



Heterologous expression of four recombinant toxins from Panamanian scorpions of the genus *Tityus* and *Centruroides* for production of antivenom[☆]

Marcos H. Salazar^{a,b,c,**}, Herlinda Clement^c, Ligia L. Corrales-García^{c,d}, Jairo Sánchez^{a,b}, John Cleghorn^a, Fernando Zamudio^c, Lourival D. Possani^c, Hildaaura Acosta^a, Gerardo Corzo^{c,*}

^a Centro de Investigación e Información de Medicamentos y Tóxicos (CIIMET), Facultad de Medicina, Universidad de Panamá, Ciudad de Panamá, Panamá

^b Facultad de Ciencias Naturales, Exactas y Tecnología, Universidad de Panamá, Ciudad de Panamá, Panamá

^c Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México

^d Departamento de Alimentos, Facultad de Ciencias Farmacéuticas y Alimentarias, Universidad de Antioquia, AA 1226, Medellín, 050010, Colombia

ARTICLE INFO

Handling Editor: Glenn King

Keywords:

Animal venom
Centruroides
 Proteomics
 Recombinant protein
Tityus

ABSTRACT

Background: The development of more effective antivenoms remains a necessity for countries where scorpionism is a public health problem. Also, the regionalization of antivenoms may be important for some countries with special scorpionism characteristics.

Objective: Production of antibodies capable of neutralizing the lethal effect of the venom of three scorpion species from Panama.

Methods: The primary structures of two neurotoxins from *T. pachyurus*, one from *T. cerroazul* and another from *C. bicolor* were elucidated using N-terminal amino acid degradation and Sanger gene cloned sequencing. The obtained mRNA transcripts were cloned and expressed using *E. coli* vectors. Different bacterial expression conditions were tested and the best culture conditions for each expressed protein is reported. The expressed scorpion toxins were purified by chromatographic methods and used as immunogens in rabbits.

Results: The antibodies produced under the reported immunization scheme show better neutralization (ED₅₀) than other reported commercial antivenoms used to neutralize similar species scorpion venoms under similar LD₅₀ conditions.

Conclusion: The information reported here shows the proof of concept for selecting recombinant immunogens with the ability to produce antibodies for neutralizing the lethal effects of the most important medical species of scorpions in Panama.

1. Introduction

Currently, there are at least sixteen species of scorpions in Panama, of which the genera *Tityus* and *Centruroides* are considered the most dangerous species (Borges et al., 2012; Miranda and de Armas, 2020). In this country, the scorpions of the genus *Tityus* are known to produce serious clinical manifestations; for example, acute lung edema, pancreatitis or cardiac arrhythmias, and even death (Salazar et al., 2018). The species *Tityus pachyurus*, *T. festae*, *T. asthenes*, *T. championi* and *T. cerroazul* are considered of medical importance because they have been associated with cases of death (Ministerio de Salud, 2017). The

species *Tityus pachyurus* was described in 1897 by Reginald Pocok, and it is considered the species associated with more cases of mortality in Panama. It has a wide distribution in the country except for the province of Darién, a large forest and mountainous area of the Panama-Colombia border, where its presence has not been reported. Even though, *T. pachyurus* presence has also been reported in the middle sector of the Magdalena River Valley (center of Colombia), through the departments of Tolima, Cundinamarca, Boyacá, Antioquia and Huila (Guerrero-Vargas et al., 2015), where it is reported as a dangerous species and of medical interest in Colombia (Otero et al., 2004). The species *Tityus cerroazul* was described in 1986 by Wilson Laurenço, its name comes

[☆] Abbreviated title: Production of antivenom from Panama.

* Corresponding author.

** Corresponding author. Centro de Investigación e Información de Medicamentos y Tóxicos (CIIMET), Facultad de Medicina, Universidad de Panamá, Ciudad de Panamá, Panamá.

E-mail addresses: Marcos_h03@hotmail.com (M.H. Salazar), corzo@ibt.unam.mx (G. Corzo).

<https://doi.org/10.1016/j.toxcx.2021.100090>

Received 2 September 2021; Received in revised form 13 December 2021; Accepted 15 December 2021

Available online 28 December 2021

2590-1710/© 2021 The Author(s).

Published by Elsevier Ltd.

This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

from the region of Cerro Azul in Panama (Laurenço, 1986). The venom of *Tityus cerroazul* has the lowest LD₅₀ compared to other *Tityus* venoms; therefore, it could be considered one of the most dangerous species in Panama (Salazar et al., 2018). It has a wide distribution in the forested areas of Panama, and also it has also been reported in Costa Rica (Viquez et al., 2005).

On the other hand, scorpion stings of the *Centruroides* genus in Panama cause high rates of morbidity, although no cases of deaths have been reported (Ministerio de Salud, 2017). The sting of *C. granosus*, *C. panamensis*, *C. bicolor*, and *C. limbatus* causes mild intoxication, generally presenting only local symptoms at the site of the sting (local pain or paresthesia). Within the genus *Centruroides*, the species *Centruroides bicolor* stands out for moderate clinical signs; for example nausea, vomiting, or tachycardia (Ministerio de Salud, 2017). *Centruroides bicolor* was described by Pocock in 1898, and its presence has also been reported in Costa Rica (Borges, 2015). In Panama, it is usually found in the provinces of Chiriquí and Veraguas.

Concerning the use of scorpion antivenoms, it is known that the early administration of antivenom (2 h after the accident) is considered the only therapy accepted by the World Health Organization (WHO) for the treatment of scorpion sting envenomings (WHO, 2016). However, scorpion antivenoms in Panama are difficult to obtain. Currently in America, Mexico produces antivenom against the sting caused by the genus *Centruroides* (Chippaux and Goyffon, 2008), Brazil against the genus *Tityus* (Candido and Lucas, 2004) as well as Argentina (de Roodt et al., 2003) and Venezuela (D'Suze et al., 2015). Panama had acquired Venezuelan antivenom (made using the venom of *Tityus discrepans*); however, data from the Universidad de Panamá (UP) in collaboration with the *Ministerio de Salud*, as well as other reports, have shown limited efficacy of such antivenom against *Tityus* species from Panama (Barona et al., 2004; Borges et al., 2012). This panorama has motivated us to assess the use of recombinant neurotoxins from the most medically important scorpions in Panama to evaluate them as immunogens to produce regional antivenoms. As background to this communication, Salazar et al. (2018) studied the venoms of four species of the genus *Tityus*, and four species of the genus *Centruroides* from Panama. The authors showed evidence that three species of *Tityus* (*T. pachyurus*, *T. festae* and *T. asthenes*) share similar toxic compounds of molecular mass around 7331.3 Da (Borges et al., 2020; Salazar et al., 2018). Similarly, studies of venom fractions of the species *T. cerroazul* and *C. bicolor* show the presence of major toxic neurotoxins with molecular masses of 6966.9 and 7485.7 Da, respectively (Salazar et al., 2018).

Therefore, in an effort to develop an antivenom for Panama, the primary structure of four neurotoxins (Sodium channel neurotoxin) was elucidated, and they were recombinantly expressed. Three neurotoxins were from the genus *Tityus*, and one was from the genus *Centruroides*. They were evaluated as immunogens for the production of antibodies capable of neutralizing the venoms of the most important medical species of the genus *Tityus* and *Centruroides* from Panama.

2. Material and methods

2.1. Venoms, venom glands and toxins

All scorpion venoms and venom glands were supplied by the *Centro de Investigación e Información en Medicamentos y Tóxicos de la Universidad de Panamá* (CIIMET-UP). Specimens were kept individually in optimal health conditions using plastic cages, at a constant temperature of 27 °C. Animals were fed with crickets and tap water *ad libitum*. Venom was extracted manually and immediately vacuum dried and stored at -20 °C until use. For transcriptomic analysis, the venom glands (48 h after venom extraction) were obtained from healthy specimens. Immediately after being removed, they were treated with RNAlater™ (ThermoFisher, Asheville, NC, USA) and stored at -70 °C until use. Toxin purification was performed according to Salazar et al. (2018). For the venom of *T. pachyurus*, the toxic peptides with molecular masses of 7331.3 Da and

7099.1 Da were named Tppa1 and Tppa2, respectively; for that of *T. cerroazul* the peptide with molecular mass of 6966.9 Da was named Tce3; and for that of *C. bicolor* the peptide with a molecular mass of 7485.7 Da was named Cbi1.

2.2. Mass spectrometry and N-terminal sequencing

The protein fractions (2–3 µg) were reconstituted in 20 µL of 60% acetonitrile with 0.1% acetic acid and directly applied to a Thermo Scientific LCQ Fleet ion trap mass spectrometer (San José, CA) using a Surveyor MS syringe pump delivery system. The eluate at 10 mL/min was split out in order to introduce only 5% of the sample into the nanospray source (0.5 mL/min). The spray voltage was set from 1.5 kV and the capillary temperature was set at 150 °C. The fragmentation source was operated at 25–35 V of collision energy, 35–45% (arbitrary units) of normalized collision energy and the scan with wide band was activated. All spectra were obtained in the positive-ion mode. The data acquisition and the deconvolution of data were performed on Xcalibur Windows NT PC data system. The average molecular masses values vary about ±1 Da, due to the limited resolution of this instrument. N-terminal Edman degradation was performed on a Shimadzu PPSQ-31 A (Shimadzu, Kyoto, Japan) automated gas-phase sequencer. Sample (60 µg) was dissolved in 10 mL of 37% CH₃CN (v/v) solution and applied to TFA-treated glass fiber membranes, pre-cycled with Polybrene (Sigma-Aldrich Co. St. Louis, MO, USA).

2.3. RNA extraction from *Tityus* and *Centruroides* species

The mRNA, from six pairs of venom glands from *Tityus* and the same number of glands from *Centruroides* species from Panama was extracted using an RNAeasy mini kit (Qiagen Inc., Germantown, MD) and the SV Total RNA Isolation System (Promega Co., Madison, WI) (Clement et al., 2019b).

2.4. Bacterial strains, enzymes and plasmids

XL1-Blue *Escherichia coli* strain was used for DNA cloning and plasmid propagation. The Shuffle® or Origami® *E. coli* strains were employed for the expression of recombinant scorpion toxins. Plasmids pCR®2.1-TOPO® (Invitrogen, CA, USA), and pQE30 (Qiagen, CA, USA) were used for cloning the scorpion toxin genes, and for production of the 6His-tagged recombinant peptides, respectively. Restriction enzymes, Taq polymerase, Factor Xa protease (FXa) and T4 DNA ligase were purchased from New England Biolabs (New England Biolabs, MA, USA).

2.5. cDNA library, construction and gene cloning

The mRNA was used to synthesize the cDNA employing an adapter primer (AP) (3' RACE System for Rapid Amplification of cDNA Ends, Invitrogen, Carlsbad, CA, USA). The reaction was carried at 42 °C for 1 h in the presence of 200 enzymatic units of Reverse transcriptase (SuperScript II RT, Invitrogen, Carlsbad, CA, USA). Since the N-terminal amino acid sequence of the toxic peptides (Tppa1, Tce3, and Cbi1) was obtained (Table S1), an oligonucleotide corresponding to its amino acid sequence was designed for gene cloning. The transcript corresponding to the N-terminal peptide from all fractions was amplified from the cDNA using Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) in combination with a degenerated primer based on N-terminal sequencing of all fractions and an equimolar concentration of the AUAP primer (GGCCACGCGTCTGACTAGTAC) (3' RACE System for Rapid Amplification of cDNA Ends, Invitrogen, Carlsbad, CA, USA).

A Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany) was used for PCR and the conditions for amplification were: 3 min denaturation at 94 °C, followed by 30 cycles at 94 °C for 40 s, 55 °C for 40 s and 72 °C for 40 s, with a final extension cycle at 72 °C for 10 min. PCR product was gel-purified from the agarose using the High Pure

PCR Product Purification Kit (Roche LifeScience, Penzberg, Germany). The resulting DNA fragment was ligated into PCR® 2.1-TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA). The ligation reaction was used to transform *E. coli* XL1 Blue chemo-competent cells. The individual clones were analyzed by colony PCR using oligonucleotides M13-Forward (5'-GTAACGACGGCCAG-3') and M13-Reverse (5'-CAGGAACAGCTATGAC-3'). The colonies with the expected DNA size were sequenced at the facilities of the *Instituto de Biotecnología*, UNAM, México.

Table S2 shows the transcripts obtained after 3' RACE. The conditions for amplifications were optimized for each toxin. PCR product was gel-purified, ligated into PCR®2.1-TOPO TA cloning vector, transformed in *E. coli* cells and then sequenced, as described previously. Table S3 shows the oligonucleotides used for constructing the expression vectors. Both the amplified gene and the expression vector pQE30 were digested with *Bam*HI and *Pst*I (New England Biolabs, Ipswich, MA, USA). After purification, a ligation reaction (10 µL) was carried out with T4 DNA ligase (Fermentas, Charlotte, CA, USA) with a 5-fold gene excess over plasmid for 16 h at 16 °C. Ten microliters of the ligation reaction were used to transfect competent *E. coli* XL1 Blue cells. Some individual clones were analyzed by colony PCR using oligonucleotides pQE30-Forward (5'-GAGCGGATAACAATTATAA-3') and pQE30-Reverse (5'-GGTCATTACTGGATCTAT-3'). The colonies with the expected DNA size were sequenced at the facilities of the *Instituto de Biotecnología*, UNAM, México. The final sequences for each recombinant toxin were named HisrTppa1, HisrTppa2, HisrTce3 and HisrCbi1, respectively (Table S4).

2.6. Expression and purification of recombinant proteins

E. coli strain Shuffle® or Origami® (New England Biolabs® Inc. MA, USA) were transformed with each plasmid and grown overnight on LB agar plates with 100 µg/mL of ampicillin at 37 °C. An isolated colony was inoculated in 50 mL of LB medium supplemented with 50 µL of ampicillin and grown overnight at 37 °C in incubation with shaking overnight. To improve the yield of biologically active toxins some extra reagents were added to the expression culture media (Table S5). The culture was added to 1 L of medium Luria-Bertulli (LB1: Tryptone 10 g, yeast extract 5 g, NaCl 10 g or LB2: Peptone 20 g, yeast extract 30 g and NaCl 21 g). The culture was grown at 37 °C with shaking until obtaining an optical density of 0.7 units of absorbance at 600 nm (OD₆₀₀) and induced with isopropyl-βD-thiogalactopyranoside (IPTG, Promega, Madison, USA) to a final concentration of 0.05–0.075 mM. The culture was kept for 14–16 h at 16 °C and the cells were harvested by centrifugation at 4424 g in JA-10 rotor, 30 min at 4 °C. The cellular pellet was recovered in washing buffer (0.05 M Tris-HCl, pH 8.0), and lysed in a French press. This material was centrifuged again (12,096 g in JA-20 rotor, for 30 min at 4 °C) and the supernatant and the insoluble fraction (inclusion bodies) were recovered. The inclusion bodies were dissolved in 6 M GndHCl in a 0.05 M Tris-HCl buffer pH 8.8.

All recombinant toxins obtained from inclusion bodies were purified using Ni-NTA (Ni-nitrilotriacetic acid) affinity column chromatography according to manufacturer instructions (QIAGEN, USA). The Ni-NTA eluting fraction with the expected molecular mass was then purified RP-HPLC using a C₁₈ preparative column (Vydac, WR Grace & Co.-Conn, USA) including a buffer A 0.1% trifluoroacetic acid (TFA) in water, and buffer B 0.1% TFA in acetonitrile (ACN), with a linear gradient ranging from 0 to 60% buffer B over 60 min. The eluting fractions with the expected molecular mass and biologically active were re-purified by RP-HPLC on analytical Biphenyl column Kinetex® 5 µm Biphenyl, 100 Å, 250 × 4.6 mm using a gradient from 0 to 20% over 5 min and 20%–60% of buffer B over 40 min at a flow rate of 1 mL/min. The presence of the recombinant protein was monitored at every step of the expression and purification processes by SDS polyacrylamide gel electrophoresis in reducing conditions (12% gel, data not shown). The molecular masses of the purified products were confirmed by mass spectrometry analysis using an LCQ Fleet apparatus from Thermo Fisher Scientific Inc. (San

Jose, CA, USA) (Fig. S1).

2.7. Secondary structure of recombinant toxins

The secondary structure of all four recombinant neurotoxins was evaluated by circular dichroism (CD). The measurement was carried out on a Jasco model J-720 spectropolarimeter (Jasco, Tokyo, Japan), from 260 to 190 nm in an aqueous solution of 60% trifluoroethanol (TFE), at room temperature, with a 1-mm pathlength cell. Data were collected at 1 nm with a scan rate of 50 nm/min, and a time constant of 0.5 s. The concentration of HisrTppa1, HisrTppa2, HisrTce3, and HisrCbi1 was 0.7, 0.7, 0.7, and 1.4 g/L, respectively. Data was the average of two separate recordings and analyzed by the software Bestsel (<http://bestsel.elte.hu/index.php>) (Micsonai et al., 2018). A table in Supplementary section shows the percentages of secondary structure obtained. The native scorpion neurotoxin Cbi1 was used as comparative controls under the same extent conditions.

2.8. Immunization

Rabbits (New Zealand, 2–2.5 kg body weight) were hyperimmunized subcutaneously with antigens HisrTppa1, HisrTppa2, HisrTce3, and HisrCbi1, for producing serum antibodies. The immunization protocols were initiated by administering a dose of 75 µg of protein mixture (HisrTppa1, HisrTppa2, and HisrTce3, 25 µg each) in PBS pH 7.2, and then a second administration of 25 µg of HisrCbi1 in PBS pH 7.2. Antigens were emulsified (1:1) with Freund's Incomplete Adjuvant (IFA, from Rockland Immunochemical Inc., Limerick, PA, USA) and alternating with aluminum hydroxide (Imject®, Thermo Scientific). The protein content of antigens for immunization had increasing doses to 2 mg in a period of twenty weeks (Table S7). The rabbit serum antibodies were purified from plasma by acid precipitation, using 5% caprylic acid (Rojas et al., 1994). A final solution of rabbit-derived immunoglobulins (IgG's) containing 30 mg/mL of protein was used for further experiments.

2.9. ELISA assay

The recognition titers of rabbit immunoglobulins were compared to commercial antivenoms by enzyme-linked immunosorbent assays (ELISA) for the presence of antibodies against scorpion venoms from Panama following the protocol from (Clement et al., 2019a; de la Rosa et al., 2019). Flat bottom MaxiSorp plates (NUNC™, thermo scientific) were treated with 100 µL solution of 5 µg/mL of either *Centruroides* or *Tityus* venoms in 100 mM sodium carbonate buffer (pH 9.6). Following overnight incubation at 4 °C, wells were aspirated and washed three times with 200 µL of washing buffer (50 mM Tris-HCl, pH 8, containing 0.5 mg/mL of Tween 20 and 150 mM NaCl). Subsequently, wells were filled with 200 µL of blocking buffer (5 mg/mL gelatin and 2 mg/mL between 20 and 50 mM Tris-HCl buffer, at pH 8). After 2 h of incubation at 37 °C, wells were washed as described above and filled with Anti-Tppa1/Tppa2/Tce3/Cbi1 were diluted 1:90 in the first column for all venoms and commercial antivenoms (Brazil 1:60 for *Tityus* venoms and 1:3 for *Centruroides* venoms, Mexican 1:3 and Venezuela 1:3 for all venoms) were diluted in the first column and 1:3 serial dilutions were made in subsequent columns using incubation buffer (50 mM Tris-HCl buffer at pH 8.0, containing 1 mg/mL gelatin, 0.5 mg/mL between 20 and 0.5 M NaCl). Finally, plates were incubated with peroxidase-conjugated goat anti-rabbit immunoglobulins 1:30,000 (Cat N. A0545, Sigma-Aldrich®) and developed with ABTS Liquid Substrate (ABTS tablets ref 11112422001, Roche). The color development reaction was arrested by the addition of 25 µL of 20% SDS, and the plate was read at 495 nm in a Microplate Reader (Tecan Sunrise IVD version), and the results were plotted using GraphPad Prism v.8.0 with non-linear regression analysis. Conventional titers were calculated from the midpoint of the curve and correspond to the IgGs dilution for half of

maximal binding, which was considered as the half maximal effective concentration (EC₅₀).

2.10. Electrophoretic analysis and Western blotting

For electrophoretic analysis and Western blotting, venom pools from the species *C. panamensis*, *C. granosus*, *C. bicolor*, *T. pachyurus*, *T. cerroazul*, *T. asthenes* and *T. festae* were included. For electrophoresis analysis, a SDS-PAGE analysis, under reducing conditions, with 12.5% gels were done according to the method proposed by Laemmli (Laemmli, 1970), then protein staining was made with Coomassie Brilliant Blue. For Western Blotting analysis, venom samples were first separated by SDS-PAGE (12.5% gels), and then transferred to a polyvinylidene difluoride membrane using a transfer apparatus Owl semi-dry system for 1 h at 400 mA. After incubation with 5% nonfat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 2 h at room temperature, the membrane was washed three times with TBST and incubated first with the rabbit IgG antibodies (1:50), washed again three times with TBST, and then the second antibody was added (goat anti-rabbit IgG coupled to alkaline-phosphatase at 1:2000). Membranes were washed once more with TBST three times and developed with 3,3',5,5'-tetramethylbenzidine (TMB) ready-to-use solution (Invitrogen Antibodies, Thermo Fisher Scientific, Asheville, NC, USA) according to the manufacturer's protocols.

2.11. Biological activities

2.11.1. Toxicity

The protocol used for assaying the activity of peptides *in vivo* was followed according to the guidelines of our Institute Committee of Animal Welfare guidelines, keeping the number of animals to a minimum. Groups of four male mice (CD-1, 18–20 g body weight) were tested by intracranial (ic) or intravenous injection (iv) (Salazar et al., 2018). Peptide fractions were diluted up to 50–100 µL, to achieve the desired concentration, with deionized water (dH₂O). The injection was performed mid-way between the left eye and the left ear (intracranial) with a 10 µL micro-syringe fitted with a glass capillary. Negative controls were performed with dH₂O only and positive controls with the neurotoxic scorpion peptide CssII isolated in our laboratory (Hernández-Salgado et al., 2009). The amounts of protein used were indicated in the headings of the table results.

2.11.2. Protecting activity of immunoglobulins

For neutralization experiments, 1.5 LD₅₀ of whole scorpion venom was pre-incubated 30 min at 37 °C with varying volumes of antiserum and then injected by iv route according to Barona et al. (2004). After 24 h, the median effective dose (ED₅₀) was calculated using the Spearman-Kärber method (Gutiérrez et al., 1990; WHO, 1981). The ED₅₀ is defined as the volume of antiserum able to protect 50% of the mice challenged. For the neutralization tests, respective venom pools of the species *T. cerroazul*, *T. pachyurus* and *C. bicolor* were used.

Table 1

Alignment of the mature amino acid sequences of the toxins used for the production of recombinant proteins.

Toxin	Amino acid sequence	Identity (%)
Tppa1	KDGYLVGNDGCKYSCLTRPGHYCASEC-SRVKGGKDGICYAW-MACYCYNMPNWKTSRSTNRCGR	100
Tppa2	KDGYLVGNDGCKYSCLTRPGHYCASEC-SRVKGGKDGICYAW-MACYCYSMPDVKWTWSRATNKC-	93.5
Tce3	KDGYLIEHRGCKYSCFFGSSWCNKEC-TLKKGSSGYC-AW-PACWCYGLPDSVKIFDSSNNKCS-	56.4
Cbi1	KDGYPMDNKGCKIACVIN-NQYCETECVTLKGGKGYCYFWKLCYCEGLPNWAKVWDRAATNKCR	48.3
	**** : * : * : * . . . * . * * * . . * * * * * . * . : * : . * : . . * *	

The amino acid sequence of Tppa1 is listed in the first line and the amino acid sequences of Tppa2, Tce3 and Cbi1 follow. The last column indicates the percentage of identity with respect to Tppa1. The last line shows amino acid identity. Gaps marked by hyphens are inserted to optimize identity. Identical amino acids are indicated by asterisks, whereas those with high or low similarities are indicated by semicolons and dots. Alignment was prepared using MUSCLE algorithm for multiple sequence alignment by MEGAX.

2.12. Statistics

Results were expressed as mean and standard deviation, or as mean with 95% confidence intervals. For all statistical methods, the software Prism 4.0 was used (Graph Pad Inc., San Diego, CA).

3. Results

3.1. N-terminal sequencing and 3' RACE

For the N-terminal sequencing analysis, the Tppa1, Cbi1 and Tce3 toxins were purified and analyzed as indicated in Section 2.2. The amino acid sequence of Tppa1, Cbi1 and Tce3 were partially sequenced at the N-terminal region, as shown in Table S1. The amino acids underlined in this table were used to design primers for 3'RACE assays. For the RNA extraction, six pairs of venom glands, for each species, were used. Only the amplified transcripts by 3'RACE (Section 2.5) with the expected size in agarose gels were purified, and DNA sequenced. Using the N-terminal designed primer of Tppa1, we obtained an additional transcript, identified as Tppa2 from the venom of *T. pachyurus* (Salazar et al., 2018). The four RNA transcripts found for the four toxins are presented in Table S2. Table 1 shows the amino acid sequences deduced from the transcripts obtained. The transcripts translation to protein sequence was performed using the bioinformatics tool Translate from ExPasy (<https://web.expasy.org/translate/>). The transcripts of the *Tityus* species strongly suggested the presence of post-translational modifications; that is, C-terminal amidation (-GK or -KK). For this reason, in the alignment shown they were not included into the sequence. The accession numbers assigned for UniProtKB were C0HLZ0 for Tppa1, C0HLZ1 for Tppa2, C0HLZ2 for Tce3 and C0HLZ3 for Cbi1.

The amino acid sequences of the four neurotoxins were compared to known scorpion toxins (Table 2) to learn more about the function/classification of the elucidated sequences (Possani et al., 1999). The amino acid sequences of Tppa1 and Tppa2 showed high identity of 96.8 and 96.7% to the sodium channel alpha-toxin To3 (P60213.3) and to the sodium channel beta-toxin To11 (H1ZZI0.1), respectively. To3 and To11 are from the venom of the scorpion *Tityus obscurus* from Brazil. The amino acid sequence of Tce3 presented sequence identity to the sodium channel beta-toxin TdNa5 (C9X4K3.1) from the venom of the scorpion *Tityus discrepans* from Venezuela, and the amino acid sequence of Cbi1 was similar to the sodium channel beta-toxin CssIX (F1CGT6.1) from the venom of the scorpion *Centruroides suffusus suffusus* from Mexico. The four toxins (Tppa1, Tppa2, Tce3, and Cbi1) showed to contain 8 cysteine amino acid residues each, responsible for making four disulfide bridges, known to be present in the sodium channel modifier scorpion toxins, and at least 16 other amino acids, which are highly preserved (Table 1). The importance of such highly conserved sixteen amino acid residues, in their primary structure, are assumed to be important for the recognition of voltage-gated sodium channels (Nav), and the consequent toxicity of these peptides.

Table 2

Alignment of the sequences from Table 1 with the amino acid sequences of the most identical reported toxins found in other scorpion venoms.

Toxin	Amino acid sequence	Identity ^a (%)
Tppa1	KDGYLVGNDGCKYSCLTRPGHYCASECSRVRKGD-GYCYAW-MACYCYSPDWVKTWSRSTNRCGR-NH ₂	96.8%
To3 NaTx14.2	KDGYLVGNDGCKYNCLTRPGHYCANECRSVRKGD-GYCYAW-MACYCYSPDWVKTWSRSTNRC	
Tppa2	KDGYLVGNDGCKYSCLTRPGHYCASECSRVRKGD-GYCYAW-MACYCYNMPNWKVKTWSRATNKC-NH ₂	96.8%
To11 NaTx14.9	KDGYLVGNDGCKYNCLTRPGHYCANECRSVRKGD-GYCYAW-MACYCYNMPNWKVKTWSRATNKC	
Tce3	KDGYIIEHRGCKYSCFPFGSSWCNKECTLKKGSS-GYC-AW-PACWCYGLPDSVKIFDSNNKCS-NH ₂	93.4%
TdNa5 -NaTx2.5	KDGYIIEHRGCKYSCFPFGTNSWCNTECTLKKGSS-GYC-AW-PACWCYGLPDNVKIFDSNNKCS	
Cbi1	KDGYPMDNKGCKIACVI-NNQYCETECVTLVKGKGYCYFWKLACYCEGLPNWAKVWDRATNKCRA	95.4%
CssIX	KDGYPMDHKGCKISCVI-NNKYCETECVTLVKGKGYCYFWKLACYCEGLPNWAKVWDRATNKCRA	

^a To3 NaTx14.2 (P60213) and To11 NaTx14 (H1ZZIO) were obtained from venom and venom gland transcript, respectively, of the species *Tityus obscurus*. Their percentage of identities are reported based on the amino acid sequence of Tppa1 and Tppa2, respectively. TdNa5 -NaTx2.5 (C9X4K3) was obtained from venom gland transcripts of *Tityus discrepans*. Its percentage of identity is reported based on the amino acid sequence of Tce3. CssiX (F1CGT6) is from the venom of *Centruroides suffusus suffusus*. Its percentage of identity is reported based on the amino acid sequence of Cbi1. Gaps were introduced to align the Cys.

3.2. Construction of vectors and gene cloning

The cDNA coding regions for Tppa1, Tppa2, Tce3 or Cbi1 were amplified by PCR, cloned using each into a pCR®2.1-TOPO® plasmid, and then with the use of restriction enzyme cleavage sites *Bam*H1 and *Pst*1 into the expression vector pQE30, which was used to produce

recombinantly such scorpion toxins. Table S3 shows the oligonucleotides used for constructing the expression vectors. The pQE30 vector was employed to produce N-terminal 6His-tagged proteins, which facilitate a rapid purification of recombinant products by nickel affinity agarose columns (NiNTA). The FXa cleavage site between the 6His-tag and the mature toxin sequences (Tppa1, Tppa2, Tce3, or Cbi1) was added in case

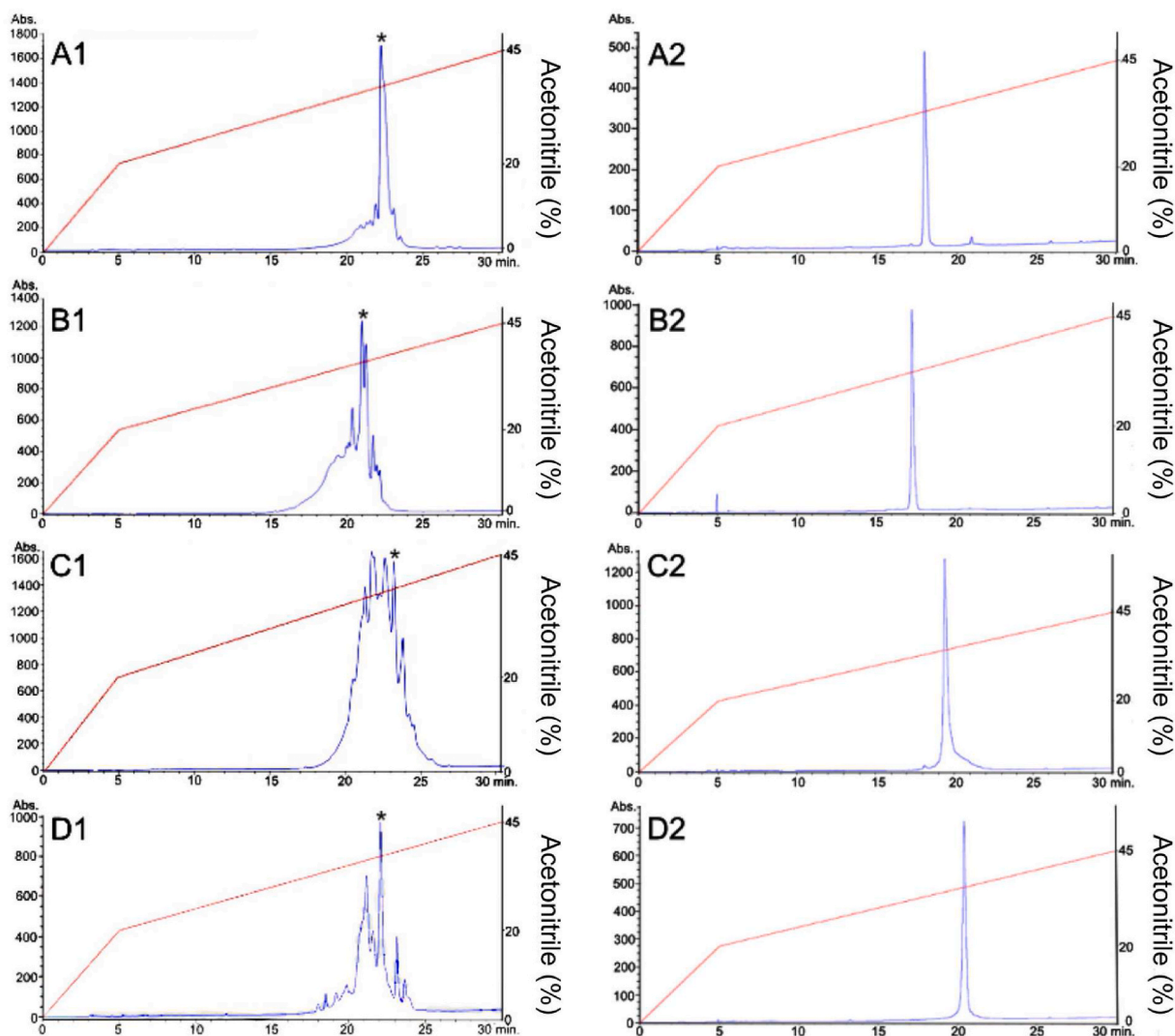


Fig. 1. Reverse phase-HPLC profiles of the purification of the recombinant proteins. HisrTppa1 (A1 and A2), HisrTppa2 (B1 and B2); HisrTce3 (C1 and C2); HisrCbi1 (D1 and D2). Reverse phase-HPLC profiles from A1 to D1 were separated using a semi preparative C₁₈ column, and reverse phase-HPLC profiles from A2 to D2 were separated using an analytical Biphenyl column. The red line depicts the acetonitrile gradient (see Materials and methods for details). The asterisk corresponds to the isoform that was active in mice.

of a harmful effect of the 6His-tag over the biological activity of toxins. The expressed proteins were named HisrTppa1, HisrTppa2, HisrTce3, and HisrCbi1, respectively. The resulting amino acid sequences, and their theoretical molecular masses are shown in Table S4. For HisrTppa2 an Arg residue was added to the C-terminal.

3.3. Expression system

Shuffle® or Origami® expression strains, different expression media, and incubation conditions were used to test the expression of the recombinant toxins. The best expression conditions found for each toxin are shown in Table S5. As a note, it was interesting to observe that toxins of the genus *Tityus* (HisrTppa1, HisrTppa2, and HisrTce3) were better expressed in the Shuffle® strain while the toxin of the genus *Centruroides* (HisrCbi1) was better expressed in the Origami® strain.

3.4. Purification and secondary structure of recombinant proteins

After protein expression, the culture medium was centrifuged at 4424 g (in JA-10 rotor) for 20 min, cells were resuspended in 50 mM Tris-HCl pH 8.0 and homogenized in a French press (Constant Systems Ltd). The lysate was centrifuged at 12,096 g (in JA-20 rotor), and the pellet containing the recombinant toxins was purified from inclusion bodies using affinity chromatography to Nickel. The recombinant proteins were desalted using C₁₈ preparative columns in a HPLC system. Finally, the recombinant proteins were purified using a biphenyl reverse phased column under similar HPLC conditions (Fig. 1). Furthermore, the expressed neurotoxins showed similar content of α -helix and antiparallel β -sheet secondary structure, indirectly confirming analogous folding to

that of the α/β structures of scorpion neurotoxins (Fig. 2). The CD spectrum of HisrCbi1 was compared to that CD spectrum of the native Cbi1 (Fig. 2B). All CD spectra were evaluated using the deconvolution software Bestsel, in order to compare the percentages of secondary structure, pertaining to all recombinant peptides. Supplementary Table S6 shows the percentages of secondary structures among the recombinant peptides and the native Cbi1. The α -helix proportion was the most conserved secondary structure among all recombinant peptides. The molecular masses of the resulting fractions were confirmed by mass spectrometry (Fig. S1), and the lethal fractions to mice were used for immunization of rabbits.

3.5. Immunization, antibody purification, evaluation and neutralization assays

Two rabbits were immunized as described in Section 2.7, and they were boosted 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, 91, 98, 105, 112, 119, 126, and 133 days later with different amounts of recombinant toxins, alternating AIF and Alumina according to the protocol in Table S7.

The antibody titers were monitored by performing direct ELISA assays every 4 weeks after taking a blood sample according to Table S7. Once the immunization process was completed, the rabbits were bled and the rabbits' blood was allowed to clot at 37 °C for at least 2 h, chilled on ice for 1 h, and centrifuged at 3836 g (in JA-14 rotor, for 20 min at 4 °C). Serum was collected and stored frozen at -20 °C until used. Finally, rabbit immunoglobulins were purified by caprylic acid (Rojas et al., 1994), which is also described and recommended in the WHO guidelines for the production, control and regulation of antivenoms (WHO, 2016). The IgGs were lyophilized in vials containing 50 mg and stored at 4 °C. For titer quantification and neutralization assays an initial concentration of 30 mg/mL of IgGs was used.

Although ELISA assays do not predict antivenom neutralization, some interesting results were found. Table 3 compares the rabbit IgGs titers obtained from the immunization with the four recombinant neurotoxins (HisrTppa2, HisrTppa1, HisrTce3 and HisrCbi1) with other commercial antivenom antibodies (see also Fig. S2). The concentration of protein in the commercial antivenoms used was 37.1 mg/mL for the Mexican Alacramyn®, 19.6 mg/mL for the Venezuelan antivenom and 80.8 mg/mL for de Brazilian antivenom, which are in the range of previous reports (Sevcik and D'Suze, 2021; Vázquez et al., 2010; Venancio et al., 2013). Table 3 shows that the Brazilian antivenom had the best IC₅₀ (low μ g/mL) values compared to the other three antivenoms. Also, it had a better recognition for components of *Tityus* venoms than that of the *Centruroides* venoms. The antibody recognition for both types of venom components from *Tityus* and *Centruroides* confirms the presence of components with structural similarities in these scorpion venoms. Conversely, the Mexican antivenom Alacramyn® had the poorest IC₅₀ (high μ g/mL) values for the *Tityus* sp. venoms, and it also had poor recognitions for *Centruroides* sp. venoms from Panama. However, it had an acceptable recognition for the venom of *Centruroides suffusus suffusus*, which is one of the venom scorpion species used for producing this antivenom. The Venezuelan antibodies had a better recognition for *Tityus* venoms than that of the Mexican Alacramyn®. However, it should be noted that the rabbit IgGs obtained here are directed towards one or two components from the *Tityus* and *Centruroides* venoms, and the antibodies from commercial antivenoms are directed for several venom components with little or no role in venom lethality. Furthermore, the antibodies from this work are rabbit IgGs with a lower mass/valence ratio compared to that of the horses F(ab')₂ of the commercial antivenoms. Although the IC₅₀ values for the IgGs obtained in this work were larger than that of such commercial antivenoms, it is clear that the IC₅₀ values are not always correlated to the neutralization activity; therefore, the median effective dose (ED₅₀) for such IgGs was performed and compared to the reported ED₅₀ of such commercial antivenoms.

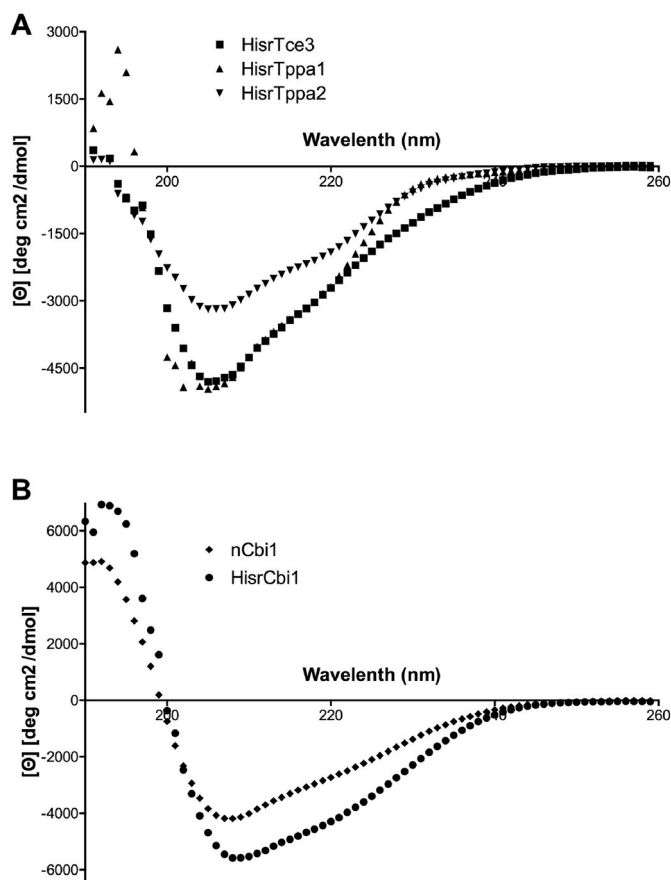


Fig. 2. Circular dichroism spectra of recombinant neurotoxins. A) Recombinant neurotoxins from the venom of *Tityus pachyurus* and *T. cerroazul*; B) Recombinant and native neurotoxin from the venom of *C. bicolor*.

Table 3IC₅₀ values for recognition of several scorpion venoms by antibodies from different commercial antivenoms and rabbit IgGs from this work.

Anti-venom	IC ₅₀ (µg/mL)						
	<i>C. s. suffusus</i>	<i>C. bicolor</i>	<i>C. granosus</i>	<i>T. pachyurus</i>	<i>T. cerroazul</i>	<i>T. asthenes</i>	<i>T. festae</i>
Alacramyn® (Mex) ^a	7.7 (6.3–9.4)	33.0 (26.8–40.7)	22.4 (18.6–27.0)	16.7 (9.9–28.1)	46.2 (29.6–72.0)	20.5 (11.9–35.3)	20.4 (14.3–29.2)
Anti-Scorpion (Ven) ^b	39.3 (32.5–47.7)	23.9 (19.4–29.5)	26.8 (21.4–33.8)	7.5 (5.8–9.6)	19.1 (17.0–21.4)	11.3 (8.7–14.5)	7.6 (6.0–9.6)
Anti-Scorpion (Bra) ^c	9.8 (7.8–12.3)	8.2 (6.5–10.4)	9.7 (7.7–12.3)	3.6 (2.9–4.5)	5.9 (4.9–7.2)	4.4 (3.5–5.6)	5.1 (4.3–6.1)
This work ^d	93.4 (87.0–101.0)	20.0 (18.5–21.5)	34.8 (30.8–39.3)	29.3 (28.5–30.2)	40.9 (39.9–41.8)	35.9 (31.9–40.5)	26.3 (21.6–32.0)

95% confidence intervals (CI) are shown in parentheses. Alacramyn® (Mex, Mexico), Anti-Scorpion (Ven, Venezuela) and Anti-Scorpion (Bra, Brazil) are F(ab')₂ from horses. The IgGs of this work are from rabbit. The antibody concentration used was a = 37.1 mg/mL; b = 19.6 mg/mL; c = 80.8 mg/mL; d = 30 mg/mL.

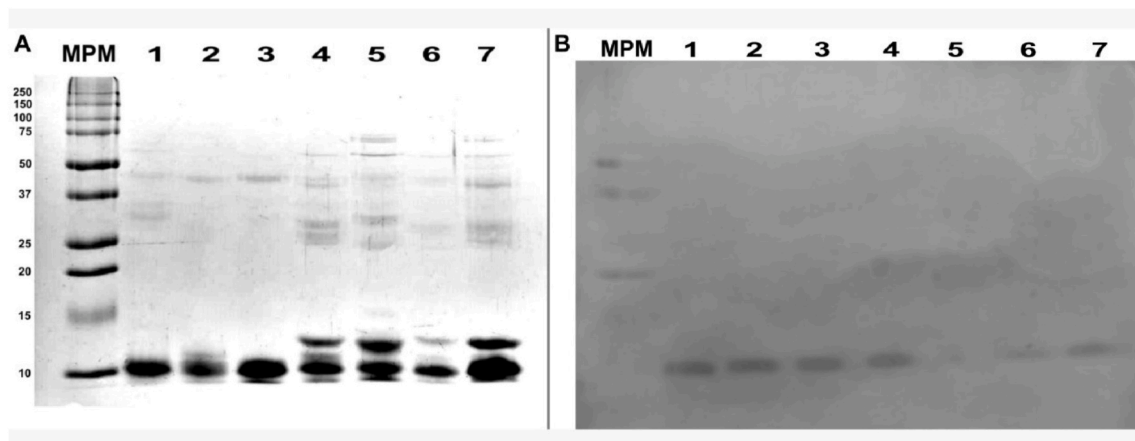


Fig. 3. SDS-PAGE of scorpion venoms from Panama and Western-blot developed with the obtained polyvalent rabbit IgGs (primary antibody). SDS-PAGE (A) and Western-blot (B) of scorpion venoms. MPM means the molecular mass markers (kDa); lanes 1 to 7 are venoms from 1 - *C. panamensis*; 2 - *C. granosus*; 3 - *C. bicolor*; 4 - *T. pachyurus*; 5 - *T. cerroazul*; 6 - *T. asthenes*; 7 - *T. festae*. The primary antibody used (1:50) was purified rabbit antibodies (IgGs), and then the second antibody was goat anti-rabbit IgG coupled to alkaline-phosphatase at 1:2000.

To evaluate the recognition of the rabbit IgGs to the Panamanian scorpion venoms, SDS-PAGE and Western-blot experiments were performed (Fig. 3). The SDS-PAGE gel shows the presence of venom components containing molecular weights of known enzymes, such as hyaluronidases (band around 37–50 kDa) for all venoms, the presence of proteases (band around 25–37 kDa) for the venom of *C. panamensis*, *T. pachyurus*, *T. cerroazul*, *T. asthenes*, and *T. festae*, the presence of phospholipases (band around 13–17 kDa for *T. pachyurus*, *T. cerroazul*; *T. asthenes*, and *T. festae*). Yet, the Western blot experiments shows that the antibodies produced recognize the components that elute around the 10 kDa band (mostly NavToxins) different from that reported by Borges et al. (2020) where the Venezuelan antivenom weakly recognizes the bands around 10 kDa, but recognizes the bands with higher molecular weights with considerable intensity.

The median effective dose (ED₅₀) of the obtained IgGs was calculated (Fig. 4) for the venoms of *T. pachyurus*, *T. cerroazul* and *C. bicolor* by implementing the 3Rs in the preclinical evaluation of antivenom (Gutiérrez et al., 2017). The purified antibodies presented an ED₅₀ of 0.74 (0.54–1.02), 0.41 (0.30–0.57) and 0.61 (0.42–0.88) mg venom/mL antivenom for *T. pachyurus*, *T. cerroazul*, and *C. bicolor*, respectively (Fig. 4). For the Panamanian scorpion venoms, the effective doses of the Venezuela antivenom were calculated in CD-1 strain of mice using 1.5 LD₅₀ as challenge dose. The results were an ED₅₀ of 0.47 (0.36–0.62), 0.40 (0.29–0.48) and 0.16 (0.12–0.21) mg venom/mL antivenom for *T. pachyurus*, *T. cerroazul*, and *C. bicolor*, which shows that with only using two toxins present in the venom of *T. pachyurus*, a better neutralization is obtained than a commercial antivenom. For the venom of *T. pachyurus* and *C. bicolor*, the 100% survival group showed only mild intoxication symptoms in the first 30 min (Salazar et al., 2018). After

inoculation, the 100% survival group injected with *T. cerroazul* venom showed symptoms of moderate intoxication that were maintained after 1 h of inoculation but survived under the parameters of the experiment. This fact may lead to the need to include within the immunization pool a second toxin from the *T. cerroazul* venom, which may cause such intoxication symptoms and it is imperative to neutralize. This toxin could be either with a molecular mass of 6995.9 Da or 7046.0 Da, which were also previously described (Salazar et al., 2018).

4. Discussion

Panama has a diverse medical important scorpion fauna corresponding to species of the genus *Tityus* and *Centruroides*. From a clinical point of view, it is very difficult to differentiate the symptoms caused by the sting of these species, which may require the need of antivenom to neutralize the venom effects of such species of these two genera of scorpions (Borges et al., 2012).

The production of recombinant proteins as antigens has been described and its application to elaborate antivenoms for animal envenomation's has been depicted in recent years (Clement et al., 2019a; de la Rosa et al., 2019; Jiménez-Vargas et al., 2017; Olvera et al., 2006). The implementation of the use of recombinant toxins as a supplement or alternative to the use of complete scorpion venom in the production of antivenoms has become a reliable alternative to obtain a commercial product with the same, or perhaps better, level of neutralization than those currently produced (Mendes et al., 2008).

The primary structures of four scorpion toxins were elucidated (Tppa1, Tppa2, Tce3, and Cbi1). All four toxins have some significant identities to known toxic peptides from scorpion venoms from Brazil,

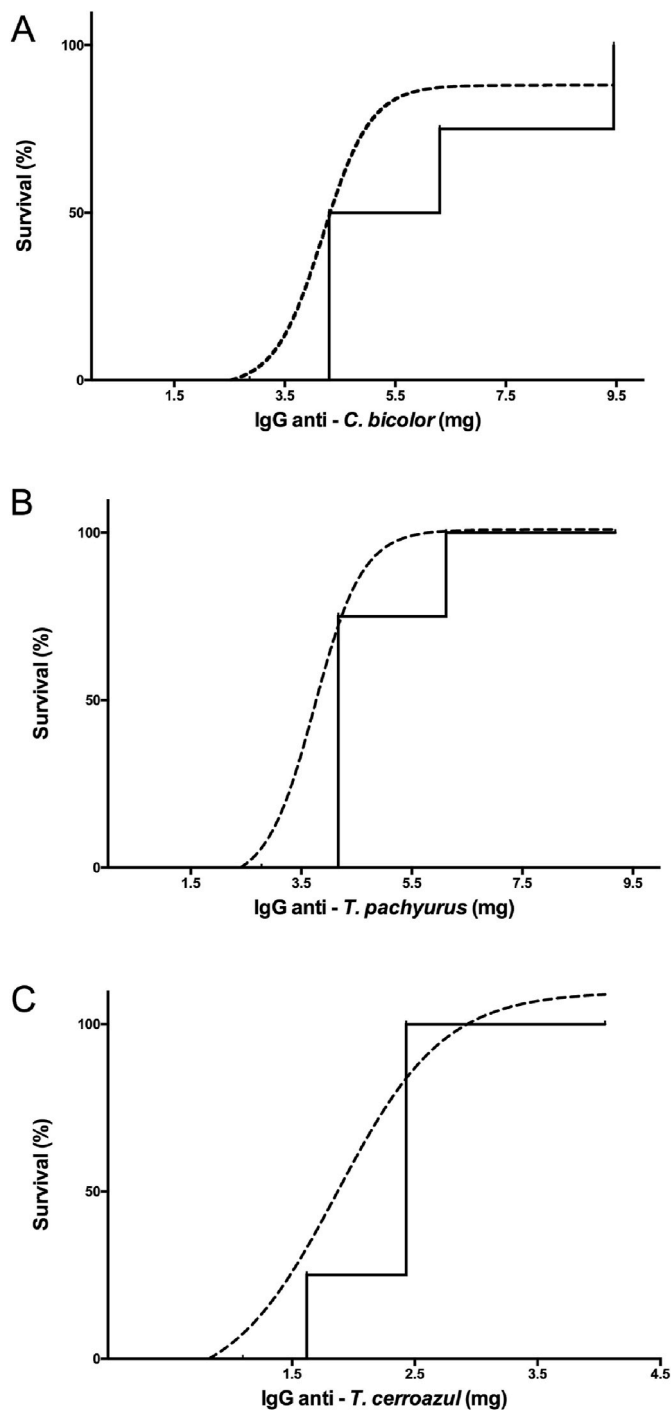


Fig. 4. Effective dose (ED_{50}) values of rabbit antibodies to neutralize *C. bicolor*, *T. pachyurus* and *T. cerroazul*. The graphs show the Kaplan-Meier survival curves (solid line) including the non-linear curves (dotted line) to calculate the ED_{50} s. The venom 1.5 LD_{50} from *C. bicolor*, *T. pachyurus* and *T. cerroazul* correspond to 94.5, 91.8 and 24.3 μ g of venom/mouse (18 g), respectively. The calculated ED_{50} for *C. bicolor* (A), *T. pachyurus* (B) and *T. cerroazul* (C) were 0.018, 0.024 and 0.013 μ g of venom/ μ g IgGs, respectively.

Venezuela and Mexico, which may be important for the molecular recognition of commercial antibodies towards the scorpion venoms from Panama. However, a good recognition of a complex antigen, like the entire venom of the scorpion, does not necessarily mean that the antibodies will neutralize the toxic effect of the venom.

All four toxins were recombinantly expressed taking in mind to obtain large amounts of toxin; however, because toxin isoforms were

generated, the yield of toxin with biological activity was very low. To improve the yield of biologically active toxins, different bacterial strains as well as modification of the expression media were tested (Table S5). These variable conditions were important for having enough material for immunization of rabbits. This is also an important point for immunization of large animals such as horses (de la Rosa et al., 2019), making possible to obtain from 2 to 5 mg of recombinant protein per liter of culture medium. The recovery was done from inclusion bodies. The expressed protein obtained from inclusion bodies was mainly aggregated and miss-folded protein that was dissolved with the help of a chaotropic agent and separated by reverse phase HPLC technique using C_{18} columns (Hernández-Salgado et al., 2012; Saucedo et al., 2012). However, with quantities of 100 μ g of protein in analytical C_{18} columns, a good separation (resolution) of the isoforms was not achieved. However, it was found that by replacing the type of the hydrophobic interaction of the stationary phase of the column (biphenyl column) a better separation was accomplished up to injection quantities of 300–350 μ g where it was possible to purify a worth quantity of a protein fraction with lethal activity (15–20 μ g ic in mice) (Fig. 1). Although other studies have shown that the use of the all protein expressed in inclusion bodies can generate neutralizing antibodies (Mendes et al., 2008), in this work, we purified the inclusion bodies and selected the toxic fraction to mice. Toxic fractions present structure epitopes, which are better immunogens than the non-active proteins (Calderon-Aranda et al., 1999).

Concerning the immunization protocol presented here, it was designed taking into account previous experience with another group of rabbits, which were immunized with all four recombinant toxins simultaneously. However, the resulting antibodies did not neutralize the lethal effect of *C. bicolor* venom but did neutralize only that of *T. pachyurus* and *T. cerroazul*. Therefore, we consider that there could be an immunodominance effect of the *Tityus* toxins in the production of antibodies, similar to that reported for *Lachesis stenophrys* venom, which reduces the equine antibody response towards *Bothrops asper* venom (Arroyo et al., 2015). For this reason, a change in the immunization protocol was considered that allowed us to obtain neutralizing antibodies for the four target neurotoxins and their respective scorpion venoms. After finishing the immunization protocol, the purified antibodies presented an ED_{50} of 0.74 mg/mL (0.54–1.02) for *T. pachyurus*, 0.41 mg/mL (0.30–0.57) for *T. cerroazul* and 0.61 mg/mL (0.42–0.88) for *C. bicolor*.

The Western blot and ELISA experiments show that the rabbit antibodies produced can recognize, besides the toxins from *T. pachyurus*, *T. cerroazul*, and *C. bicolor*, also components of the venoms from *T. festae*, *T. asthenes*, and *C. granosus* (Fig. 3). This may be due to the fact that Tppa1 is also present within the venoms of *T. asthenes* and *T. festae* (Borges et al., 2020; Salazar et al., 2018), and also it may be due to the significant sequence similarity of Tppa1 and Tppa2 to other *Tityus* sp. toxins. The cross-neutralization effect has already been studied previously for the venom of *T. pachyurus* from Colombia and the commercial antivenoms from Brazil (ED_{50} = 0.29 mg/mL (0.23–0.37), Mexico (ED_{50} 0.33 mg/mL (0.26–0.41 mg/mL) and Venezuela (ED_{50} not determined by low potency) (Barona et al., 2004). For Panamanian scorpion venoms, the effective doses of the Venezuela antivenom were calculated in CD-1 strain of mice using 1.5 LD_{50} as challenge dose. The results were an ED_{50} of 0.47 mg/mL (0.36–0.62) for *T. pachyurus* from Panama, an ED_{50} of 0.40 mg/mL (0.29–0.48) for *T. cerroazul* and an ED_{50} of 0.16 mg/mL (0.12–0.21) for *C. bicolor*, which shows that with only using two toxins present in the venom of *T. pachyurus*, a better neutralization is obtained than a commercial antivenom.

5. Conclusions

The data presented here demonstrate the proof of concept that the use of the most toxic neurotoxins present in scorpion venoms, obtained by recombinant means, and used as immunogens can produce neutralizing antibodies against the lethal effect caused by venoms of three

species of scorpions of medical importance in Panama. To improve the neutralization towards the venom of *T. cerroazul*, *T. asthenes* and *T. festae* with a view for producing IgGs in horses, it may be necessary to include more recombinant toxins from these scorpion venoms in the immunization process. According to our experience, they could be the neurotoxins either with molecular masses of 6995.9 Da or 7046.0 Da for *T. cerroazul*, 7073.6 Da for *T. festae* and 7101.5 for *T. asthenes* (Salazar et al., 2018). These IgGs produced in horses must be tested using challenge doses equivalent to at least 3 LD₅₀ of each venom. Future studies of the transcriptome or proteome of these Panamanian scorpions will be necessary to elucidate the sequences of the recommended toxins.

Ethical statement

No experiments with humans were performed. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed, and procedures performed in the present study involving animals were done so in accordance with the bioethical standards at the “Instituto de Biotecnología - UNAM”.

Consent to participate

All researches listed voluntarily agree to participate in this research study.

Consent for publication

All researches agree for publication.

Availability of data and materials

All data generated during this study will be available upon request.

Credit author statement

Marcos H. Salazar: Conceptualization, Methodology and Formal analysis, Writing, Herlinda Clement: Methodology and Formal analysis, Ligia L. Corrales-García: Methodology and Formal analysis, Jairo Sánchez: Methodology and Formal analysis, John Cleghorn: Methodology and Formal analysis, Fernando Zamudio: Methodology and Formal analysis, Lourival D. Possani: Funding acquisition, Writing – review & editing, Hildauro Acosta: Writing – review & editing, Funding acquisition, Gerardo Corzo: Conceptualization, Writing, Writing – review & editing, Funding acquisition.

Funding statement

This work was financed by grants from Panamanian funds from SENACYT (COL10-045, INF10-051), Ministerio de Ambiente (UNDP-GEF ABS Global Project) and Vicerrectoría de Investigación y Postgrado, Universidad de Panamá; and Mexican funds from the Dirección General de Asuntos del Personal Académico (DGAPA-UNAM) grant number IN202619 to LDP and grant number IT200321 to GC. Also, we acknowledge the funds from CONACyT-FORDECYT (grant number 303045) awarded to the Alagon-Becerril-Corzo-Possani research group consortium.

Marcos H. Salazar is a Ph.D. fellow from *Programa de Doctorado en Ciencias Biomédicas (PDCB)* from Universidad Nacional Autónoma de México (UNAM) and he was recipient of a PhD scholarship (#518494470) from CONACyT.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors acknowledge to M. Sc. Timoteo Olamendi-Portugal, Mr. Ricardo Mondragon, BSc. Felipe Olvera, and Dra. Gloria Saab for their help in protein enzymatic digestions, immunization of rabbits, IgGs purification, and circular dichroism experiments, respectively. The authors also thank Carolina Guevara and Maribel Barría for in field collection of scorpions, keeping them in captivity and venom extraction. The patent filling advice from M. Sc. Martin Patiño and MBA Mario Trejo is also greatly acknowledged. We thank the Instituto de Biotecnología of UNAM for giving us access to its computer cluster.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.toxcx.2021.100090>.

References

- Arroyo, C., Solano, S., Herrera, M., Segura, Á., Estrada, R., Vargas, M., Villalta, M., Gutiérrez, J.M., León, G., 2015. *Lachesis stenophrys* venom reduces the equine antibody response towards *Bothrops asper* venom used as co-immunogen in the production of polyspecific snake antivenom. *Toxicol* 103, 99–105.
- Barona, J., Otero, R., Núñez, V., 2004. Aspectos toxicológicos e inmunológicos del veneno del escorpión *Tityus pachyurus* Pocock de Colombia: capacidad neutralizante de antivenenos producidos en Latinoamérica. *Biomedica* 24, 42–49.
- Borges, A., 2015. Scorpionism and dangerous scorpions in Central America and the caribbean region. In: Gopalakrishnakone, P., Possani, L.D., F Schwartz, E., Rodríguez de la Vega, R.C. (Eds.), *Scorpion Venoms*. Springer Netherlands, Dordrecht, pp. 215–244.
- Borges, A., Miranda, R.J., Pascale, J.M., 2012. Scorpionism in Central America, with special reference to the case of Panama. *J. Venom. Anim. Toxins Incl. Trop. Dis.* 18, 130–143.
- Borges, A., Lomonte, B., Angulo, Y., Acosta de Patiño, H., Pascale, J.M., Otero, R., Miranda, R.J., De Sousa, L., Graham, M.R., Gómez, A., Pardal, P.P.O., Ishikawa, E., Bonilla, F., Castillo, A., de Avila, R.A.M., Gómez, J.P., Caro-López, J.A., 2020. Venom diversity in the Neotropical scorpion genus *Tityus*: implications for antivenom design emerging from molecular and immunochemical analyses across endemic areas of scorpionism. *Acta Trop.* 204, 105346.
- Calderon-Aranda, E.S., Selisko, B., York, E.J., Gurrola, G.B., Stewart, J.M., Possani, L.D., 1999. Mapping of an epitope recognized by a neutralizing monoclonal antibody specific to toxin Cn2 from the scorpion *Centruroides noxious*, using discontinuous synthetic peptides. *Eur. J. Biochem.* 264, 746–755.
- Candido, D.M., Lucas, S., 2004. Maintenance of scorpions of the genus *Tityus koch* (scorpiones, buthidae) for venom obtention at Instituto butantan, são paulo, Brazil. *J. Venom. Anim. Toxins Incl. Trop. Dis.* 10, 86–97.
- Chippaux, J.P., Goyffon, M., 2008. Epidemiology of scorpionism: a global appraisal. *Acta Trop.* 107, 71–79.
- Clement, H., Corrales-García, L.L., Bolanos, D., Corzo, G., Villegas, E., 2019a. Immunogenic properties of recombinant enzymes from *Bothrops ammodytoides* towards the generation of neutralizing antibodies against its own venom. *Toxins* 11, 702.
- Clement, H., Corzo, G., Neri-Castro, E., Arenas, I., Hajos, S., de Roodt, A.R., Villegas, E., 2019b. cDNA cloning, heterologous expression, protein folding and immunogenic properties of a phospholipase A2 from *Bothrops ammodytoides* venom. *Protein Expr. Purif.* 154, 33–43.
- de la Rosa, G., Olvera, F., Archundia, I.G., Lomonte, B., Alagon, A., Corzo, G., 2019. Horse immunization with short-chain consensus alpha-neurotoxin generates antibodies against broad spectrum of elapid venomous species. *Nat. Commun.* 10, 3642.
- de Roodt, A.R., Garcia, S.L., Salomon, O.D., Segre, L., Dolab, J.A., Funes, R.F., de Titto, E. H., 2003. Epidemiological and clinical aspects of scorpionism by *Tityus trivittatus* in Argentina. *Toxicol* 41, 971–977.
- de Salud, Ministerio, 2017. Situación Epidemiológica de las Picaduras de Alacrán en la República de Panamá. Años: 2000-2016.
- D', Suze, G., Castillo, C., Sevcik, C., Brazón, J., Malave, C., Hernandez, D., Zerpa, N., 2015. Scorpionism and dangerous species of Venezuela. In: Gopalakrishnakone, P., Possani, L.D., F Schwartz, E., Rodríguez de la Vega, R.C. (Eds.), *Scorpion Venoms*. Springer Netherlands, Dordrecht, pp. 273–298.
- Guerrero-Vargas, J.A., Rodríguez Buitrago, J.R., Ayerbe, S., Flórez Daza, E., Beltrán Vidal, J.T., 2015. Scorpionism and dangerous species of Colombia. In: Gopalakrishnakone, P., Possani, L.D., F Schwartz, E., Rodríguez de la Vega, R.C. (Eds.), *Scorpion Venoms*. Springer Netherlands, Dordrecht, pp. 245–272.
- Gutiérrez, J.M., Rojas, G., Lomonte, B., Gené, J.A., Chaves, F., 1990. Evaluación de la capacidad neutralizante de los antivenenos en América. Instituto Clodomiro Picado, Universidad de Costa Rica, San José, CR, pp. 1–21.
- Gutiérrez, J.M., Solano, G., Pla, D., Herrera, M., Segura, Á., Vargas, M., Villalta, M., Sánchez, A., Sanz, L., Lomonte, B., León, G., Calvete, J.J., 2017. Preclinical evaluation of the efficacy of antivenoms for snakebite envenoming: state-of-the-art and challenges ahead. *Toxins* 9.

- Hernández-Salgado, K., Estrada, G., Olvera, A., Coronas, F.I., Possani, L.D., Corzo, G., 2009. Heterologous expressed toxic and non-toxic peptide variants of toxin CssII are capable to produce neutralizing antibodies against the venom of the scorpion *Centruroides suffusus suffusus*. *Immunol. Lett.* 125, 93–99.
- Hernández-Salgado, K., Possani, L.D., Corzo, G., 2012. 174. In vitro folding of a recombinant beta-scorpion neurotoxin: the influence of N-terminal hydrophobic regions. *Toxicon* 60, 185.
- Jiménez-Vargas, J.M., Quintero-Hernández, V., González-Morales, L., Ortiz, E., Possani, L.D., 2017. Design and expression of recombinant toxins from Mexican scorpions of the genus *Centruroides* for production of antivenoms. *Toxicon* 128, 5–14.
- Laurenço, W.R., 1986. *Tityus cerroazul*, nouvelle espèce de Scorpion de Panama (Scorpiones, Buthidae). *Bull. Mus. Natl. Hist. Nat.* 8, 637–641.
- Mendes, T.M., Dias, F., Horta, C.C., Pena, I.F., Arantes, E.C., Kalapothakis, E., 2008. Effective *Tityus serrulatus* anti-venom produced using the Ts1 component. *Toxicon* 52, 787–793.
- Micsonai, A., Wien, F., Bulyaki, E., Kun, J., Moussong, E., Lee, Y.H., Goto, Y., Refregiers, M., Kardos, J., 2018. BeStSel: a web server for accurate protein secondary structure prediction and fold recognition from the circular dichroism spectra. *Nucleic Acids Res.* 46, W315–W322.
- Miranda, R.J., de Armas, L.F., 2020. A new species of *anateris* (scorpiones: buthidae) from Panama. *Euscorpium* 1–7.
- Olvera, A., Ramos-Cerrillo, B., Estevez, J., Clement, H., de Roodt, A., Paniagua-Solis, J., Vazquez, H., Zavaleta, A., Arroz, M.S., Stock, R.P., Alagon, A., 2006. North and South American *Loxosceles* spiders: development of a polyvalent antivenom with recombinant sphingomyelinases D as antigens. *Toxicon* 48, 64–74.
- Otero, R., Navio, E., Cespedes, F.A., Nunez, M.J., Lozano, L., Moscoso, E.R., Matallana, C., Arsuza, N.B., Garcia, J., Fernández, D., Rodas, J.H., Rodriguez, O.J., Zuleta, J.E., Gómez, J.P., Saldarriaga, M., Quintana, J.C., Núñez, V., Cárdenas, S., Barona, J., Valderrama, R., Paz, N., Diaz, A., Rodriguez, O.L., Martinez, M.D., Maturana, R., Beltrán, L.E., Mesa, M.B., Paniagua, J., Flórez, E., Lourenço, W.R., 2004. Scorpion envenoming in two regions of Colombia: clinical, epidemiological and therapeutic aspects. *Trans. R. Soc. Trop. Med. Hyg.* 98, 742–750.
- Possani, L.D., Becerril, B., Delepiepierre, M., Tytgat, J., 1999. Scorpion toxins specific for Na-channels. *Eur. J. Biochem.* 264, 287–300.
- Rojas, G., Jimenez, J.M., Gutierrez, J.M., 1994. Caprylic acid fractionation of hyperimmune horse plasma: description of a simple procedure for antivenom production. *Toxicon* 32, 351–363.
- Salazar, M.H., Arenas, I., Corrales-García, L.L., Miranda, R., Velez, S., Sanchez, J., Mendoza, K., Clegghorn, J., Zamudio, F.Z., Castillo, A., Possani, L.D., Corzo, G., Acosta, H., 2018. Venoms of *Centruroides* and *Tityus* species from Panama and their main toxic fractions. *Toxicon* 141, 79–87.
- Saucedo, A.L., del Rio-Portilla, F., Picco, C., Estrada, G., Prestipino, G., Possani, L.D., Delepiepierre, M., Corzo, G., 2012. Solution structure of native and recombinant expressed toxin CssII from the venom of the scorpion *Centruroides suffusus suffusus*, and their effects on Nav 1.5 sodium channels. *Biochim. Biophys. Acta* 1824, 478–487.
- Sevcik, C., D'Suze, G., 2021. New insights on the pharmacokinetics of venoms and antivenoms. In: Gopalakrishnakone, P., Ferroni Schwartz, E., Possani, L.D., Rodríguez de la Vega, R.C. (Eds.), *Scorpion Venoms: Scorpion Venoms*. Springer Netherlands, Dordrecht, pp. 1–25.
- Vázquez, H., Chávez-Haro, A., García-Ubbelohde, W., Paniagua-Solis, J., Alagón, A., Sevcik, C., 2010. Pharmacokinetics of a F(ab')₂ scorpion antivenom administered intramuscularly in healthy human volunteers. *Int. Immunopharm.* 10, 1318–1324.
- Venancio, E.J., Portaro, F.C.V., Kuniyoshi, A.K., Carvalho, D.C., Pidde-Queiroz, G., Tambourgi, D.V., 2013. Enzymatic properties of venoms from Brazilian scorpions of *Tityus* genus and the neutralisation potential of therapeutical antivenoms. *Toxicon* 69, 180–190.
- Viquez, C., de Armas, L.F., Lourenço, W.R., 2005. Presencia de *Tityus cerroazul* Lourenço, 1986 Scorpiones: buthidae en Costa Rica y descripción del macho. *Boletín de la Sociedad Entomológica Aragonesa* 36, 93–96.
- WHO, 1981. Progress in the Characterization of Venoms and Standardization of Antivenoms. World Health Organization, Geneva, Switzerland.
- WHO, 2016. In: Organization, W.H. (Ed.), *World Health Organization Guidelines for the Production Control and Regulation of Snake Antivenom Immunoglobulins*. World Health Organization, Geneva, Switzerland.