Toxic peptides and genes encoding toxin γ of the Brazilian scorpions *Tityus bahiensis* and *Tityus stigmurus*

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Seven toxic peptides from the venom of *Tityus bahiensis* and *Tityus stigmurus* were isolated and sequenced, five of them to completion. The most abundant peptide from each of these two species of scorpion was 95% identical with that of toxin γ from the venom of *Tityus serrulatus*. They were consequently named γ -b and γ -st respectively. The genes encoding these new γ -like peptides were cloned and sequenced by utilizing oligonucleotides synthesized according to known cDNA sequences of toxin γ , and amplified by PCR on templates of DNA purified from both *T. bahiensis* and *T. stigmurus*. They contain an intron of approx. 470 bp. Possible mechanisms of processing and expressing these peptides are discussed, in view of the fact that glycine is the first

residue of the N-terminal sequence of *T. stigmurus*, whereas lysine is the residue at position 1 of toxin γ from *T. serrulatus* and *T. bahiensis*. In addition, chemical characterization of the less abundant toxic peptides showed the presence of at least four distinct families of peptides in all three species of the genus *Tityus* studied. There is a large degree of similarity among peptides from different venoms of the same family. By using specific horse and rabbit antisera, the venoms of *T. bahiensis*, *T. serrulatus* and *T. stigmurus* were compared. They showed an extended degree of cross-reactivity. Thus these three species of scorpion have similar toxic components, the genes of which are similarly organized, processed and expressed.

INTRODUCTION

The information presented here deals with biological and chemical aspects of toxic peptides and the cloning of the genes encoding toxin γ from the three most dangerous species of Brazilian scorpions: *Tityus bahiensis*, *Tityus stigmurus* and *Tityus serrulatus*. They cover an immense geographical area of the country, and represent an important public health problem causing fatal accidents in humans [1–3]. Aside from the medical interest, scorpion toxins are valuable tools for the study of ion channels [4–7]. A large amount of information on their diversity, structure, specificity and molecular mechanism of action has been published. The most thoroughly studied aspects are the isolation and chemical and physiological characterization of these toxic peptides [8–11]. The best-known class of scorpion toxins contains peptides that recognize Na⁺ channels [8,12–14]. Recent reports of cloning from cDNA have appeared in the literature [15–17]; however, little information is available about these genes at the genomic level [18,19]. In this paper we describe in detail for the first time the cloning of three genes from genomic DNAs of scorpions of the genus *Tityus* from Brazil. From the venom of *T. bahiensis* and *T. stigmurus* new toxic peptides have been purified and their amino acid sequences determined, showing the presence of peptides similar to toxins γ , III-8, IV-5 and TsTX VI of *T. serrulatus* [20,21]. The same types of peptides were found in all three species studied. Immunological studies confirm the presence of common epitopes, and suggest the possibility of mutual protection by using antisera produced against any of the three species reported here.

MATERIALS AND METHODS

Source of biological materials

Venoms, animals and horse anti-arachnid serum were a gift from the Instituto Butantan, Sao Paulo, Brazil (approved project no. 702.1809/90). Crude dried venom was solubilized in water and centrifuged at 15000 g for 30 min. The supernatant was either directly used for fractionation or lyophilized and kept at –20 °C for subsequent dilution in appropriate buffers for assays.

Source of chemicals

Only analytical-grade solvents and reagents were used. Sephadex G-50 was from Pharmacia Fine Chemicals (Uppsala, Sweden), and CM-cellulose CM-32 from Whatman (Clifton, NJ, U.S.A.). TosPheCH₂Cl-treated trypsin and protease from *Staphylococcus aureus* V8 were purchased from Sigma (St. Louis, MO, U.S.A.) and Boehringer-Mannheim (Mannheim, Germany) respectively. Acetonitrile and trifluoroacetic acid for HPLC were obtained from Pierce (Rockford, IL, U.S.A.). Chemicals and solvents for peptide sequencing were from Millipore Co. (Milford, MA, U.S.A.).

Primers for PCR, hybridization and sequencing were synthesized as described previously [17,19]. Vent polymerase for PCR and its buffer were from New England Bio-Labs (Beverly, MA, U.S.A.). Restriction enzymes and their reaction buffers were purchased from Boehringer-Mannheim. Kit for DNA sequencing (Sequenase version II) was obtained from US bio-chemical (Cleveland, OH, U.S.A.). Cloning and sequencing

Figure 1 Purification of toxin γ from *T. bahiensis* by column chromatography

(a) A Sephadex G-50 Superfine column (0.9 cm $\times$ 200 cm) was equilibrated, loaded and eluted in presence of 0.15 M NaCl/20 mM ammonium acetate buffer, pH 4.7, with 100 mg of soluble venom from *T. bahiensis*. Tubes containing 1.5 ml of eluate were collected, and fractions I–VIII were pooled as indicated by the horizontal bars. T, toxic; L, lethal. (b) A CM-cellulose column (0.9 cm $\times$ 30 cm) was loaded with 21.7 mg of lethal fraction VI from (a), after dialysis against 20 mM ammonium acetate buffer, pH 4.7. This buffer was used for equilibration and elution from the column with a linear gradient from 0 to 0.55 M of NaCl (250 ml each) (■); fractions of volume 2.5 ml were collected. Subfractions (1–6) were collected as indicated by the horizontal bars. L, start of the loading of material; G, start of the gradient. (c) Toxic subfraction VI-6 from (b) (5.3 mg) was dialysed and further separated on a CM-cellulose (0.9 cm $\times$ 30 cm) equilibrated with 50 mM sodium phosphate buffer, pH 6.0. The gradient was developed from 0 to 0.4 M NaCl (250 ml each) (■) in the same buffer. Inset: HPLC application (C_8 reverse-phase column) of subfraction VI.6.2 from (c) (about 100 μ g of protein), elution was attained with a gradient from 0.12% trifluoroacetic acid in water to 60% acetonitrile in the presence of 0.1% trifluoroacetic acid (solvent B), developed over 1 h. L, elution position of the lethal toxin γ from *T. bahiensis*.

vector (pBluescript phagemid; pKS), M13-20 and M13 reverse sequencing primers came from Stratagene (La Jolla, CA, U.S.A.). Water double-distilled over quartz was used throughout the experimental work.

Lethality tests

Albino mice (strain CD1) were used to test the lethality of the venoms and their components. Fractions containing up to 50 μ g of protein diluted in the appropriate buffers were assayed by intraperitoneal injection. Intoxicated animals usually show the following symptoms: excitability, restlessness, salivation, sialorrhoea, dyspnoea, apnoea, paralysis of rear limbs, diarrhoea and death. Three designations were used to characterize the degree of intoxication: 'lethal' means that the animal showed some, if not all, of the symptoms described, and died within 24 h of application; 'toxic' means that the mouse showed some of the symptoms but survived; 'non-toxic' means that the mouse showed same behaviour as after injection of buffers or saline alone. Animals were treated humanely and the number of mice used for lethality tests was kept to a minimum according to approved protocols.

Separation procedures

Soluble venom from *T. bahiensis* was applied to a Sephadex G-50 (Superfine) column and eluted with 20 mM ammonium acetate

buffer, pH 4.7. Fractions were pooled according to the absorbance profile at 280 nm and assayed for toxicity. Further fractionation was performed by column chromatography, using the cation-exchange resin CM-cellulose, equilibrated in the same mobile phase as used for gel filtration. Elution was achieved by means of a linear gradient from 0 to 0.55 M NaCl, as previously described [21,22]. Lethal and toxic fractions were further purified by subsequent application to a CM-cellulose column, equilibrated and developed with 50 mM sodium phosphate buffer, pH 6.0, using a linear gradient from 0 to 0.38 M NaCl. Before the separation at pH 6.0, fractions were dialysed against double-distilled water (3 $\times$ 30 min) followed by an additional 30 min against the buffer used for the next column, using Spectrapor 3 M membranes (Spectrum Medical, Los Angeles, CA, U.S.A.). When necessary, a final separation by HPLC on a C_4 or C_{18} reverse-phase column (Vydac, Hesperia, CA, U.S.A.) was conducted.

Soluble venom from *T. stigmurus* was directly separated by HPLC, using a C_{18} reverse-phase column (Vydac 218TP54; 5 μ m; 4.6 mm $\times$ 250 mm; thermostatically controlled at 30 $^{\circ}$ C) using a Waters Associates solvent delivery system, WISP sample processor and a Hewlett-Packard hp1040A photodiode array detector. Protein content was determined by either amino acid analysis or measurement of A_{280} , assuming that 1 absorbance unit in a 1 cm pathlength cuvette equals 1 mg/ml protein.

Purity of components was assessed by three independent means: HPLC of small aliquots on a C_{18} reverse-phase microbore

Figure 2 Primary structure of five novel toxins from *T. bahiensis* and *T. stigmurus* venom

The overlapping sequences were obtained from peptides purified by HPLC, after the following treatment (indicated by labels under the corresponding primary structures): direct Edman degradation (–d–); chymotrypsin (=CH=); trypsin (=T=); protease V8 (.V8a. and .V8b.); Asp-N (=AspN=); Lys-C (.LysC.); amino acid analysis (aa) and confirmed by nucleotide sequence (nt).

column (2 mm internal diameter) in the Waters system mentioned above; PAGE in acidic conditions [23] or in the presence of SDS [24]; microsequencing as described below.

Reduction, alkylation and cleavage of peptides

Reduction of toxins with dithiothreitol and alkylation with iodoacetic acid were performed as previously described [25]. Cleavage of peptides with trypsin, chymotrypsin and protease V8 were performed as described by Zamudio et al. [26]. Cleavage of reduced and alkylated toxin with lysyl C-endopeptidase (Lys-C) (Boehringer-Mannheim) was performed in 200 μ l of 24 mM Tris/HCl buffer, pH 7.2, containing 1 mM EDTA, using an enzyme/peptide ratio reduced and alkylated of 1:200. Similarly, cleavage of toxin with aspartyl N-endopeptidase (Asp-N) (also from Boehringer-Mannheim) was performed in 200 μ l of 50 mM sodium phosphate buffer, pH 8.0, at an enzyme/peptide ratio of 1:100. Peptides produced by enzymic hydrolysis were separated by HPLC, as described previously [25,26], and used for sequence determination or amino acid analysis.

Amino acid analysis and microsequencing

Amino acid analysis was performed on samples hydrolysed in 6 M HCl with 0.5% phenol at 110 °C in evacuated sealed tubes as described previously [21]. Microsequence determination of native toxins, reduced and carboxymethylated toxins and their peptide fragments was carried out with a 6300 Microsequencer from MilliGen (Division of Millipore) or a model 6400/6600 ProSequencer from the same company using either the peptide adsorbed or covalently attached protocols, by means of Sequelon membranes (arylamine or 1,4-phenylene di-isothiocyanate) as previously described [21,25,26].

DNA purification

Purification of genomic DNA from scorpions *T. bahiensis* and *T. stigmurus* was performed using entire animals as described for *T. serrulatus* [17].

Synthesis of oligonucleotides

Three different oligonucleotides were synthesized, as described previously [17]: 1, (5'-TCGATCTGAACGATGAAAGG-3') corresponding to the nucleotide sequence from positions 1–20; 2, (5'-GCCAACTTATTACAGTTCATTG-3') corresponding to the complementary sequence of nucleotides of positions 766–790; 3, (5'-GTGATCCATGAGATAACCTT-3') corresponding to the complementary sequence of nucleotides 552–571. Oligonucleotides 1 and 2 were used for sequencing and PCR, whereas oligonucleotide 3 was used only for sequence determination. The final nucleotide sequence data were determined from both strands.

PCR and cloning

PCR, cloning and DNA sequencing were performed as described previously [17,19].

Preparation of antisera

Horse antiserum against arachnids ('Soro Antiaracnido', batch N. 9212294) was a gift from the Butantan Institute (Sao Paulo, Brazil). In addition, we prepared two different antisera in the laboratory. The first was a mouse anti-(horse Fab) serum. This was required to visualize the various venom components recognized by the Butantan Institute antiserum. The Butantan Institute horse serum was processed to its Fab fragments. Mouse antiserum was prepared using five Balb/c mice, which were immunized with 100 μ g of protein (horse Fab fragments) 4 times at intervals of 15 days. The first injection was performed using Freund's complete adjuvant, and subsequent injections were performed using Freund's incomplete adjuvant. Mice were bled 11 days after the last immunization.

The second antiserum obtained in the laboratory was an anti-(toxin γ) serum from *T. serrulatus*. This serum was prepared as described above except that one rabbit (New Zealand White) was used and 20 μ g of toxin γ was injected each time.

ELISA

Polyvinyl plates (catalogue no.2596; Costar, Cambridge, MA, U.S.A.) were coated with 0.3 μ g/well soluble venom from *T. serrulatus*, *T. bahiensis*, *T. stigmurus* and *Centruroides noxius*, as well as with purified toxin γ (0.15 μ g/well) from *T. serrulatus*, dissolved in 0.02 M NaHCO₃ buffer, pH 9.6. The plates were incubated overnight at 4 °C. Non-specific binding sites were blocked with 1% BSA in 0.015 M sodium phosphate buffer, pH 7.4, and PBS. Double serial dilutions of horse anti-arachnid serum and rabbit anti-(toxin γ) serum were assayed, using the protocol described previously by our group [27].

For the experiments with the serum from the Butantan Institute the mouse anti-(horse Fab fragment) serum was used at a dilution of 1:2000, and the reaction was developed with a third antibody (horseradish peroxidase-labelled goat anti-mouse IgG from Bio-Rad Laboratories, Richmond, CA, U.S.A.). Negative controls were performed by omitting the horse anti-arachnid serum in the case of the Butantan Institute serum and by using preimmune rabbit serum in the case of toxin γ . Venom from *C. noxius*, a Mexican scorpion, was used as a control for an unrelated species.

PAGE and immunoblot analysis

SDS/PAGE [24] in a continuous 10–20% gradient of acrylamide was used to separate the venoms from *T. bahiensis*, *T. serrulatus* and *T. stigmurus* and purified toxin γ from *T. serrulatus*. After electrophoresis, slab gels were stained with Coomassie Blue G-250 for identification of protein bands or transferred to 0.45 μ m nitrocellulose membrane (Schleicher & Schuell, Keene, NH, U.S.A.) as previously described [28] for antibody recognition. Non-specific binding sites were blocked with 1% BSA, and the antigens were identified with horse anti-arachnid serum from the Butantan Institute (dilution 1:500) or rabbit anti-(toxin γ) serum (dilution 1:1000). For the first test, the second antibody was mouse anti-(horse Fab fragment) serum (diluted 1:2000), visualized with horseradish peroxidase-labelled goat anti-mouse IgG, and for the next test (toxin γ), the visualizing antibody was horseradish peroxidase-labelled goat anti-rabbit IgG (Bio-Rad Laboratories). Protocols used here are the same as previously described [27]. Negative controls were the same as described above for ELISA.

RESULTS AND DISCUSSION

Purification and amino acid sequencing of toxic peptides

Figure 1 shows the chromatographic profiles of components from the venom of *T. bahiensis*. Fractions IV, V and VII (Figure 1a) are toxic, and fraction VI is lethal to mice at the dose assayed (under 50 μ g per mouse). From fraction VI a final pure toxin was obtained by three (Figures 1b, 1c and inset) additional chromatographic steps described in the Figure legend. The purified peptide was lethal to mice at the dose used (50 μ g). This is the most abundant toxin of *T. bahiensis* venom, corresponding to 10% of the whole soluble venom. PAGE confirmed the homogeneity of this component (results not shown), and its amino acid sequence was determined (Figure 2). This peptide was called toxin γ -bahiensis (abbreviated to γ -b), because its amino acid sequence is 95% identical with that of *T. serrulatus*. Three additional components from *T. bahiensis* venom were isolated by HPLC and CM-cellulose chromatography, as shown in Figure 3. They were called toxin III-8-bahiensis (abbreviated to III-8b), toxin IV-5b and TbTX-VI respectively. Interestingly, toxin TbTX-VI

Figure 3 Chromatographic separation of toxins from *T. bahiensis*

(a) HPLC separation of toxic subfraction VI.4 from Figure 1(b) (100 μ g) on a C_8 reverse-phase column, using the same conditions as those described for the inset of Figure 1(c). T, toxin III-8b. (b) Subfraction VI.2 from Figure 1(b) (7.6 mg of protein) was applied to a CM-cellulose column (0.9 cm $\times$ 30 cm) equilibrated with 50 mM sodium phosphate buffer, pH 6.0. The gradient was developed from 0 to 0.3 M NaCl (250 ml each) (■), in the same buffer; tubes containing 2.5 ml of eluate were collected. L, lethal toxin IV-5b. Component 3 from this column was toxin TbTX-VI.

Figure 4 Chromatographic separation of *T. stigmurus* venom and toxic fractions

(a) Soluble venom from *T. stigmurus* (100 μ g) was separated by HPLC, using a C_{18} reverse-phase column, as described in the Materials and methods section. Component 1 is toxin III-8st, and component 2 is γ -st. (b) Fraction IV from Sephadex G-50 column separation of *T. stigmurus* (results not shown), applied and eluted using conditions identical with those described for Figure 1(a), was further subjected to HPLC fractionation, producing pure toxin IV-5st (labelled with an asterisk).

If we compare the amino acid sequences of the nine toxins for which the full primary structure is known, a consensus sequence is obtained (Figure 5). Only 21 of 66 residues are conserved in identical positions, the most important ones being the cysteine residues and the stretches at positions 2–8, 36–41, 45–53 (Figure 5), which correspond to segments assumed to be responsible for the β -sheet structures of these toxins [33]. Several conservative mutations have also occurred, among the basic (b), acidic (a), hydrophobic (h) and aromatic (r) amino acid residues, which have been substituted by an amino acid with similar side chains

Figure 7 ELISA of antisera against scorpion venoms and toxin γ of *T. serrulatus*

Cross-reactivity of the Butantan Institute anti-arachnid serum and rabbit anti-(γ toxin) serum was detected by ELISA using coated plates with 0.3 μ g/well scorpion venoms and 0.15 μ g/well purified toxin γ of *T. serrulatus*. Results are mean values of triplicates. (a) Venoms from *T. serrulatus* (●), *T. bahiensis* (○) and *T. stigmurus* (X) were tested using the Butantan Institute anti-arachnid serum at the dilutions indicated. (b) Venom from *C. noxius* (Δ) and toxin γ from *T. serrulatus* (■) were assayed with the Butantan Institute anti-arachnid serum. (c) Same as (a) but assayed with rabbit anti-(toxin γ) serum. (d) Same as (b) but assayed with rabbit anti-(toxin γ) serum.

(Figure 5). Finally, for the γ -like peptides it seems that the last residue (cysteine) is amidated, as shown for toxin VII [31,34]. Our cloning results also support this view, not only for toxin γ of *T. serrulatus* [19], but also for the γ -b and γ -st toxins, as discussed next.

Cloning γ -like toxins from DNAs of *T. bahiensis* and *T. stigmurus*

Nucleotide sequences of the genomic regions encoding toxin γ -b (*T. bahiensis*) and γ -st (*T. stigmurus*) are shown in Figure 6. They are compared with the homologous sequence from *T. serrulatus*, used as a reference [19]. Analysis of these nucleotide sequences permits the identification of two exons (nt 1–55 and nt 531–790) and an intron (nt 56–530) corresponding to toxin γ from *T. serrulatus*. The intron sizes are 474 bp and 465 bp for *T. stigmurus* and *T. bahiensis* respectively. The nucleotide sequences of the two exons from the gene encoding toxin γ of *T. serrulatus* coincide exactly with the cDNA sequence reported for toxin γ of the same scorpion (toxin VII from ref. [31]), except for positions 745 and 760. We have found a cytosine (genomic sequence [19]) in both cases instead of thymine reported from the cDNA library [31]. Intron 5' and 3' boundaries are very well conserved among these three sequences. These boundaries have the regular features of all eukaryotic introns thus far reported [19]. Intron sequences are more variable than those of the exons. Intron sequences from

T. serrulatus and *T. stigmurus* are more similar to each other than to that of *T. bahiensis*.

The exon sequences of the γ toxins studied are very well conserved. Exon 1 (nt 1–55) is 100% homologous in all three species. Exon 2 (nt 531–790) demonstrates some variability. One apparent hot spot for mutation is the codon for residue number 28 (nt 629–631), which is different in the three sequences. The next most variable point is the codon interrupted by the intron (nt 531–532). The nucleotide at position 622 is also changed giving rise to a substitution of arginine for serine in the case of *T. bahiensis*. The remaining changes are single-base mutations, usually at the third base position, without modification of the encoded amino acid residue. These observations might indicate a selective pressure on the γ -like toxins from these three scorpion species.

One distinctive feature of γ -st toxin is that it starts with glycine instead of lysine (which in this case corresponds to residue number 2). Analysis of the sequence encoding this *T. stigmurus* signal peptide suggests a possible explanation for this feature. A single-base mutation in the second position of the codon for glutamic acid of *T. serrulatus* (position –2 of the signal peptide; nt 542–544) caused a substitution of glycine for glutamic acid in *T. stigmurus*. All signal peptides thus far reported for scorpion toxins have a valine at position –3 [17,31], suggesting that the residue in this position is important for the cleavage of the signal

peptide. Positions -3 and -4 in the γ toxins reported here are valine residues, suggesting that, in the case of a change in position -1, the cleavage site would be displaced one residue towards the N-terminus of the signal peptide, position -4 now becoming position -3, as demonstrated by these results. In this manner, *T. bahiensis* and *T. serrulatus* γ toxins have a 20-amino-acid-long signal peptide whereas that from *T. stigmurus* has only 19 residues. At the C-terminal region, it appears that γ toxin precursors are in fact processed after synthesis. As shown in Figure 6, before the termination codon in the translated peptide there is a glycine followed by two lysines. Thus the cysteine at the end of the mature toxin may be amidated after C-terminal processing, as earlier suggested [15,31,35].

We have isolated, cloned and sequenced the genomic regions encoding γ toxins from *T. serrulatus*, *T. stigmurus* and *T. bahiensis*. We have shown that these genomic regions have the same organization. It seems that transcription of these genomic regions generates pre-mRNAs of approx. 800 nt containing an intron of approximately 470 nt. The processing of these pre-mRNAs might give a mature molecule of approx. 330 nt. Translation of the mRNAs produces a precursor of 83–84 amino acid residues, which is processed at both ends (amino and carboxyl). At the N-terminus, a 19–20-amino acid signal peptide is released by a signal peptidase. At the C-terminus, both lysines are eliminated by a carboxypeptidase and finally the amino group of the preceding glycine is utilized to amidate the cysteine by an amidase. The final mature toxin γ contains 61 (*T. serrulatus* and *T. bahiensis*) or 62 (*T. stigmurus*) residues, with a cysteinamide at the C-terminus in both cases.

Immunological similarities between scorpion venoms from the genus *Tityus*

In Brazil, there are three main species of scorpions dangerous to man: *T. serrulatus*, *T. bahiensis* and *T. stigmurus* [1–3, 36]. The Butantan Institute prepares a horse antiserum using venom from *T. serrulatus* as an antigen. This serum has been successfully used in humans stung by scorpions [2,3]. However, an important question has arisen as to whether this antiserum could be used to treat people stung by either of the other two species. The results in Figures 7 and 8 show that the three species of *Tityus* studied have identical venoms in terms of the horse serum titre and cross-reactivity using the Butantan Institute anti-arachnid serum (see the Materials and methods section), confirming the data of Nishikawa et al. [36].

Figure 7(b) shows that the titre of this serum against the venom of another genus of scorpion, *C. noxius*, is low (absorbance of only 0.2 unit with a dilution of 1:100), suggesting a lack of similarity among the various antigenic determinants. However, if purified toxin γ from this venom (*T. serrulatus*) is used in the ELISA, the Butantan Institute anti-arachnid serum produces a positive reaction. This means that toxin γ is one of the major antigenic components recognized by this antiserum, as expected from the neutralizing capacity of the antiserum [2,3]. Figures 7(c) and 7(d) further confirm the antigenicity and cross-reactivity of γ -like toxins in the venoms of the genus *Tityus*. These experiments were conducted with rabbit anti-(toxin- γ of *T. serrulatus*). In Figure 7(c) the plates were independently coated with venoms from *T. serrulatus* (homologous venom), *T. bahiensis* and *T. stigmurus*, and tested with rabbit anti-(toxin γ) antibodies, whereas in Figure 7(d) the plates were loaded with toxin γ or *C. noxius* venom. The latter results show that the anti-(toxin γ) serum has a good autologous titre, as expected. However, it was also able to recognize a putative γ -like toxin in the venom of *C. noxius*. It is known that in *C. noxius* venom there are Na⁺-

Figure 8 Western blots of venoms from the genus *Tityus* and purified toxin γ from *T. serrulatus*

SDS/PAGE separation of venoms (30 μ g/lane) and toxin γ from *T. serrulatus* (5 μ g/lane) was performed using a gradient from 10 to 20% acrylamide, as follows: lanes 1, 2 and 6, *T. serrulatus* venom; lanes 3 and 7, *T. bahiensis* venom; lanes 4 and 8, *T. stigmurus* venom; lane 5, purified toxin γ from *T. serrulatus*. After electrophoresis the protein content was transferred to nitrocellulose membranes [28] and revealed using anti-arachnid serum from the Butantan Institute (lanes 2, 3 and 4) and rabbit (anti-toxin γ) serum (lanes 5–8). Lane 1 was the negative control, revealed without antisera. Arrows on the right indicate positions of molecular-mass markers. Abbreviations: A.P., Application point; T.D., tracking dye., K, kDa.

channel toxins, which share some similarities with *T. serrulatus* toxins [26]. The electrophoretically separated venoms, revealed by immunoblot (Figure 8), show that: (1) the cross-reactivity of the Butantan Institute antiserum to the venoms from *T. serrulatus*, *T. bahiensis* and *T. stigmurus* is striking (similar proteins in terms of molecular mass and charge are recognized in all the species studied); (2) one of the most important families of proteins recognized in the immunoblot experiment is located at a position that corresponds to peptides of molecular mass about 7500 Da, exactly where toxin γ from *T. serrulatus* migrates, as well as other known Na⁺-channel-blocking toxins from the venom of the other *Tityus* species (very heavily staining band in Figure 8, lanes 2–4); (3) several prominent bands occur in positions that correspond to proteins of higher molecular mass and are probably non-toxic components of the scorpion venoms used as antigens for serum production.

Additional evidence supporting this conclusion is presented here, by showing the similarities of the toxic peptides isolated and characterized from *T. bahiensis* and *T. stigmurus* (Figure 2) compared with those from *T. serrulatus* (Figure 5). Although direct evidence, via serotherapy in humans, is still needed to confirm that serum produced against any individual species of venom from the genus *Tityus* is capable of neutralizing the venom of another species, these results are very encouraging and might become medically important. The present work strongly supports the findings of Nishikawa et al. [36] suggesting that any of these venoms might be satisfactorily used to produce effective antisera against the venom of the other two species of scorpions.

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