 and XmyogU2 from *Xenopus laevis* (XmyogU2) (Charbonnier et al., 2002) that contains one and two consensus motifs for a poly(A$^+$) signal, respectively. These results suggest the presence of at least, two different poly(A$^+$) signals in XmyogU2, generating two transcripts with different 3′ ends. Similarly, the presence of two signals of polyadenylation in Gyroxin-like B1.3 clone, suggests that two mRNAs could be transcripted with longer or smaller 3′UTR.

### 3.2. Dendrogram of serine proteases based on mature toxin sequences

Some SVSPs affect pathways of the coagulation cascade by activating specifically blood components involved in blood coagulation, fibrinolysis and platelet aggregation. These thrombin-like enzymes have both fibrinogenolytic and fibrinolytic activities, but many of them cleave only fibrinogen, releasing preferentially either FPA or FPB or both FPA and FPB, what promotes coagulation (Serrano and Maroun, 2005).

In order to group the predicted Gyroxin-like serine proteases in relation to other SVSPs, a dendrogram was generated based on the amino acid sequence and function of each SVSPs shown in Fig. 5.

The group I, Thrombin-like enzymes group contains SVSPs that cleave fibrinogen and is divided into subgroups. The mature toxins encoded by Gyroxin-like B2.1, B1.3, B1.4 homologues belong to subgroup Ia that contains most of the well-known venom clotting enzymes that convert fibrinogen into a fibrin clot, e.g. Batroxobin, Ancrod, Crotalase and Acutobin. These toxins are characterized by being highly glycosylated Aα fibrinogenases.

The mature peptide codified by Gyroxin-like B1.7 clone belongs to the subgroup Ib including toxins like CPI enzyme and Protein C. The CPI enzyme-2 (capillary permeability-increasing enzyme-2) was isolated from *Agkistrodon caliginosus* venom and liberates permeability-increasing peptides from rabbit fibrinogen (Shimokawa and Takahashi, 1997). Protein C was isolated from *Agkistrodon contortrix contortrix* (Stocker et al., 1987) and denominated ACC-C (commercialized as Protac). Its activation was measured by the prolongation of the activated partial thromboplastin time due to the proteolytic degradation factors Va and VIIIa by activated protein C.

The subgroup Ic – comprises some kininogenases like KN-BJ that have been characterized as being capable of cleaving kininogen to produce bradykinin, leading to a hypotensive effect (Serrano et al., 1998). Halystase and Calobin had been described as weakly clotting molecules, cleaving both the Aα and Bβ fibrinogen chains (Wang et al., 2001).

The group II encompasses Plasminogen activators and contains the TSV-PA toxin, which facilitate the solubilization of fibrin clots.

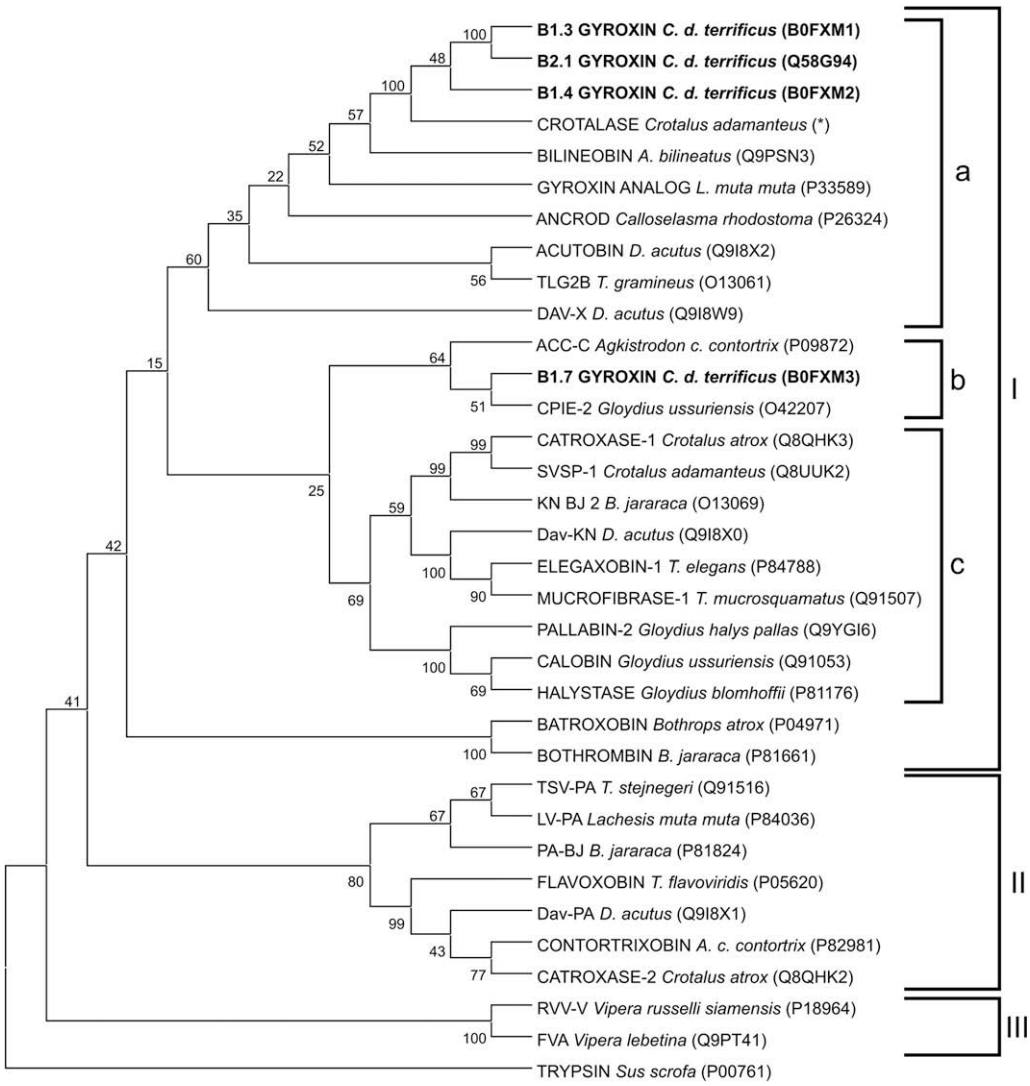



**Fig. 5.** Dendrogram of 34 snake venom serine proteases mature toxins. Toxin names were indicated in bold followed by snake species in italic and the Swissprot accession numbers were represented in parenthesis. (*) Crotalase toxin sequence was based on Henschen-Edman et al. (1999). All positions containing gaps and missing data were eliminated from the dataset. There were a total of 188 positions in the final dataset. The distance was calculated by number of amino-acid differences. The optimal tree with the sum of branch length = 796.39 and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches.

The group III is related to Factor V activators, whose thrombin activity is mimicked by SVSPs. RVV-Vα was shown to activate Factor V to the same extent as α-thrombin. It mediates Factor V activation by single peptide bond cleavage, generating the heavy and light chains of Factor Vα (Tokunaga et al., 1988).

### 3.3. Expression and purification of a recombinant Gyroxin B2.1

Usually, snake venom toxins are polypeptide chains with several disulfide bonds. Most of them form insoluble inclusion bodies when expressed in *E. coli*, as in the case of Batroxobin from *B. atrox* (Maeda et al., 1991) and Acutin from *Agkistrodon acutus* (Pan et al., 1999).

In the case of serine protease (Tm-5) from *Trimeresurus mucrosquamatus* expressed in *E. coli*, *in vitro* renaturation of the fusion protein from inclusion bodies produced a yield of only 2% of an active fibrinogenase, indicating the low efficiency in the refolding process (Hung and Chiou, 2000).

Gyroxin purified from the venom *C. d. terrificus* was shown to contain a sugar moiety of 3.8% by weight (Camillo et al., 2005). The recombinant Gyroxin B2.1 can be glycosylated only at Asn 81 and the presence of the sugar seems to be important for the selectivity of its target, and consequently for biological activity.

Due to the presence of 12 cysteine residues in the cDNA of Gyroxin B2.1 and the fact that the native Gyroxin is glycosylated, this toxin was expressed in eukaryotic system (COS-7 cells) due to its potential to make post-translational

modification (PTM) like the proteolytic processing, glycosylation of precursors and secretion to the culture medium.

There are two SVSPs, AaV-SP-I and AaV-SP-II from the venom of *Agkistrodon acutus*, that have putative glycosylation sites at Asn 35. The proportions and types of carbohydrates within these two proteases are: 9% for AaV-SP-I formed by trisaccharide $NAG^{301}$-$FUC^{302}$-$NAG^{303}$ molecule and 4% for AaV-SP-II formed by monosaccharide $NAG^{301}$ molecule. It was seen that AaV-SP-I has higher amidolytic activity than AaV-SP-II, and the distinct saccharides have different influence on the structure of the active site (Zhu et al., 2005). Therefore, alteration of amidolytic activity can result from different types and proportions of carbohydrates in the toxins.

The thrombin-like enzymes, Ancrod from the *Agkistrodon rhodostoma* and Batroxobin from *B. atrox moojeni* venom, are N-glycosylated and contain about 31% and 5.85% (by mass) in neutral carbohydrates respectively (Lochnit and Geyer, 1995). Interestingly, both toxins have hemotoxic effect, but are sold commercially as defibrinogenolytic agents: Ancrod (Arvin™) and Batroxobin (Defibrase™) (Koh et al., 2006). However, differently from Batroxobin, Ancrod induces the barrel rotation syndrome (Alexander et al., 1988).

Then, for the expression of recombinant SVSPs, eukaryotic systems should be the method of choice. Hence, we decided to express Gyroxin transiently in COS-7 cells in order to select the vector construct which promotes the highest level of production of this toxin. Gyroxin was cloned into pSecTag2 HygroA and pED vectors and transfected individually in COS-7 cells and recombinant Gyroxin was detected by immunoblotting using anti-crotalic serum from Butantan Institute in transfected COS-7 cells total extract and conditioned culture medium.

The Fig. 6A and B shows a western blot of COS-7 cells extract and Fig. 6C and D shows a SDS-PAGE and respectively the western blot of secreted recombinant Gyroxin purified by affinity with Benzamidine Sepharose 6B resin from conditioned culture medium over 24 h.

In Fig. 6A the lane 1 shows a Low-Range Rainbow Molecular Weight Marker (45 K to 2.5 KDa), lane 2 contains the COS-7 cells transfected with pSecTag2-Gyro total extract and a recombinant protein band was detected in the western blot. The lane 3 shows the COS-7 cells transfected with pED-Gyro total extract and a correspondent band was detected in western blot. As one can see, COS-7 cells transfected with pED-Gyro (lane 3) express Gyroxin more efficiently than those transfected with pSecTag2-Gyro

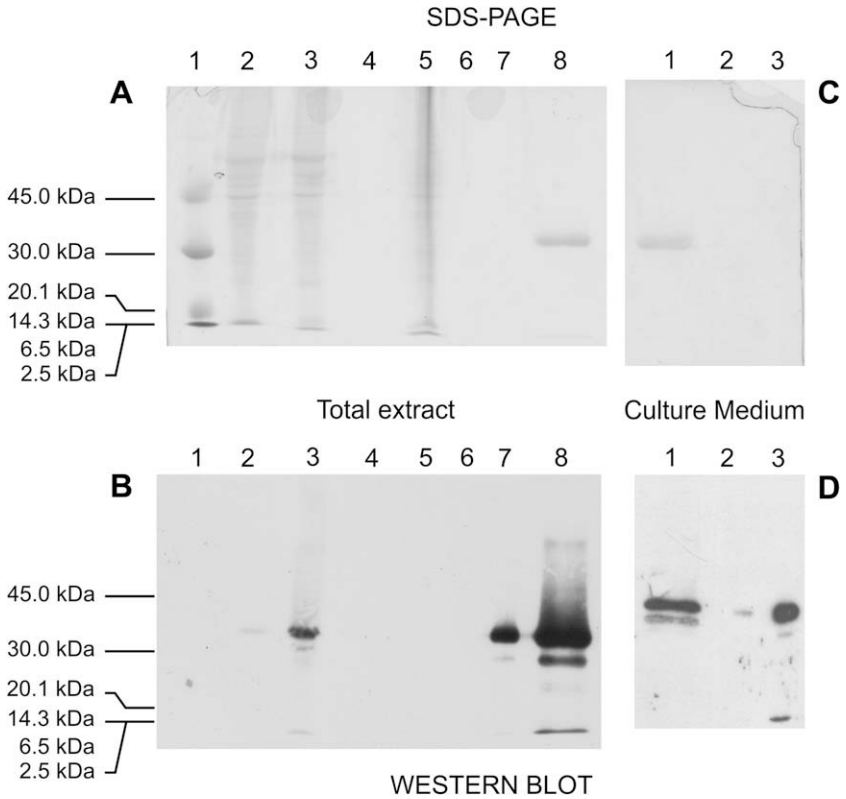



**Fig. 6.** SDS-PAGE and Western blot of recombinant protein expressed by pED-Gyro and pSecTag2-Gyro in COS-7 cells and secreted in culture medium over 24 h purified by affinity with Benzamidine Sepharose 6B resin from conditioned. A – SDS-PAGE and B – Western blot of total extract COS-7 cells: 1) Marker (M.W); 2) COS-7 cells total extract transfected with pSecTag2-Gyro; 3) COS-7 cells extract transfected with pED-Gyro; 4) empty; 5) untransfected COS-7 cells (negative control); 6) empty; 7) 0.5 μg of Gyroxin purified from *C. d. terrificus* venom (positive control); 8) 5 μg of Gyroxin purified from the *C. d. terrificus* venom (positive control). C – SDS-PAGE and D – Western blot of recombinant Gyroxin purified from conditioned culture medium. 1) 3 μg of purified recombinant Gyroxin, 2) 0.05 μg of Gyroxin purified from *C. d. terrificus* venom (positive control); 3) 0.5 μg of Gyroxin purified from *C. d. terrificus* venom (positive control). The primary antibody was anti-crotalic serum from Butantan Institute and the reaction was detected with secondary antibody conjugated to horseradish peroxidase.

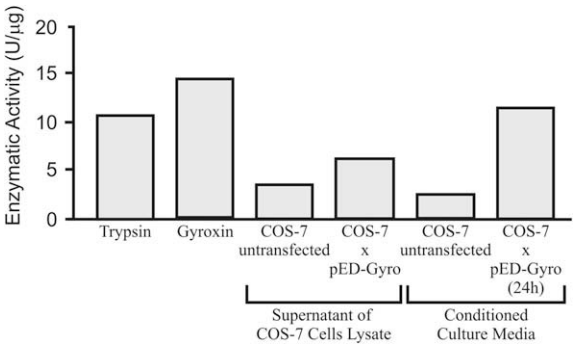



**Fig. 7.** Esterase activity assay of recombinant Gyroxin purified by Benzamidine Sepharose from the supernatant of lysate and conditioned culture medium over 24 h of transfected COS-7 cells with pED-Gyro. Supernatant lysate and conditioned culture medium of untransfected COS-7 cells were used as negative controls. Trypsin and Gyroxin purified from the *C. d. terrificus* venom were used as positive controls.

vector (lane 2). In the lane 5, untransfected COS-7 cells total extract was applied as a negative control and this extract does not react with anti-crotalic serum from Butantan Institute as expected.

The lanes 7 and 8 (6A) represent positive controls and contain 0.5 μg and 5 μg of native Gyroxin respectively and this protein was recognized by anti-crotalic serum in western blot (6B). Native Gyroxin is not well dyed by Coomassie blue and requires at least 3 μg of the protein to be visualized, so in the SDS-PAGEs it was not possible to visualize the native Gyroxin (positive control) at concentrations of 0.5 μg (lane 7 – 6A, lane 3 – 6C) and 0.05 μg (lane 2 – 6C), but it was detected by immunoblottings (6B and 6D). In the SDS-PAGE (6C) lane 1 shows 3 μg of secreted recombinant Gyroxin purified by affinity with Benzamidine Sepharose 6B resin from conditioned culture medium over 24 h and is recognized in western blot (lane 1 – 6D) by anti-crotalic serum from Butantan Institute.

The recombinant Gyroxin in total cell extracts and the secreted recombinant Gyroxin in the culture medium purified by Benzamidine Sepharose shows the same electrophoretic pattern of the native Gyroxin purified from the venom.

Transient expression offers a convenient means to compare different vectors, by ensuring that an expression plasmid is adequated to establish a stable expressing cell line. Thus the expression of Gyroxin, in a transient system, using COS-7 cells was a rapid way to choose the vector to be used in a future transfection for establishment of a stable Gyroxin-expressing cell line. pED-Gyro is a dicistronic mRNA expression vector in which Gyroxin cDNA is located upstream of the amplifiable marker gene, dihydrofolate reductase (DHFR), which can be used for transfection of *dhfr* deficient cells (CHO DX-B11 cells). Using this system there is a possibility of pED-Gyro amplification and consequently of attaining higher level of Gyroxin expression, as described for other proteins using such expression system (Chura-Chambi et al., 2004; Peroni et al., 2002; Soares et al., 2000). Thus, stable transfection is being conducted to obtain higher and constitutive expression with CHO DX-B11 *dhfr*$^{-}$ cells.

Gyroxin cDNA was cloned in fusion with the murine Igκ signal peptide nucleotide sequence, which allows its secretion in the cell culture medium. Recombinant Gyroxin has been purified using Benzamidine Sepharose 6B affinity chromatography, mainly from the conditioned medium over 24 h from COS-7 cells transfected with pED-Gyro, in an relative amount that is similar to one purified from crude *C. d. terrificus* venom. To confirm that recombinant Gyroxin was fully active, an enzymatic assay using TAME as substrate was done.

The esterase activity of conditioned culture medium over 24 h transfected with pED-Gyro was higher than supernatant of COS-7 cells lysate transfected with pED-Gyro (Fig. 7) indicating that the murine Igκ signal peptide in this pED-Gyro was capable of driving active recombinant Gyroxin out of cell. The recombinant toxin showed esterase activity in the culture medium as indicated in Fig. 7.

## 4. Conclusion

In the present work we described the cloning of five serine protease precursor genes from *C. d. terrificus* venom gland cDNA library: clones B2.1, B1.3, B1.4, B1.5 and B1.7. The dendrogram based on amino acid sequences and biological activities showed that these clones belong to two subgroups showing the divergence between clone B1.7 (subgroup Ib) and the others belonging to subgroup Ia. The clone B1.5 is a truncated form of cDNA precursor, with a deletion of 146 bp, which was not included in the functional dendrogram for analysis.

The transient expression of active Gyroxin was obtained by transfecting COS-7 cells and this product was secreted into the culture medium with an esterase activity, suggesting a correct folding of recombinant toxin. This rapid and direct methodological approach opens up new possibilities to express heterologous snake toxins in mammalian cells. Moreover, such strategy will allow one to proceed the characterization of their structure and activities, by obtaining the protein in the culture medium with high level of purity and specificity.

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## Conflict of interest

The authors declare that there are no conflicts of interest.

## Appendix. Supplementary information

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.toxicon.2009.03.022.

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