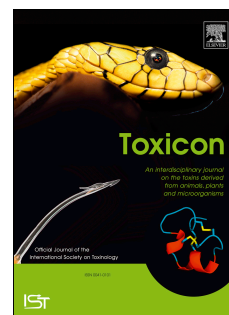


Accepted Manuscript

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PII: S0041-0101(18)30006-0

DOI: [10.1016/j.toxicon.2018.01.006](https://doi.org/10.1016/j.toxicon.2018.01.006)

Reference: TOXCON 5795

To appear in: *Toxicon*

Received Date: 21 November 2017

Revised Date: 9 January 2018

Accepted Date: 10 January 2018

Please cite this article as: Batista, C.V.F., Martins, J.G., Restano-Cassulini, R., Coronas, F.I.V., Zamudio, F.Z., Procópio, R., Possani, L.D., Venom characterization of the Amazonian scorpion *Tityus metuendus*, *Toxicon* (2018), doi: 10.1016/j.toxicon.2018.01.006.

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Venom characterization of the Amazonian scorpion *Tityus metuendus*

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Summary

The soluble venom from the scorpion *Tityus metuendus* was characterized by various methods. *In vivo* experiments with mice showed that it is lethal. Extended electrophysiological recordings using seven sub-types of human voltage gated sodium channels (hNav1.1 to 1.7) showed that it contains both α - and β -scorpion toxin types. Fingerprint analysis by mass spectrometry identified over 200 distinct molecular mass components. At least 60 sub-fractions were recovered from HPLC

separation. Five purified peptides were sequenced by Edman degradation, and their complete primary structures were determined. Additionally, three other peptides have had their N-terminal amino acid sequences determined by Edman degradation and reported. Mass spectrometry analysis of tryptic digestion of the soluble venom permitted the identification of the amino acid sequence of 111 different peptides. Search for similarities of the sequences found indicated that they probably are: sodium and potassium channel toxins, metalloproteinases, hyaluronidases, endothelin and angiotensin-converting enzymes, bradykinin-potentiating peptide, hypothetical proteins, allergens, other enzymes, other proteins and peptides.

Keywords: amino acid sequence; Na⁺-channel; proteome analysis; scorpion venom; *Tityus metuendus*

1. Introduction

Scorpions of the family Buthidae are known to contain toxic peptides that can be dangerous to humans (Chippaux and Goyffon, 2008). From the Latin American continent there are at least two genera from this family that might cause intoxication symptoms: *Centruroides* distributed basically in the North region (mainly Mexico and United States of America) and *Tityus* found from Central America to South American countries (distributed from Panamá to Argentina) (Borges, 2015; Santibáñez-López et al., 2015).

The focus of this communication refers to the genus *Tityus* (here after abbreviated *T.*). However, since this is not a review article we mention only some representative references to help the reader to be familiar with the subject. From the genus *Tityus* the best studied scorpion venom components are from *T. serrulatus*, *T. stigmurus* and *T. bahiensis* (Possani et al., 1981, 1985, 1991; Becerril et al., 1996, 1997; Martin-Eauclaire et al., 1994; De Lima and Martin-Eauclaire, 1995; Maragoni et al., 1995; Pinheiro et al. 2003; Batista et al., 2007; Petricevich et al., 2008; Martin-Eauclaire et al., 2016). Another well studied scorpion is *T. discrepans* from Venezuela (D'Suze et al., 1996, 1999, 2004; Batista et al., 2006; Prochnika-Chalufour et al., 2006). *T. cambridgei* and its synonymous species *T. obscurus* is another known scorpion venom (Batista et al., 2000, 2002a, 2002b, 2004; Murgia et al., 2004; Guerrero-Vargas et al., 2012). From Colombia one of the species studied is *T. pachyurus* (Barona et al., 2006; Guerrero-Vargas et al., 2012). *T. costatus* from Brazil was

also studied (Diego-Garcia et al., 2005, 2007). *T. trivittatus* is the best studied from Argentina (Coronas et al., 2003, 2014; Roodt et al., 2010). The Amazonian region contains many species of *Tityus* but they are not well studied, even regarding the systematic classification of their names (Lourenço, 2002 and 2006). One of these scorpions belongs to the species *T. metuendus*. This scorpion is known to cause human intoxication (Souza et al., 1995, Queiroz et al., 2015), but its venom, to the best of our knowledge, has not been described in detail (see review by Pucca et al, 2015). Concerning the venom components of the dangerous species of scorpions, many toxic peptides have been reported (see review Possani et al., 1999, 2000). These are known to be relatively short peptides. There are at least two types of peptides that are responsible for the symptomatology caused by their venoms: the short chain peptides with approximately 40 amino acids that affect K^+ -channels (mostly channel blockers), and the long chain peptides comprising 59 to 72 amino acids that are modulators of Na^+ -channels, affecting the mechanism of channel gating (Rodríguez de la Vega and Possani, 2004, 2005).

Here we report a general proteomic analysis of the soluble venom from the scorpion *T. metuendus*, the separation of the venom components by high performance liquid chromatography, the amino acid sequence of some of the short and long chain peptides, as well as the experimentally determined sequences of 111 peptides obtained by tryptic hydrolysis of the whole soluble venom. A substantial electrophysiological analysis of this soluble venom on sodium ion-channels is also included here. This is the first time that an in depth physiological analysis of venom is performed for any of the species of genus *Tityus* of the Amazonian Region of Brazil. Earlier, Cologna et al (2012) have reported electrophysiological effects of toxin Ts2 from *T. serrulatus*, which occurs more in the Central part of Brazil.

2. Material and Methods

2.1 Venom collection

Scorpions were collected in the city of Manaus, state of Amazonas, Brazil (3° 03'46" S 59° 59' 20" W) with permission from the *Ministerio do Meio Ambiente*, number 56748-1. The specimens were identified according to Lourenço (2002). The venom of 40 male and

female specimens were milked for venom by electrical stimulation (12 V) and freeze-dried. The sample was fractionated and characterized in the Mexican laboratory. The dried venom was dissolved in 3.95 mL of water and centrifuged 15000 *g* for 15 min at 4 °C. The whole soluble protein recovered from the supernatant of this sample was estimated to be 26.07 mg protein, assuming one unit of absorbance at 280 nm equal to 1 mg/mL.

2.2 Biological assays *in vivo*

The whole soluble venom was assayed *in vivo*, using mice strain CD1 (25 g body weight) by intraperitoneal injection. The protocol used for assaying the activity of peptides *in vivo* was followed according to the guidelines of our Institute Committee of Animal Welfare, keeping the number of animals to a minimum, in order to avoid inflicting pain to mammalian animals. Venom amounts varying from 35 to 200 micrograms per animal were assayed. Confirmatory activities were conducted by electrophysiological assays *in vitro*, as described in section 2.5.

2.3 High performance liquid chromatography separation

The venom was separated by high performance liquid chromatography (HPLC) using a C₁₈ reversed-phase column (Vydac, Hiperia, California, USA). The HPLC profile was obtained each time by fractionating aliquots of venom of 2.15 mg protein content. The gradient run from solution A (0.12% trifluoroacetic acid in water) to 60% solution B (0.10% TFA in acetonitrile), over 60 min. Fractions were collected manually and directly used or freeze-dried for future analysis.

2.4 Amino acid sequence of peptides

Purified peptides (*circa* 0.5 nmol) were sequenced by direct Edman degradation using a PPSQ-31A Protein Sequencer from Shimadzu Scientific Instruments, Inc. (Columbia, Maryland, USA). The amino acid sequence was obtained by using both native peptide and reduced and alkylated samples, prepared as earlier described by our group (Olamendi-Portugal et al., 2016). In order to get homogeneous peptides for sequencing analysis,

several fractions were alkylated and further purified by reversed-phase HPLC in the same conditions as described in item 2.3.

2.5 Electrophysiological recordings

Electrophysiological experiments were performed essentially as earlier described (Restano-Casulini et al., 2017). Cells in culture expressing various sub-types of sodium channels were a gift from Professor Enzo Wanke from the University of Milano-Bicocca, Italy. The cells were maintained in incubation at 37 °C with 5% CO₂ and 95% humidity. Dulbecco's Modified Eagle Medium (DMEM) (SIGMA, Toluca Mexico), supplemented with 10% Fetal Bovine Serum (FBS) (Byproducts, Guadalajara Mexico) were used for culture. HEK cells expressing human sodium channels (hNav) sub-types 1.1 to 1.6 and CHO cells expressing hNav 1.7 were used.

Extracellular solution contained in mM: 130 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, 5 glucose, pH 7.3 adjusted with NaOH. Intracellular solution contained in mM: 105 CsF, 27 CsCl, 5 NaCl, 2 MgCl₂, 10 EGTA, 10 HEPES, pH 7.3 adjusted with CsOH.

Sodium currents were elicited by means of a step depolarization during 100 ms, from -120 mV to 40 mV with 10 mV increments, followed by a 50 ms step at full-activation potential (-10 mV or -30 mV in the case of hNav 1.5 channels). Holding potential was set at -120 mV and a short strong depolarization pre-pulse (5 ms at 50 mV) was applied 50 ms before the depolarization steps. For the activation process, the channel conductance (G) at each potential was calculated from the relation: $G = I / (V_m - E_{Na})$ where I is the peak current elicited at V_m potential and E_{Na} is the Nernst equilibrium potential for Na⁺, experimentally calculated for each cell. Normalized conductance (G/G_{max}) was graphed against test potentials. For the inactivation process, current was tested at full activation potential after a depolarization at different potentials. Normalized current (I/I_{max}) was graphed against the preconditioning potentials. For all sodium channels (except hNav 1.5), data from at least 3 cells were averaged and then fitted using a Boltzmann equation or the sum of two Boltzmann equation, as earlier described by Schiavon et al., 2006. Current acquisitions were performed by means the MultiClamp 700B amplifier along with the analog-digital converter Digidata 1440A and software pClamp10 (Molecular Devices, Sunnyvale CA,

USA). Data were analyzed with the software Clampfit10 (Molecular Devices Sunnyvale CA, USA) and Origin 7 (OriginLab, Northampton MA, USA).

2.6 Mass spectrometry analysis

Two different instruments were used. Quick information regarding identification of molecular masses of fractions obtained by HPLC used a Thermo Scientific LCQ Fleet™ ion trap mass spectrometer (San Jose, CA) was used. The samples were dried on a Savant Speed-Vac (Thermo Scientific, San Jose, CA) and reconstituted in 60% acetonitrile with 1% acetic acid and directly applied (5 µL) into the equipment. The spray voltage was set to 1.6 kV and the capillary temperature was set to 180 °C. All spectra were obtained in the positive-ion mode. For the complete proteomic analysis a LC-MS/MS system composed by a nano-flow pump Dionex UltiMate 3000 and an Orbitrap Velos mass spectrometer with a nano-spray ion source both from Thermo-Fisher Scientific (San Jose, CA) was used.

2.6.1. Mass fingerprint determination

The mass fingerprint of the venom components was obtained by LC-MS analysis of 1.0 µg of soluble venom. An online separation of the components using a 30 cm length C₁₈ reversed-phase micro-column (0.5 µm particle size) made in house was performed before molecular mass determination. The full MS data was collected using 60,000 of resolution-power into an Orbitrap Velos mass spectrometer. Molecular masses up to 3 kDa were reported as monoisotopic mass and masses higher than 3 kDa as average mass according Xcalibur-Xtract program for automatic deconvolution.

2.6.2. Sample preparation for LC-MS/MS analysis

Lyophilized venom was reconstituted with 50 mM ammonium bicarbonate, centrifuged at 14,000 rpm for 10 minutes and the supernatant was reduced with 10 mM dithiothreitol (DTT), alkylated with 50 mM iodoacetamide and enzymatically digested in solution with trypsin (Promega, Madison, WI). Finally, the solution containing the peptides was desalted by C₁₈ ZipTips (Millipore), concentrated in a Savant SPD 1010 SpeedVac (Thermo-Fisher

Scientific, San Jose, CA) and reconstituted with 5 µl of 0.1% formic acid for LC-MS/MS analysis.

2.6.3. LC-MS/MS analysis

Complex mixtures of tryptic peptides generated by in solution digestion were analyzed by a LC-MS system using a reversed-phase (RP) C₁₈ capillary column constructed in house (30 cm length; 0.5 mm ID), which was filled with a C-18 Jupiter silica with 4 µm particle size from Phenomenex (Torrance, CA). Gradient elution was performed from 5% to 80% solvent B during 120 min, maintaining a flowrate of 300 nl/min. All spectra were collected in a Orbitrap Velos using positive ion mode and data dependent acquisition allowing the fragmentation from z: 2+ to z: 5+ ion charges. Acquisition method was constructed with dynamic exclusion set to maximum 120 ions, 30 s for pre-exclusion and 90 min for exclusion. The other acquisition parameters were 60,000 for resolution power, 3.0 Da of isolation width, 35 arbitrary units of normalized collision energy, 0.250 of Q-activation and 10 ms of activation time. CID (Collision Induced Activation) and HCD (High-energy Collision Dissociation) were used alternately and the spectra of both were integrated for data analysis and visualization.

2.6.4. MS data analysis

The raw data file was, firstly, analyzed by Peaks Studio and then manually analyzed for assignment of the amino acid sequences. All derived sequences were individually submitted to BLASTP program (NCBI) for protein identification using Scorpiones (taxid:6855) as database.

3. Results and Discussion

3.1 Biological effects and electrophysiological characterization

The soluble venom was shown to be very toxic to mammals. This venom was not assayed in other animals, although it is known that venom from this genus of scorpion does contain toxins specific for insects. *In vivo* experiments conducted with mice showed that the venom

is deadly. For this assay an aliquot of the recovered whole soluble venom lyophilized was weighed in a microbalance (1,960 micrograms). When dissolved in 1 mL water the absorbance reading at 280 nm was 2.07 units. This means that one unit at A280 nm contains 946 μ g of venom. From this whole soluble venom fraction we injected mice intraperitoneally (i.p.) with 20, 100, 200 and 300 micrograms equivalent of venom. Major intoxication symptoms were restless, piloerection, sialorrhea, hyperactivity, respiratory difficulties, partial paralysis of limbs, exophthalmus, loss of equilibrium, convulsions and dead. Minor intoxication was noticed with 20 μ g, but starting with 100 μ g the symptomatology of intoxication was very clear and with 300 μ g the mouse was dead within 38 minutes after injection, whereas that injected with 200 μ g died within 80 min after injection.

In addition to experiments performed with the whole soluble venom, we have separated the venom by chromatographic procedures, as described in section 2.3 and various sub-fractions were tested for *in vivo* symptomatic effects of intoxication.

Fractions of components eluting at 28.33 min (see Fig.1 in section 3.2) injected with protein equivalent to 37.5 μ g showed the mouse having respiratory problems with paralysis of rear limbs. A mouse injected with 278 μ g of fraction eluting at 34.59 min showed convulsions and died after 8 minutes of injection. A mouse injected with fraction eluting at 43.56 min showed paralysis of rear limbs and loss of equilibrium. Fraction elution at 54.25 min was not toxic.

Since earlier descriptions in the literature have reported that scorpion venoms contain specific toxic peptides that affect ion channel function in excitable cells (Possani et al., 1999), it was decided to assay the effect of the whole soluble venom using an electrophysiological system.

Soluble venom (TV) of the scorpion *Tityus metuendus* was tested for its activity on seven subtypes of human voltage gated sodium channels (hNav 1.1-hNav 1.7), using a concentration of 30 μ g/mL. Currents were elicited at different potential for obtaining the voltage dependence of the activation process, and then a pulse at full activation voltage was applied to obtain the voltage dependence of the inactivation process. In these experimental conditions, venom was able to change the gating properties of almost all analyzed sodium channel subtypes. In most cases, both α - and β -toxin effects were present, except for

hNav1.4 (not evident) and hNav1.7 that showed both effects (Fig.2, letters A-F). Specifically, if it is true for neuronal channels, it is not the same for hNav 1.4 and hNav 1.5 channels that are skeletal and cardiac muscular type respectively. The current of hNav1.5 is slightly modified by the venom with only small reduction of peak current of about 10% (panel E). The current of the hNav 1.4 suffers beta effect changing the voltage dependence of activation to more negative potential ($V_{1/2}$ shifts from -27.5 ± 0.3 mV to -30.9 ± 0.8 mV, see also Table 1). For this channel, the alpha effect was not visible (panel D). Among neuronal channels, hNav 1.3 was the most affected by the venom. In this current the shift of the activation curve was about 45 mV to more negative potential; also the inactivation curve changes to more negative potential (about 17 mV), while the inactivation time process became slower (panel C). The gating processes of hNav 1.1, hNav 1.2, hNav 1.6 and hNav 1.7 were sensible to the venom in a similar manner. All these channels showed a consistent beta and alpha effect (panel A, B, F, G). Parameters of the fittings are reported in the Table 1.

To the best of our knowledge this is the first study of the effect of a *Tityus* species of Amazonian scorpion venom, for which an extended electrophysiological recording is reported. Here we used the venom from *T. metuendus*, assayed in Na^+ channels hNav1.1 to hNav1.7. Toxicity to humans stung by this species of scorpion, certainly is due in great extent, to toxic peptides that affect the open and closing mechanism (gating) of Na^+ channels, known as the α - and β -scorpion toxin effects, as described here.

3.2 HPLC separation and amino acid sequence of peptides

The profile of venom separation is shown in Fig.1. At least 60 chromatographic peaks were resolved. Some components from prominent peaks were subjected to molecular weight determination by mass spectrometry and components labeled 1 to 5 (Fig.1) were used for sequence analysis. Fraction 1 eluting at 24.41 min in Fig. 1 was homogeneous, as judged by mass spectrometry measurements, allowing the determination of the primary structure. As shown in Fig.3 the amino acid sequence obtained for peptide 1, by direct Edman degradation, permitted obtaining most of the sequence, up to Glycine 34. The expected molecular mass for the segment of the peptide directly sequenced (from amino acid number 1 up to 34) is 3742.23 Da. The average molecular mass experimentally determined for this

peptide was 3927.26 Da, thus the only amino acid missing has the molecular weight corresponding to the amino acid tryptophan (of mass 186.21 Da) for a final molecular weight of 3928.30 Da, which is within the experimental error of our equipment. The physiological function of this peptide is not known. However it is closely related to peptides that usually bind to K⁺-channels. Actually this sequence is identical to one earlier deduced from DNA analysis of venom gland from the related species *T. obscurus*, accordingly to data deposited at EMBL/GenBank (UniProtKB - A0A1E1WVZ4 (A0A1E1WVZ4_TITOB), by Oliveira et al., 2015, but not yet formally published. Fraction 2 eluting at 24.66 min in Fig.1 seemed to be homogeneous by mass spectrometry, showing an average molecular mass of 1735.00 Da. It was sequenced by Edman degradation, allowing the identification of 17 amino acids, as indicated in Fig.3. The theoretical expected molecular mass for the first 17 residues is 1605.81 Da. The only missing amino acid for completion of the theoretical expected molecular weight of this peptide was glutamic acid, which adds to 1734.92 mass units, within the correct molecular mass expected. A segment of an identical peptide was found in a putative bradykinin-potentiating peptide reported in UniProtKB-A0A1E1WVX0 (A0A1E1WVX0 TITOB) of the scorpion *T. obscurus* (Oliveira et al., 2015)

Fraction 3 eluting at 28.33 min in Fig.1 was also homogeneous by mass spectrometry analysis, giving an experimentally determined average molecular mass of 4004.61 Da. A reduced and alkylated sample of this peptide was sequenced by automatic Edman degradation allowing the identification of the first 34 amino acids, up to residue lysine, as indicated in Fig. 3, which adds up to 3817.29 Da. In order to match the expected molecular weight of the native peptide a tryptophan was missing, for a final molecular weight of 4003.61 Da, within the experimental error of our equipment. This sequence is also identical to one earlier deduced from DNA analysis of venom gland from the related species *T. obscurus*, accordingly to deposited data (A0A1E1WVX0 TITOB) ,(Oliveira et al., 2015).

The physiological function of this peptide is not known, but it was investigated using Shaker-type ion-channels (data not shown), without positive results..

The component labeled 4 in Fig.1 eluting at 30.07 min, showed two peptides with experimental molecular masses of 7796.19 Da and 7767.80 Da. A sample containing 70 µg of this fraction was reduced and alkylated with iodoacetamide. The HPLC separation of the

peptides allowed obtaining the major component in pure form for sequence determination. The peptide has 69 amino acids, and the full amino acid sequence was obtained by direct Edman degradation in the first place. The reduced and alkylated peptide has a molecular mass of 8260.89 Da, corresponding to the native peptide of 7796.19 Da. It shows that the 8 cysteines are forming four disulfide linkages. The first 54 amino acids were identified (labeled -direct-), shown in Fig.3. The reduced and alkylated peptide was digested with endopeptidase V8 from *Staphylococcus aureus* (Olamendi-Portugal et al., 2016) and six peptides were obtained by HPLC separation (data not shown). The sequence of the peptides was determined by mass spectrometry. The overlapping sequences are shown in Fig.3, where the C-terminal sequence was obtained from residues Ser38 up to Val69. This peptide is similar, although not identical to the sequence of a peptide identified by DNA sequencing of putative peptides of the Amazonian scorpion *T. obscurus* (Oliveira et al., 2015). Different amino acids were found in the following corresponding positions: 3, 29, 37, 60, 61 and 69, which is not Arg, as that of *T. obscurus*. (UniprotKB - H1ZZI3 (SCX14_TITOB)).

Component labeled 5 in Fig.1 also contained two peptides with molecular weight of 7318.00 Da and 7385.00 Da. The sample was reduced and alkylated with iodoacetamide. The more abundant component, which corresponds to the peptide with 7318.00 Da of molecular weight, was fully sequenced. The N-terminal amino acid sequence was obtained for component of 7318.00 Da, as shown in Fig.3 (see peptide numbered 5). The N-terminal sequence up to residue number 30 (glutamic acid) was obtained by direct Edman sequencing. The reduced and alkylated peptide was also digested with endopeptidase V8 (Olamendi-Portugal et al., 2016) and the corresponding peptides were identified by mass spectrometry. The various segments are indicated according to the elution profile from the column after digestion. It has 65 amino acid residues with 4 disulfide bridges. The amino acid sequence is identical to a peptide, whose amino acid was deduced from DNA analysis of the parent scorpion *T. obscurus* (Oliveira et al., 2015), as shown in Fig.4. This figure shows amino acid sequence comparison of the two new long-chain peptides of Fig.3 (named query 1 and query 2) with other known peptides from scorpions of the genus *Tityus*. Only two sequences were selected; one with 96% and 100% identity and one with 58% and 64% identity, respectively.

In addition, several sub-fractions were reduced, alkylated and have had their N-terminal amino acid sequence determined. The principal component of fraction eluting at 24.41 min with molecular weight of 3927.00 Da was sequenced up to the amino acid 26, with the following sequence: TPFRYCNPRNCAKECQGRGKETTYCD. The component eluting at 37.26 min with molecular weight of 6961.00 Da, gave the following amino acid sequence: KEGYLLGSRGCKMNCLTxPGNYCELECSLVGGxNG. The component eluting at 42.10 min whose molecular mass was 7153.00 units, gave the following amino acid sequence: SRRGYPTQKGxVYSSFWGS N. Non identified amino acids are labeled x.

3.3 Complete proteomic analysis of whole soluble venom

The whole soluble venom of this scorpion was introduced into the Orbitrap Velos mass spectrometer (see Material and Methods) producing a complete fingerprint of the molecular masses of the venom components. As shown in Table 2, 222 molecular masses were identified after running the soluble venom for 290 min into the system. The molecular masses identified vary from 697.45 to 27056.46 Da. The great majority of components display a molecular mass around 3,000 to 4,000 Da, which correspond to peptides that bind to K^+ -channels and between 7,000 to 8,000 Da which correspond to peptides that affect Na^+ -channels, as it can be seen in Fig.5. The molecular masses obtained for purified or partially purified fractions were all present in Table 2, with minor differences due to the experimental error of the two machines used for their identification (data not shown). The proteomic analysis of the total soluble venom of this scorpion, after digestion with trypsin and sequencing allowed the identification of different classes of components, mainly toxins affecting ion channels, lectins, cysteine rich- proteins and enzymes such as metalloproteinases, hyaluronidases, among others. In total, 111 MS/MS derived sequences were obtained, which are presented in the Supplementary Table 1. The sequences identified contain at least 8 to more than 30 amino acids. Comparing the sequences found with Scorpion (taxid:6855) database by BLASTP program we found that many sequence alignments resulted in putative or hypothetical proteins already known to exist in scorpion venoms. Most of the precursor ions selected for fragmentation and whose sequences were generated had z : 2⁺, except for 12 precursor ions with z : 3⁺, 4⁺ and 5⁺.

As it can be observed most peptide fragments identified have sequence similarity with toxins or proteins already purified and characterized from scorpions of the genus *Tityus*. Comparing the sequences found (Supplementary Table 1) with known components from scorpion venoms, it was possible to identify: 43 peptides containing amino acid sequences similar (not necessarily identical) to known sodium channel specific toxins, 26 sequences with similarities to known metalloproteinases, 7 peptides with amino acid sequences similar to known potassium channel specific toxins, 11 peptides of hypothetical proteins, 4 hyaluronidases, 4 endothelin and angiotensin-converting enzymes, 2 allergens and 14 other enzymes, proteins and peptides. Our results do not mean that each peptide is from a different venom protein/peptide component of the venom. It is quite possible that some peptides are segments of the same protein, as surely it is the case for the metalloproteinases. The full metalloproteinase enzyme is hundreds of amino acids in length, and the same protein was cleaved in distinct segments by trypsin, thus resulting in apparent high number of peptides, but from the same protease. One of the peptides (number 5 of Supplementary Table 1) was identical to the sequence of the N-terminal amino acid sequence of peptide 3 of Fig.3. Similarly the peptide number 68 of the Supplementary Table 1 was identical to the N-terminal sequence of peptide 4 of Fig.3. Peptides 6 and 16 of the Supplementary Table 1 are internal segments of the peptide 4 of Fig.3. The various components identified correspond to different functions. From a gene ontology view (UniProtKB source) the percentage of protein types found in the proteomic analysis of the venom from *T. metuendus* was estimated to be: Molecular functions (47%), Cellular components (30%) and Biological processes (23%).

The results described here contribute to the biological and chemical characterization of the venom components from this scorpion, for which, to the best of our knowledge, nothing was known thus far.

4. Conclusion

In conclusion this is the first report on the biological and chemical characterization of the venom from the species *Tityus metuendus* from the Amazonian Region of Brazil. The venom is toxic to mammals, confirming earlier reports of the literature (Pucca et al, 2015). Fine electrophysiological assays using seven sub-types of Na⁺-channels clearly show the

presence of peptides that modify the gating mechanism of these ion-channels, comprehending both the α - and β -scorpion toxin types. The venom contains components with over 220 different molecular weights. Several amino acid sequences of some of the peptides were obtained by automatic Edman degradation and mass spectrometry determination of soluble venom digested with trypsin allowed the identification of 111 peptides that show sequence similarities with other known venom components, mainly similar to those found in the related species *T. obscurus* from the Amazonas.

Acknowledgements

The authors acknowledge Lorena Hernández-Oriuela and Erika Patricia Meneses from IBT-UNAM Proteomics Laboratory for technical assistance. The technical assistance of L.I. Servando Aguirre Cruz is also recognized. This work was partially supported by grant IN203416 of *Dirección General de Asuntos del Personal Académico* UNAM, given to LDP.

Author contributions: CVFB, JGM, RP, LDP, conceived the experiments, JGM obtained the scorpion venom, RRC, FZZ, FICV did most of the experimental work, CVFB was in charge of mass spectrometry data, LDP, CVFB, RRC, JGM analyzed the data and wrote the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Figure legends

Figure 1: HPLC of whole soluble venom of the scorpion *T. metuendus*

An aliquot of soluble venom (2.15 mg) was separated by high performance liquid chromatography (HPLC) using a C₁₈ reversed-phase column. The gradient run from solution A (0.12% trifluoroacetic acid in water) to 60% solution B (0.10% TFA in acetonitrile), over 60 min. Fractions labeled 1 to 3 were further analyzed.

Figure 2: *Tityus metuendus* total venom (TV) effect on hNav sodium channels.

Panels A-G shows the activity of the venom in human sodium channels of the sub-type 1.1-1.7 (same order of the panels letters). Panels depict voltage dependence of the activation process (round symbols) and the inactivation process (square symbols) and control (black symbols) and after application of TV at 30 μ g/ml (empty symbols). Light gray lines are the results of the fitting obtained by using the Boltzmann equation. For hNav 1.1, hNav 1.2 and hNav 1.6, the activation process in presence of TV was fitted with the sum of two Boltzmann equations. Insets on the right of each panel, shows an example of the current traces corresponding to the stimulation with the sub-threshold potential and the full activation potential, in the control (black line) and after application of the toxin (gray line). Sub-threshold potentials are indicated by the arrows. Scale values: 1 ms in the x-axis and 1 nA in the y-axis.

Figure 3: Amino acid sequence determination of peptides from *T. metuendus* scorpion venom

Underlabeled “direct” means sequence obtained by loading the peptide directly to the sequencer for Edman degradation. “Ms” means amino acid identified by its molecular weight or directly sequenced on the Orbitrap Velos apparatus after HPLC separation (the elution time from the column is indicated).

Figure 4: Sequence comparison with closely related sequences of other peptides of *Tityus* scorpion venoms

Two best Blast hits of two novel sequences (Query 1 and Query 2) obtained from the venom of the scorpion *T. metuendus*. The full description of the best two protein matches are described as follow: To14 (RecName: Full=Toxin To14; AltName: Full=T-alpha* NaTx3.10; Flags: Precursor); SC (Recombinant long-chain sodium channel specific toxin 8, synthetic construct); To2 (RecName: Full=Alpha-toxin To2; AltName: Full=Alpha-toxin

Tc48a; AltName: Full=Alpha-toxin To48a); Td1 (RecName: Full=Toxin Td1; AltName: Full=PT-beta* NaTx13.1; Flags: Precursor).

Figure 5: Molecular mass distribution of components found in the fingerprint-proteomic analysis of the venom from *T. metuendus*

Seven consecutive molecular mass ranges were chosen for classification of the main components present in the venom. Mass ranges of 1-3 and 3-5 kDa are typical of K⁺-channels blocker toxins; ranges of 5-7 and 7-10 kDa contains Na⁺-channel modifier toxins, whereas components with molecular masses from 10-20 kDa mainly contain phospholipase enzymes.

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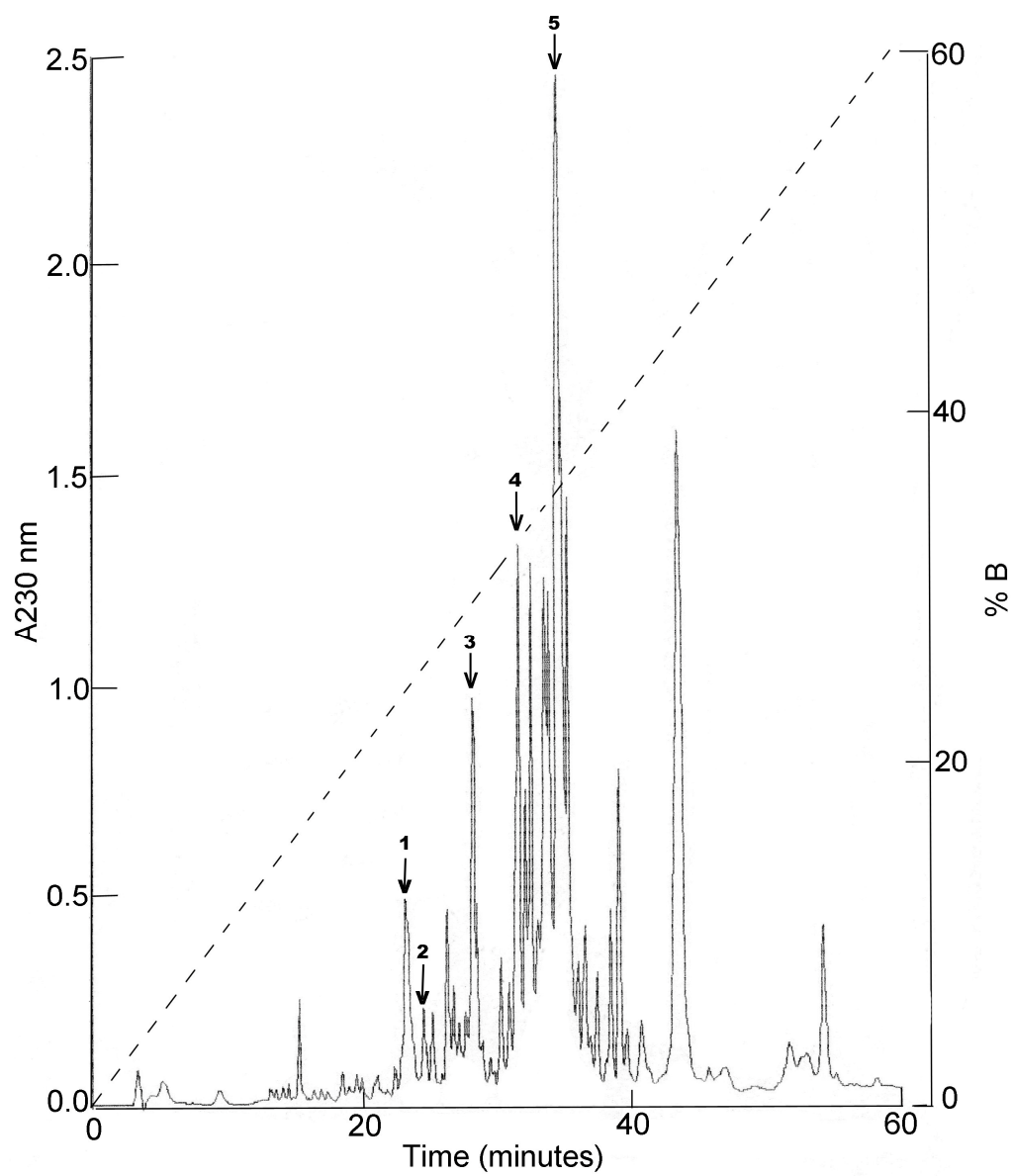
Table 1

	<i>V_{1/2} activation ± S.E. (mV)</i>		<i>V_{1/2} inactivation ± S.E. (mV)</i>	
	control	total venom	control	total venom
hNav 1.1	-26.1 ± 0.6	-49.2 ± 3.9	-62.8 ± 0.2	-66.7 ± 0.3
hNav 1.2	-23.8 ± 1	-41 ± 3	-57 ± 0.5	-62 ± 0.5
hNav 1.3	-18.6 ± 0.9	-61.9 ± 1.8	-58 ± 0.7	-74.9 ± 0.3
hNav 1.4	-27.5 ± 0.3	30.9 ± 0.8	-69.1 ± 0.1	-71.2 ± 0.8
hNav 1.5	-39.6 ± 0.6	40.6 ± 0.4	-77.2 ± 0.6	-76.7 ± 0.7
hNav 1.6	-25.8 ± 0.3	-38.5 ± 12	-62.3 ± 0.5	65.1 ± 1
hNav 1.7	-22.8 ± 0.6	-31.2 ± 0.4	-66.7 ± 0.1	-62 ± 0.6

Potentials of middle activation ($V_{1/2}$ activation) and inactivation ($V_{1/2}$ inactivation) before and after exposure to the venom, as results from data fitting.

Table 2: Fingerprinting *Tityus sp.* soluble venom

RT	Molecular masses (Da)
0-20	697.45; 725.44; 800.48; 896.51; 951.56; 1195.69; 1533.8; 1733.88; 2387.34; 2445.09; 2642.13; 2734.20; 3023.28; 3367.46; 3563.42; 3653.36; 3732.49; 3780.46; 3807.86; 3849.70; 3926.61; 4002.65; 4087.78; 4158.84; 4287.86; 4517.86; 4692.03; 4836.08
20-40	2043.17; 3365.46; 4457
40-60	1276.67; 1378.67; 1654.79; 1772.83; 2664.08; 3521.42; 3547.43; 4481.07; 5620.70; 6403.77; 7920.48; 8050.61
60-80	1034.46; 1141.69; 1229.68; 1544.82; 1836.98; 1984.05; 2041.08; 2170.11; 2283.20; 2339.17; 2398.22; 2513.25; 2628.28; 2743.31; 2905.36; 3635.4; 3821.61; 4306.92; 4435.02; 5899.27; 6010.85; 6364.71; 7485.41; 7734.53; 7821.59; 7942.67; 8214.63
80-100	742.46; 1278.23; 1559.68; 1716.97; 1912.99; 2154.15; 2267.24; 2317.15; 2382.26; 2431.12; 2497.29; 2755.38; 3311.03; 3496.56; 3511.56; 3661.58; 4298.89; 4324.86; 4403.68; 4688.86; 4750.14; 5007.29; 5206.42; 5321.45; 5449.55; 5529.54; 6085.87; 7185.08; 7441.25; 7665.38; 7794.39; 8053.53; 11025.96; 16537.66
100-120	733.38; 814.44; 921.41; 1037.55; 1193.19; 1506.85; 2342.35; 2737.37; 3896.87; 4807.15; 4906.18; 5027.29; 5434.24; 5604.46; 6031.93; 6193.97; 6880.01; 7022.03; 7155.12; 7319.15; 7769.36; 7883.35; 7911.35; 8073.40; 11121.31
120-140	1503.76; 1600.98; 1828.14; 3414.54; 5869.92; 6271.06; 6589.81; 6771.35; 6867.08; 7007.09; 7149.59; 7193.16; 7269.14; 7955.65; 8168.48; 8205.50; 8330.53; 8867.81; 10377.44; 10744.90; 11107.32; 11265.45; 11515.28
140-160	1094.44; 1365.91; 1395.79; 1576.90; 1603.91; 1805.82; 1830.08; 1863.82; 2038.94; 2239.40; 2424.33; 2495.36; 2610.40; 2681.57; 2709.58; 2739.44; 3006.78; 3165.83; 3637.90; 4150.91; 4646.63; 4801.78; 4874.03; 5004.09; 5497.42; 6569.27; 6836.8; 6975.98; 7513.20; 10215.01; 10531.05; 10617.06; 10681.12; 10962.22
160-180	1335.76; 1398.16; 1789.82; 1847.83; 1886.78; 1917.11; 2016.18; 2129.27; 2216.30; 2587.40; 2672.76; 2853.42; 2911.43; 5453.38; 6907.84; 6949.86; 7668.00; 10320.21; 10594.08; 10667.06; 10893.27; 11047.30; 11192.32; 13899.70; 16328.15
180-200	3871.88; 5922.18; 10611.04; 10803.07; 10876.25
200-220	1550.66; 1688.71; 1714.75; 2295.98; 3667.88; 5124.04; 10106.95; 10411.00; 25637.17; 27116.93
220-290	13708.93; 16988.90; 24776.32; 25575.04; 26609.60; 27056.46



10 20 30 35
1 TPFRCNPRN CAKECQGRGK ETTYCDEVC KCSGW
-----direct-----
ms

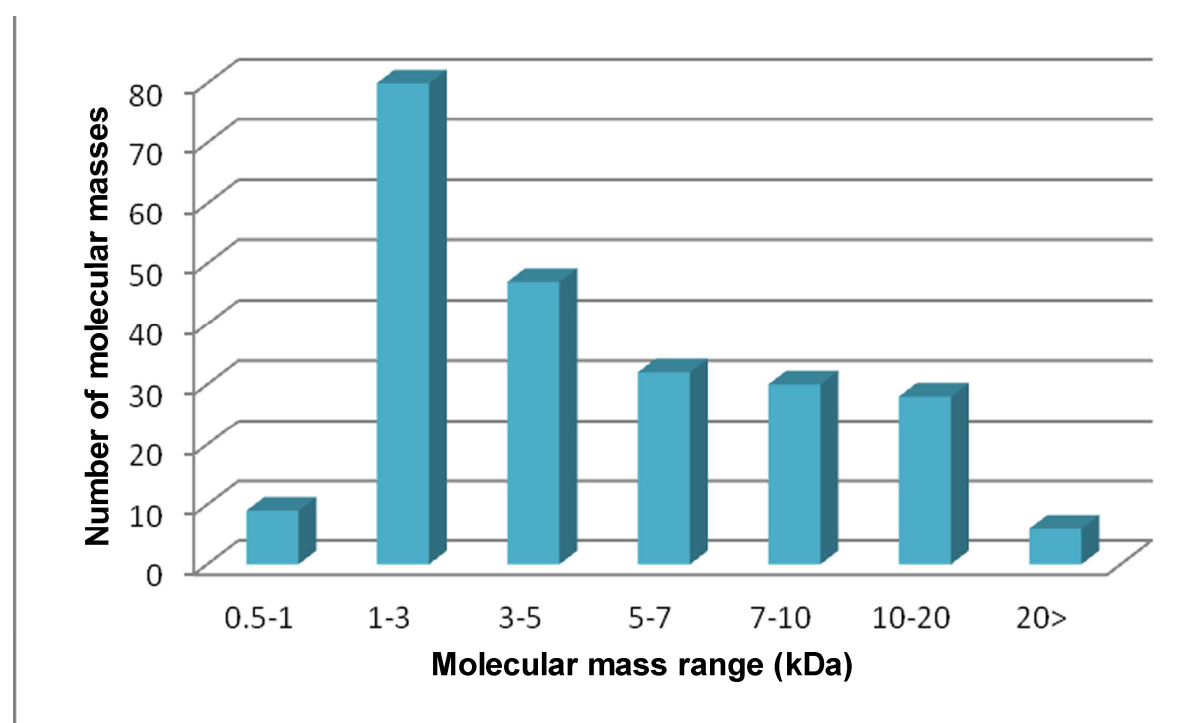
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2 KVLAPAEAP AEAPAAE
---direct-----
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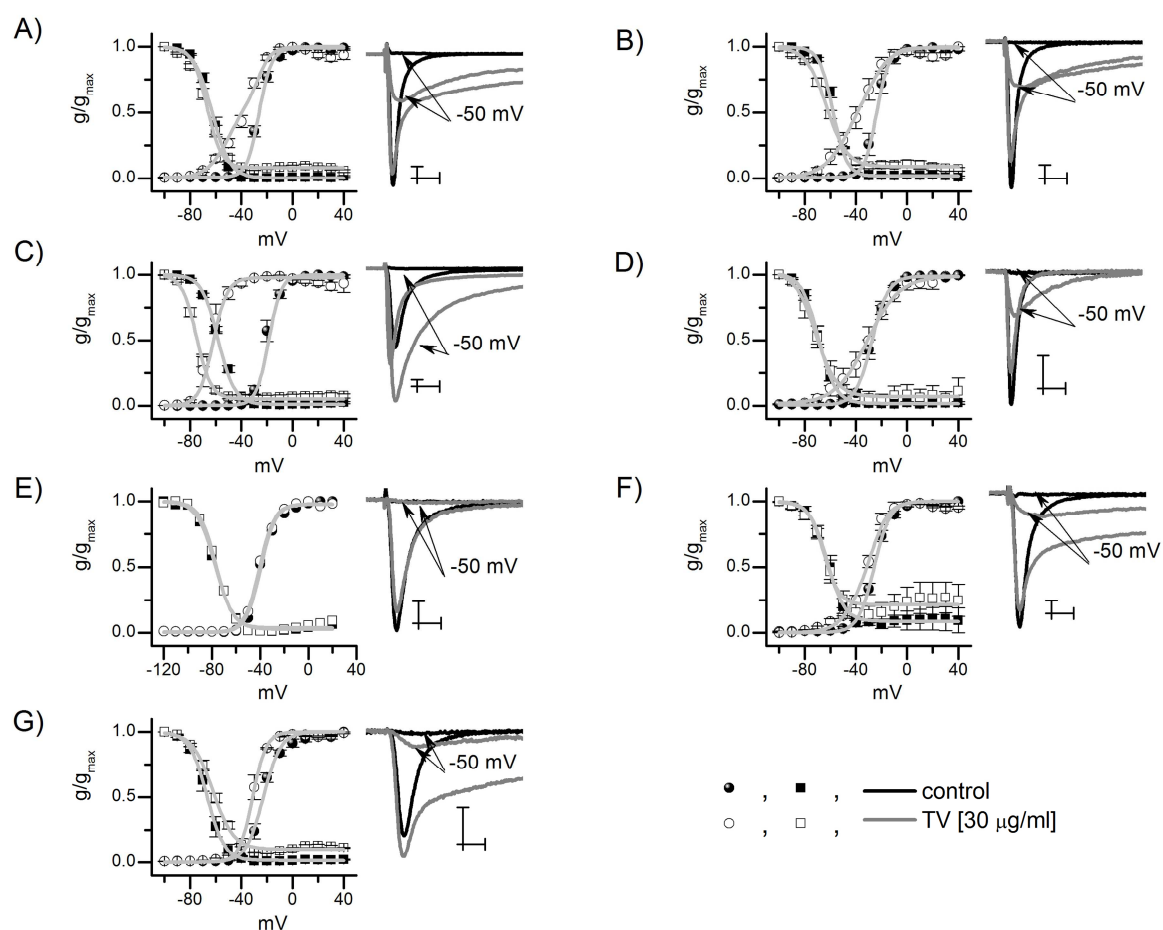
10 20 30 35
3 TAIGNCNPFT CDKECKTKGN KRGYCENYNC ECSKW
-----direct-----
ms

10 20 30 40 50 60 69
4 KKNDYPVDTA KRNCMLDCNV WDDEGYCDNF CKGRKAESGY CYKLKAACYC YGLPDDSPK TSGRCNPV
-----direct-----
-----V8 at 25.78-----

10 20 30 40 50 60 65
5 NKDGYLMEGD GCKMGCLTRK ASYCVDQCKE VGGKDGICYA WLSCYCYNMP DSVEIWDSKN NKCGK
-----direct-----
---30.96, 32.77--- ---27.12--- ----18.35---

Sequence ID	Protein name	Sequence	Identity (%)
-	<i>Query 1</i>	KKNDYPVDTAKRNCMLDCNVWDDGYCDNFCKGRKAESGYCYKLKAACYCYGLPDDSPKTSGRCPNV	
H1ZZI3.1	To14	KKDDYPVDTAKRNCMLDCNVWDDGYCDKFCCKGRKADSGYCYKLKAACYCYGLPDDSPKTSGRCPNV	66/69(96%)
ACD35699.1	SC	KKDGYFVDHA--NCKYEC--WYDDKYCDDLCKKRKADSGYCYKLNIISCYCLGLPDNAAIKDYGRCRP	39/67(58%)
-	<i>Query 2</i>	NKDGYLEMGDGCKMGCLTRKASYCVDQCKEVGGKDGICYAWLSCYCYNMPDSVEIWDKNNKCGK	
P60212.3	To2	NKDGYLEMGDGCKMGCLTRKASYCVDQCKEVGGKDGICYAWLSCYCYNMPDSVEIWDKNNKCGK	65/65(100%)
Q1I180.2	Td1	-KDGYLEMENGCKRGCLTRPARYCPNECSRLLKGDGYCYLWLACYCYNMPESAPVWERATNRCGK	41/64(64%)





HIGHLIGHTS

1. Venom from *Tityus metuendus* scorpion was separated and a complete fingerprint of components were identified by mass spectrometry
2. Five peptides were fully sequenced by Edman degradation and mass spectrometry analyses
3. Various peptides were sequenced at the N-terminal region by Edman degradation
4. Physiological action of whole soluble venom on 7 sub-types of Na⁺-channels were performed by patch clamp techniques
5. A total of 111 tryptic digested peptides from soluble venom were sequenced by mass spectrometry and identified