Mem. Inst. Butantan
v. 54, n. 2, p. 35-52, 1992

STRUCTURAL AND FUNCTIONAL CHARACTERISTICS OF TOXINS PURIFIED FROM THE VENOM OF THE BRAZILIAN SCORPION *Tityus serrulatus* LUTZ AND MELLO.*

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ABSTRACT: Venom from the scorpion *Tityus serrulatus* collected in four distinct geographical localities of Brazil was studied by gel electrophoresis, immunoelectrophoresis, Sephadex G-50 gel filtration and ion exchange chromatography. Lethality tests in mice were also conducted. None of these methods revealed a significant difference among these samples of venom. The Instituto Butantan venom of *T. serrulatus* has shown constant and reproducible biochemical and pharmacological properties, in our laboratories, for 18 years. The venom has several polypeptides toxic in mice, among the major toxic components are toxin gamma, III-8 and IV-5, which affect sodium channel permeability. The molecular weight of these toxins is in the order of 7,000. One of the

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*Part of this article was used for the master of Sciences degree of G.S. Rode, Facultad de Químicas, UNAM, (1984).

Recebido para publicação em 9.10.1990 e aceito em 11.6.1992.

minor components is toxin II-9A, a shorter peptide (approx. 4,200 mol. weight) that modifies potassium permeability in axons. At least four additional minor toxic components were also purified with very similar amino acid sequences to the major components. *Tityus* venom was shown to exert an important secretagogue effect on guinea pig pancreas.

KEYWORDS: Scorpion toxins, *T. serrulatus*, pancreatitis, amino acid sequence.

INTRODUCTION

Toxins from the venom of the Brazilian scorpion *Tityus serrulatus* Lutz and Mello have been purified and studied by several independent groups of investigators[15,37,3,27,35], and several reviews[10,26] have been written. Tityustoxin and toxin gamma are the most thoroughly characterized. Toxin gamma was the first toxin for which a considerable part of the N-terminal amino acid sequence was reported[27,29] and the only one, thus far, for which the total amino acid sequence has been determined[31,6]. A brief summary of the different types of toxins present in the venom of *T. serrulatus* was reviewed by Possani[26]. Pharmacological and neurochemical studies were initially performed with Tityustoxin[10,14]. In recent years, both Tityustoxin and toxin gamma have been more extensively studied, and have been shown to affect Na^+ channel permeability of a variety of tissues[18,41] (and review)[20]. Initial work conducted by Bartholomew[5] reported that stings by scorpions of the genus *Tityus* can cause acute pancreatitis in humans.

Also the effect of the venom from *T. serrulatus* was demonstrated to cause pancreatitis in dogs[22]. Pioneer work by Novaes *et al*[25]. performed with tityustoxin has shown a clear effect of this purified toxin on the pancreatic secretion of the rat. Our preliminary experiments have shown several purified toxins to be active in secretory discharge of zymogen proteins of pancreatic lobules[12]. In the past it has been suggested that some of the differences found in the venom of *T. serrulatus* could be due to heterogeneity from scorpions of the same species collected in different geographical localities of Brazil[34]. There is need for a definitive study on the different toxic components present in the venom of *T. serrulatus* used at the Butantan Institute (Brazil). The purpose of this communication is to present the analysis of the venom of *T. serrulatus* from scorpions collected at different locations in Brazil by Butatan Institute, and at the same time to report some chemical and physiological charecteristics of toxins isolated from this venom.

MATERIALS AND METHODS

Venom Source.

Venom from *T. serrulatus* was obtained from the Instituto Butantan, São Paulo, Brazil. The scorpions were collected in several geographical localities of the State of Minas Gerais (Santa Barbara, Corrego Novo and Cataguases) and from the State of São Paulo (Aparecida do Norte). In Minas Gerais scorpions often are found in elevated places, mountains covered by low vegetation and grasses or in cracks and crevices of eroded land. They are also found in wooden support for the railroad track. In São Paulo they were mostly collected near houses.

Venom used for immunization at the Butantan Institute is normally obtained from scorpions of different areas, mainly from Santa Barbara and Corrego Novo, but for

this specific work, special care was taken to keep the venom separate for each area (see above). Venoms were dried in vacuum and kept refrigerated. Venom from *T. discrepans* was obtained from animals collected in the mountains surrouding Caracas City, in Venezuela. The venom was lyophilized and kept at -20°C. The dried venoms were dissolved in water and centrifuged 18,000 x g, for 10 min. The supernatants were directly used for comparative studies of whole venoms or for fractionation and characterization of toxic components.

Source of Chemicals.

Sephadex G-50 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and carboxymethyl-cellulose resins (CM-32) and diethylaminoethyl cellulose resins (DE-32) were from Whatman Inc., (Clifton, New Jersey). Spectrapor type 3 dialysis tubing was obtained from Spectrum Medical Industries (Los Angeles, CA.). Radiolabeled Iodoacetic acid came from New England Nuclear (Boston, Mass.), while dithiothreitol (DTT) was from Calbiochem Behring Corp. (La Jolla, CA). Most reagents for high performance liquid chromaography were from Pierce Chemical Co. (Rockford, Illinois) and chemicals and solvents for amino acid sequencing were from Beckman Instruments Inc. (Palo Alto, CA.), or from the sources indicated[29,31,23]. Additional chemicals were analytical grade reagents purchased from the following sources: paraformaldehyde, Eastman Kodak Co., (Rochester, NY); glutaraldehyde, Electron Microscopy Sciences (Fort Washington, PA); osmium tetroxide, Ernest F. Fullam, (Latham, NY); magnesium uranyl acetate, K&K Laboratories (Plainview, NY); epoxy resin, LX-112, Ladd Res. Industries (Burlington, VT); acrylamide and other reagents for gel electrophoresis, BioRad Laboratories (Richmond, CA).

Animals.

Adult albino mice (CD1 strain), approximately of 20 g body weight were used for lethality tests. Male albino Dunkin-Hartley guinea pigs weighing 250-350 g were used for microscopy studies.

Source of Anti-venom.

Horse anti-venom was obtained from Butantan Institute. The anti-venom (whole serum, undigested) was separated from the blood of horses immunized against the standard venom of *T. serrulatus*.

Lethality Tests.

Lethality tests in mice were conducted as described[27]. LD_{50} values were graphically determined from percentage of death mice versus logarithm of venom concentration. Basically, aliquots were injected intraperitoneally into 20 g mice that were observed for toxic effects. Among the symptoms of toxicity are: hyperexcitability, lacrimation, apnea, partial paralysis of the rear limbs, diarrhea, respiratory arrest and death.

Gel Electrophoresis and Immunoelectrophoresis.

The gel electrophoresis system of Reisfeld *et al.*[33], was used for comparison of venoms and assess purity of samples. Immunodiffusion and immunoelectrophoresis were carried out in 1% agarose gels[17], and precipitates were visualized after dye staining of dried and washed gels[4].

Protein quantification.

Unless otherwise stated, protein content was determined spectrophotometrically, assuming that one unit at 280 nm (1 cm pathlength) is equivalent to 1 mg/ml protein concentration.

Separation Procedures.

Separation of soluble venom was basically performed according to previous publication by our group[29]. Briefly, the procedure includes a size exclusion chromatography and two consecutive steps of weak cation exchange chromatography through carboxymethyl-cellulose (CM-cellulose) resins. The first separation is performed in a column (0.9 x 200 cm) of Sephadex G-50 (medium), equilibrated with 20 mM ammoniumm acetate buffer, pH 4.7, from which four main fractions are obtained. Each one of the toxic fractions (II to IV) are further separated in CM-Cellulose resins at two different pH's. The first CM-Cellulose column (0.9 x 30 cm) is developed in ammonium acetate buffer, pH 4.7. In this step toxic components II-11, III-8 and III-10, and IV-5 are separated, corresponding to the main toxic components of the venom[29].

Each one of these components is subsequently purified by rechromatography of the dialyzed fractions through a second CM-Cellulose column equilibrated with 50 mM sodium phosphate buffer, pH 6.0. A linear gradient from 0 M to 0.5 M NaCl in the same buffer is used to elute the toxic proteins in homogeneous form.

Enzymatic digestion mixtures of reduced and alkylated proteins used for amino acid sequencing were chromatographically separated into their component peptides by reverse-phase high performance liquid chromatography (HPLC) on octadecylsilane columns developed with linear gradients up to 60% acetonitrile (containing 0.1% trifluoracetic acid). Isolated fractions were evaporated to dryness under vacuum in a Savant Speed-Vac Concentrator prior to amino acid analysis and sequenator application.

Hyaluronidase Purification.

Hyaluronidase was assayed by the method of Tolksdorf *et al.*[38]. The enzyme was purified from the venom of *T. serrulatus* by three consecutive steps: Sephadex G-50 gel filtration, ion-exchange chromatography through diethylaminoethyl-cellulose (DE-Cellulose) resins and affinity chromatography using Sepharose-concanavalin A. The hyaluronidase activity elutes in the first fraction of the Sephadex G-50 column (see Results). This fraction was loaded in a DE-Cellulose column, after dialysis against 50 mM TRIS-HCL buffer, pH 7.6. To the unbound material containing the hyaluronidase activity salts were added to give the final concentration of 1 mM CaCl, 1 mM MnCl and 1.0 M NaCl, in the same TRIS-HCl buffer, pH 7.6. This material was chromatographed through a Sepharose-concanavalin A column (10 ml) equilibrated with the same buffer mentioned above. Elution was obtained with stepwise gradients of the equilibration buffer containing 0.04 M and 0.5 M alpha-methyl-mannoside. Most of the enzyme was eluted with the second buffer (0.5 M mannoside).

Amino Acid Composition and Sequence Determination.

Amino acid composition was obtained with acid hydrolysates of purified proteins analyzed with a Durrum/Dionex D-502 automatic amino acid analyzer, as described[27,32]. Prior to amino acid sequence determination the toxins were either reduced and carboxymethylated or pyridylethylated, as described[27,29,31]. Determination of half-cystine was obtained after performic acid oxidation[24]. Tryptophan content was deduced from sequence data. Carboxypeptidase digestionn for the determination of carboxyl-terminal amino acid residues was carried out in 50 mM sodiumm acetate buffer, pH 3.5, with carboxypeptidase P (Boehringer Mannheim Biochemicals, Indianapolis, IN). Fragmentation of reduced and alkylated toxins by enzymatic cleavage with protease V8 from *Staphylococcus aureus* and/or tryp-

sin was obtained as described previously[31]. Peptides were separated by HPLC as mentioned above.

Primary sequence determination by automatic Edman degradation[11], was carried out either on a Beckman 890M (Palo Alto, CA) microsequencer or on polybrene-treated fiber disks in an automated gas phase sequenator (Mod. 470A, Applied Biosystems, Foster City, CA), equipped with an online PTH-analyzer, as described[31,23].

Light and Electron Microscopy.

Pancreatic lobules were prepared according to the method described by Scheele and Palade[36].

They were immersed in Karnovsky's fixative[16] (1% formaldehyde-2% glutaraldehyde in 0.1M sodium cacodylate, pH 7.4) and diced into 1.0 mm cubes, and allowed fo fix overnight at 4°C.

Tissues were postfixed 3 hours at 4°C in 1% osmium tetroxide in 0.1M sodium cacodylate, pH 7.4. Following *en bloc* stain in 0.5% magnesium uranyl acetate overnight, tissue blocks were dehydrated and embedded in LX-112 epoxy resin[21].

For light microscopy, sections 0.5 μm in thickness were stained with 1% toluidine blue in 1% sodium borate[21] and photographed in a Zeiss Standard 16 microscope equipped with a Zeiss MC63a photomicrographic camera.

For electron microscopy, thin sections of 800 A were stained with alkaline lead citrate[40], then examined and photographed in a JEOL 1200EX electron microscope.

RESULTS

The five different venom samples obtained from *T. serrulatus* collected in Aparecida do Norte, Cataguases, Corrego Novo, Santa Barbara and the standard Butantan venom (mainly a mixture of Santa Barbara and Corrego Novo) were assayed for toxicity in mice and for enzymatic activities. They were analyzed by electrophoresis in beta-alanine-urea-acetate gels and immunoelectrophoresis for comparative purposes. They were each separated under identical conditions by gel filtration and ion-exchange chromatography. Lethal dose (LD_{50} determinations performed with CD1 albino mice, by intraperitoneal injections, gave essentially the same results for each of the venom samples. For example, the venom from Santa Barbara has a LD_{50} of 21 $\pm$ 1μg/20 g mouse weight, while the standard venom gave 22 $\pm$ 1μg/20 g mouse weight. The only enzymatic activity found in all 5 samples of venom was hyaluronidase, which was further characterized (see below). The results of electrophoresis separation of venom from scorpions collected in different geographical localities are presented in Fig. 1. The same amount of protein (100 μg) was applied in each cylindrical tube for polyacrylamide gel separation. At least 14 components are visible when stained with coomassie blue; numbers 5, 10, 11, 12, 13 and 14 being the more intensely colored. The most basic components with faster eletrophoretic mobility correspond to toxic peptides. The venoms have very similar electrokinetic properties. A slight variation in the realtive intensity of certain colored bands might be present. Compare for example, band 2 and 10 of lane *c* with the others. In Fig. 2 are the immunoelectrophoretic analysis of the different venoms using horse anti-venom as the serum source. The longitudinal slot contained the serum, while the venoms were placed inthe lateral wells. Two plates were run in parallel. The results of Fig. 2A show no appreciable immunological differences among the venoms. Fig. 2B shows the result of *T. ser-*

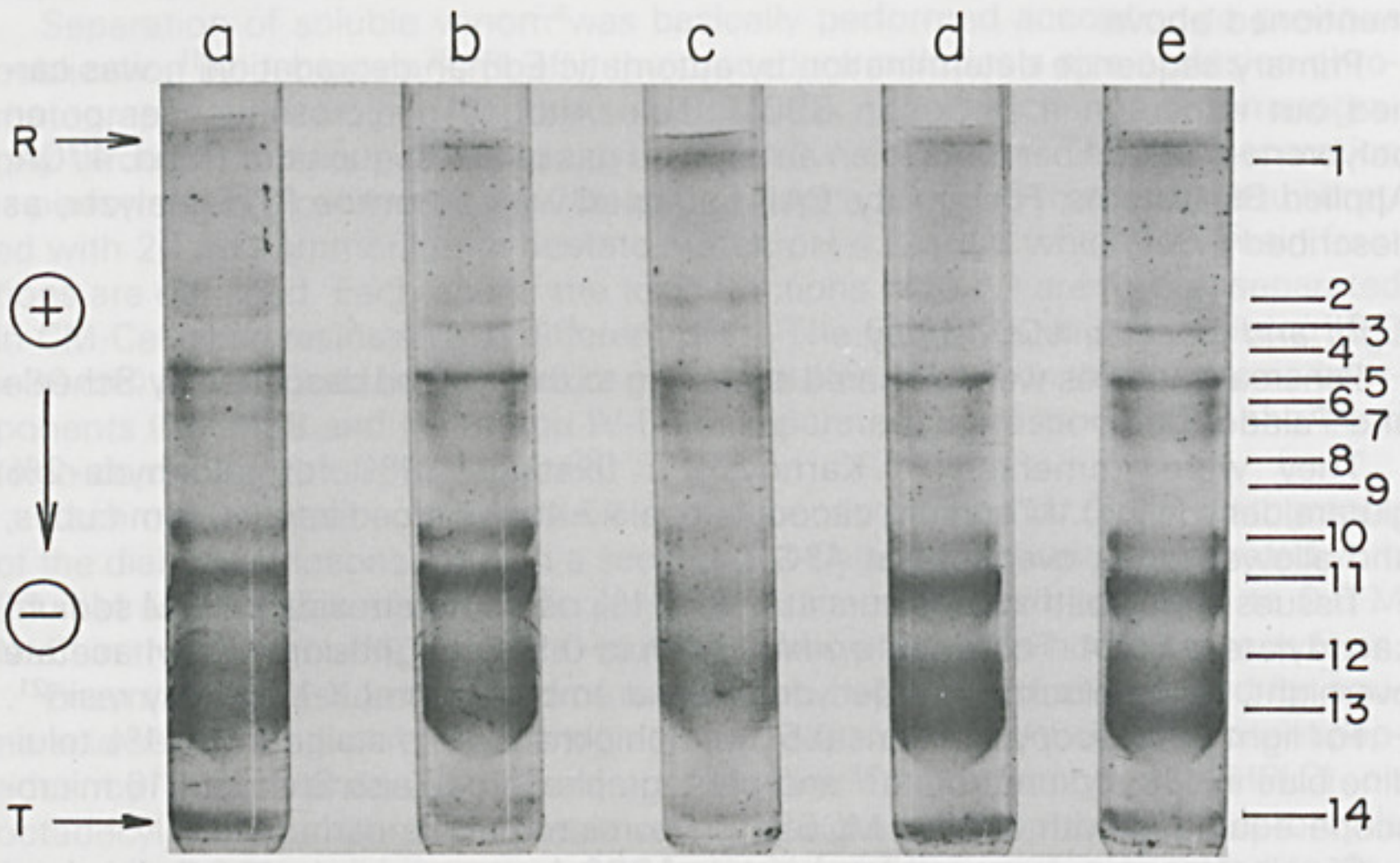

Fig.1. Gel electrophoresis of *T. serrulatus* venoms.
T. serrulatus venom from scorpions collected in Corrego Novo and Santa Barbara (a), Santa Barbara(b), Corrego Novo(c), Aparecida do Norte(d) and Cataguases(e) was applied to cylindrical gels of polyacrylamide (100 ug soluble venom in each lane) and run in the beta-alanine-acetate-urea electrophoresis system of Reisfeld *et*al[33]. Proteins and peptides run towards the cathode. A least 14 bands are visible; the most intensevely colored are number 5 and 10 to 14. R. designates origin of running gel, and T designates tracking dye.

rulatus venom and a control from another species of the same genus of scorpion *Tityus discrepans*). This figure (2B) shows a marked difference between *T. serrulatus* venom and *T. discrepans* collected in Venezuela. Fractionation of soluble venom by gel filtration on Sephadex G50 separates the venom from *T. serrulatus* into four main components (I to IV), see Fig. 3A, also Possani *et al.*[29] Very similar results were observed with venoms obtained in different geographical areas (data not shown). In Fig. 3 (B to D) are the results of further fractionation of the toxic fractions II, III and IV in CM-Cellulose columns.

Ten toxic sub-fractions were obtained: II-9 to II-11, III-7 to III-10, IV-5, IV-6 and IV-8, all toxic for mice. As discussed further below the toxic sub-fractions (ten) are not necessarily all different.

By this ion exchange separation method no appreciable differences were found among venoms of *T. serrulatus* collected in different geographical localities (data not shown). Final separation of pure polypeptides requires an additional chromatographic step. Usually a CM-Cellulose column equilibrated and run in 50 mM sodium-phosphate buffer, pH 6.0 is used. Dialyzed components II-11, III-8, III-10, and IV-5 were purified under these conditions to homogeneity.

Separation was obtained with a salt gradient from 0 to 0.5 M NaCl (see example in Fig. 3E).

During this chromatography small contaminants are eliminated; and the major toxic component is the main fraction of the chromatogram. The amino acid com-

position of the major toxins is compiled in Table I. Toxin II-11 (also called gamma toxin[27], and toxin III-10 are identical.

Recoveries obtained during these separation procedures are indicated in the legend of Fig. 3.

The homogeneity of toxic peptides was assessed by several criteria: shape of ion exchange columns (symmetrical peaks), gel electrophoresis[28], amino acid composition, profile of Sephadex G50 separation of reduced and alkylated tox-

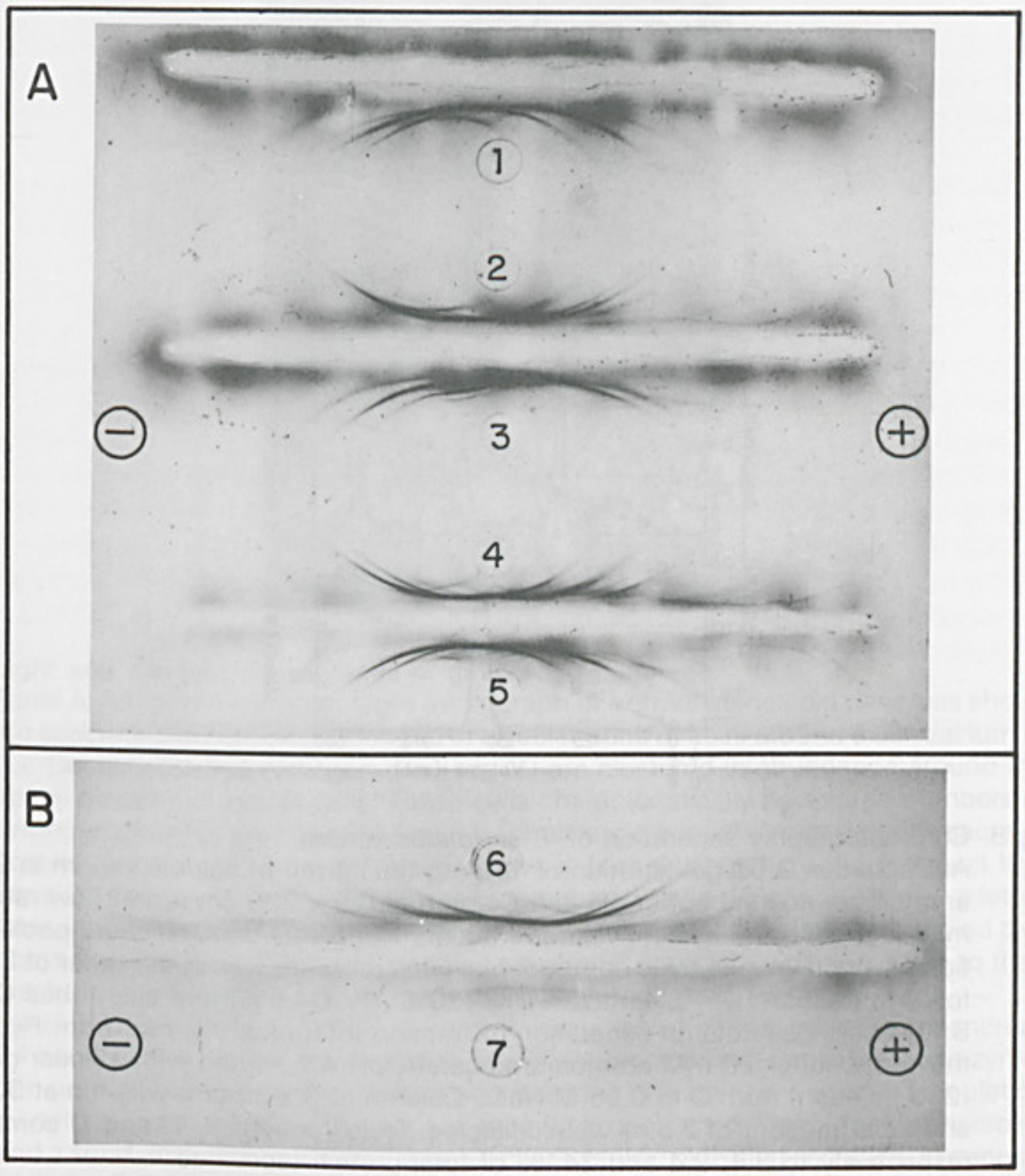

Fig.2. Immunoelectrophoresis of venoms from *T.serrulatus*.
PANEL A: Venom from scorpions collected in 5 different places were applied in agarose gel in 25 mM TRIS-Glycine buffer, pH 8.3 for electrophoresis and immunodiffusion, according to King *et al*17 . Wells contained 100 μg (in 10 ul) of each venom: Corrego Novo and Santa Barbara (1), Santa Barbara (2), Corrego Novo(3), Aparecida do Norte (4) and Cataguases (5). In the central slots 50 μl of horse anti-serum were used for immudiffusion.
PANEL B: Comparison of standard venom of *T. serrulatus* (6) and venom from T. *discrepans* (7) applied in the same conditions as in panel A.

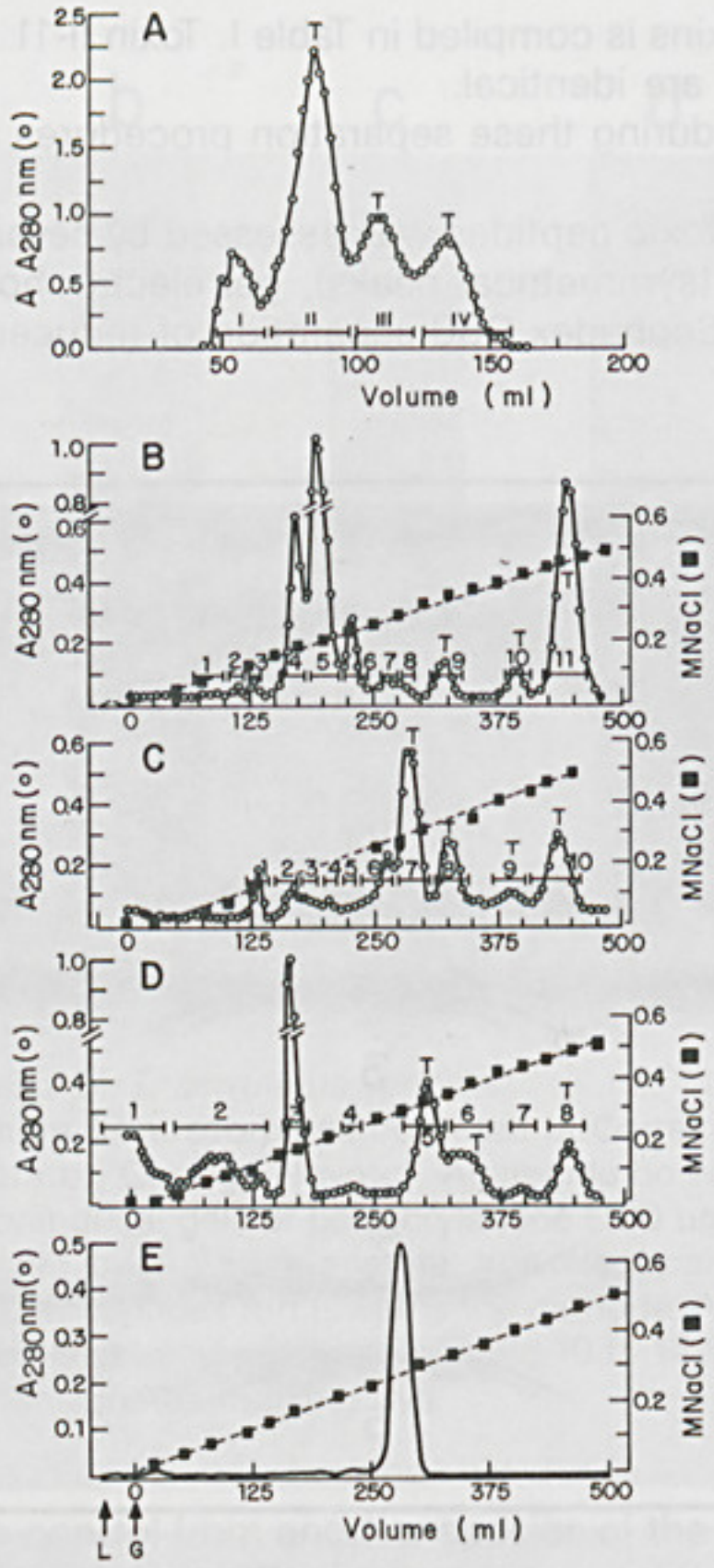

Fig.3. Chromatographic separation of *T. serrulatus* venom.
A: Sephadex G-50 gel filtration of 160 mg (in 1.4 ml) of soluble venom in 20 mM ammonium acetate buffer pH 4.7. Column of 0.9 x 200 cm run at flow rate of 15 ml/h and collected in 2 ml volume-fraction. Horizontal bars indicated pooled fractions (I-IV). T means toxic components. Final recovery was in the order of 83% as follows: fraction I (14%), II (32%), III (20%), IV (14.5%) and side tubes (2,5%).
B: CM-cellulose column separation of fraction II (approx. 42 mg) from Fig. 3A in the same buffer 20 mM ammonium acetate, pH 4.7, eluted with a linear gradient (250 ml each) from O to 0.55 M NaCl. Column (0.9 x 30 cm) was run at 30 ml/h, and tube-fractions of 2.5 ml were collected. Toxic fractions 9, 10 and 11 correspond respectively to 3.4, 2.4 and 24.1% of total protein recovered.
C: Same as in B for fraction III (26 mg) of Fig.3A Toxic fractions 8,9 and 10 correspond respectively to 15.5., 6.3 and 16.4% of protein recovered. Fractions 6 and 7 (toxic) were pooled because they were not well separated, and correspond to 32,4% of total material recovered.
D: Same as in B fraction IV (19 mg) of Fig.3A. Toxic fractions 5, 6 and 8 correspond respectively to 10.5, 5.9 and 4.9% of material recovered.
E: Final purification of gamma toxin from sub-fraction II-11 of Fig 3B (10 mg) in 50 mM phosphate buffer pH 6.0. Column, ion exchanger, flow rate, tube-fractions and salt gradient similar to Fig. 3B. Purified toxin is approximately 84% of the material applied to the column.

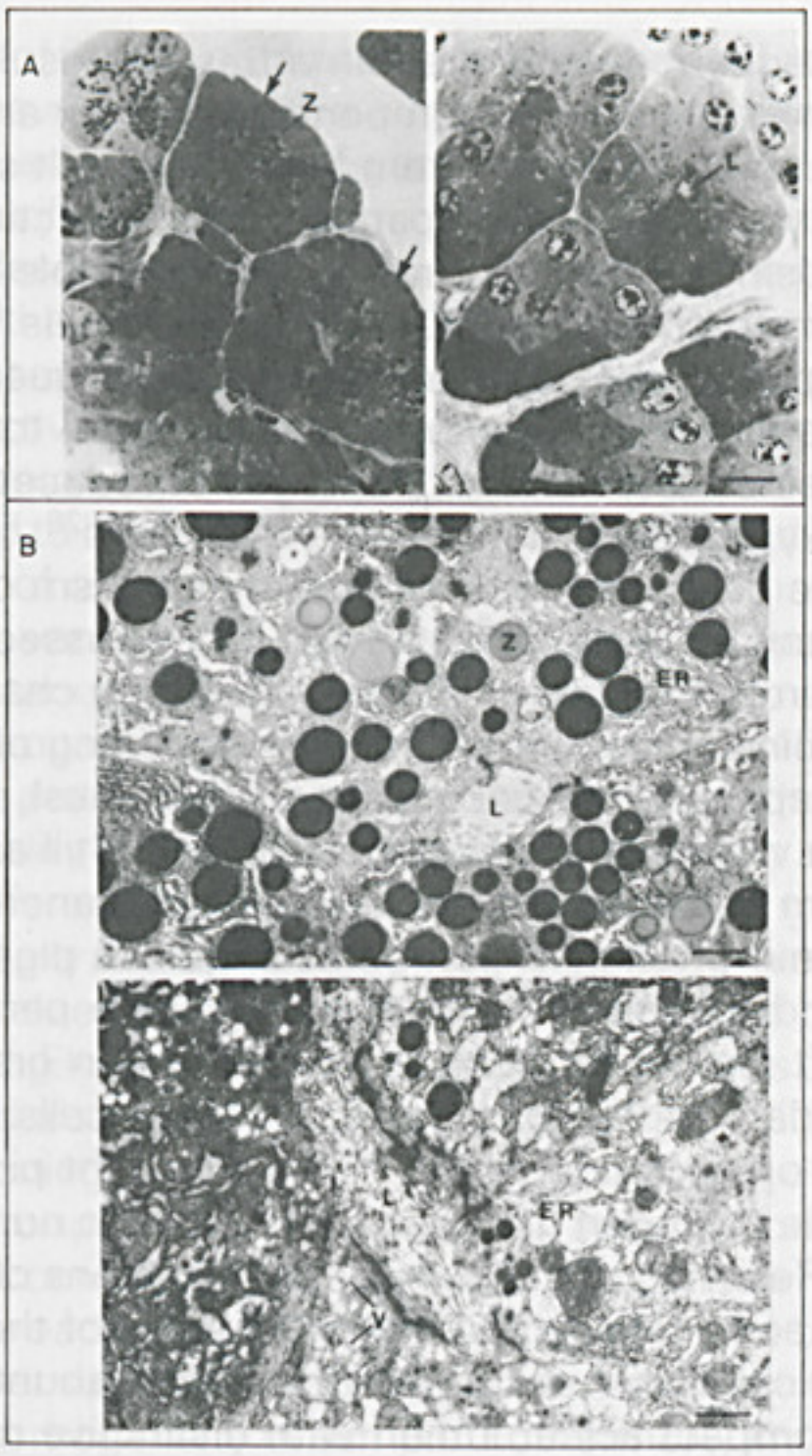

Fig.4. Light and electron micrographs of guinea pig pancreas.
Panel A: left panel (control): Light micrograph of normal guinea pig pancreas showing exocrine cells (arrows) arranged in alveolar units of acini around a central lumen (L). Densely stained zymogen granules (Z) are clustered in abundance around the apical portions of acinar cells. These cells characteristically have large numbers of zymogen granules prior to stimulation by cholinergic or peptidergic exocrine secretagogues. Another portion of the picture shows an Islet of Langerhans (IL). Bar igual 10μ.
Panel A: right panel (venom): Light micrograph of guinea pig pancreas after a lethal dose of the Brazilian scorpion *Tityus serrulatus* whole venom. The animal died two hours postinjection. Zymogen granules are almost completely depleted due to their discharge from stimulation by the venom secretagogues. Bar igual 10μ.
Panel B: Upper panel (control): Electron micrograph of normal guinea pig pancreas 24 hours after a control injection of Krebs-Ringer Bicarbonate. Densely stained zymogen granules (Z) are packed at the apical portions of acinar cells prior to stimulation by natural secretagogues. The acinar lumen (L) is contracted with numerous microvilli and a small amount of secretory material characteristic of resting secretion. In general, both the Golgi complexes and the endoplasmic reticulum (ER) are normal in appearance. Bar igual 1μ
Panel B: Lower panel (venom): Electron micrograph of guinea pig pancreas from an animal injected with a lethal dose of venom from the Brazilian scorpion, *Tityus serrulatus*. At death, 2.8 hours post-injection, the zymogen granules had been discharged in response to stimulation by the venom secretagogues. The acinar lumen (L) is distended with densely stained crystalline secretory material. The two cells at right of picture have rough ER cisternae grossly dilated with secretory proteins and may have sustained some structural damage. Numerous apical vesicles (V) present in the cells at bottom and upper left are evidence that exocytosis had occurred. Bar igual 1μ

ins, and amino acid sequence. Table II summarizes the results of amino acid sequence determination of all the toxic components thus far analyzed. Also included for comparative purposes are data from the literature. It should be noted that toxin II-9A and II-9B were originally separated after reduction and alkylation of fraction II-9 (ref.[30]). Using a different strategy we were able to obtain toxin II-9A in homogeneous form to show the effect on K^+ channels [8] and Valdivia, H.H. and Possani, L.D., unpublished. Complete amino acid sequence of toxin gamma (II-11) was obtained after HPLC of enzymatically cleaved toxin[31]. Toxin III-8 sequence was also obtained after several cleavages of reduced and alkylated peptide, including carboxypeptidase time course hydrolysis[28].

Purified toxins have been used to verify the mechanism of action in a variety of preparations [41,18] and reviews[10,14,20]. As will be discussed below two classes of toxins were found in the venom of *T. serrulatus*, long chain polypeptides (approximately 60-62 amino acids), which impair functioning of Na^+ channels, and short-chain peptides (approximately 40 amino acid residues), which modify potassium channels from a variety of excitable membranes. Still another known effect of *T. serrulatus* venom is the secretagogue effect on pancreas[5,12,22,25]. For this reason the results of morphology experiments in guinea pigs reported in Fig. 4A (light microscope) and 4B (electron microscope) were performed. It is clearly shown in Fig. 4A (left picture for control) that zymogen granules in pancreatic acinar cells are abundant in the apical areas of these cells. The tissue displays its typical cytological organization, while in Fig. 4A (right picture for animals injected with venom) the zymogen granules are reduced in number or even absent in some acinar cells. Referring of the higher magnifications of the electron micrographs in Fig. 4B the acinar tissue demonstrates some of the distinctive characteristics of normal apical acinar cell organization with abundant electron-dense zymogen granules, compact acinar lumen with distinctive microvilli, and stacks of endoplasmic reticulum (ER) cisternae. In Fig. 4B (lower part, venom-treated pancreas) the apical region is largely devoid of mature zymogen granules, and a few granules of reduced size remain. The discharge of zymogen granules is evident from the abundance of crystalline and amorphous secretory content in this lumen. Fig. 4B shows in addition to the lack of zymogen granules that the ER cisternae are greatly distended with proteins vectorially destined for discharge. Apical vesicles are evidence of recycling of zymogen granule membrane after exocytotic release in response to venom-secretagogue stimulation. Other micrographs (not shown) demonstrate luminal surfaces partially effaced with loss of microvilli on part of the acinar plasmalemma.

The only enzymatic component isolated in homogeneous form from this venom was the hyaluronidase, which was non-toxic. For this purpose we have used three chromatographic separation steps (see Material and Methods). The enzyme activity is very stable in the whole venom, but during purification, especially in the last step, the activity is lost within a few hours.

The enzyme was obtained in pure form, as judged by polyacrylamide gel electrophoresis, and its molecular weight was calculated to be 42,000, with the aid of SDS polyacrylamide gels[19] (data not shown).

DISCUSSION

T. serrulatus venom from the Instituto Butantan (São Paulo, Brazil) used by our group during the last 18 years has always given very consistent biochemical and pharmacological results.

Numerous batches of venom obtained from the Butantan Institute during the period of 1973 to 1990 have been used in our laboratories, initially for purification of several toxins[2] and characterization of gamma toxin[27,13]. Since 1981 the strategy we have followed for purification and chemical characterization of toxins is very similar to the one presently described[29,31], which we assume is the most convenient one. At least in our hands the venom from the Butantan Institute is highly appreciated because of its constant and reproducible biochemical and pharmacological behavior. One of the main objectives of this communication is to compare venom obtained from *T. serrulatus* scorpion of different geographical localities of Brazil; at least from regions not considered to be ''restricted areas'', as mentioned by other authors[10,34]. A careful observation of our results (Fig. 1 to 3) indicates that there are no significant differences in the venom of *T. serrulatus* collected in topological areas greater than 400 Km. The distance of Santa Barbara, Corrego Novo and Cataguazes from Belo Horizonte, the capital of the State of Minas Gerais, is 100, 400 and 460 Km respectively, while the distance of Aparecida do Norte from the capital of São Paulo State is 200 Km. Lethality tests conducted with venom from scorpions of different localities, using 10 different doses of venom, in 10 different groups of mice (strain CD1, i.p.) gave essentially the same value (see Results), which in turn is the same or very similar to the value initially reported by Bücherl[7], and by our group[27], of 25 μg/20 g mouse weight. Electrophoretic separation of venoms by the system of Reisfeld *et al*[33], suggested also that there was no appreciable difference in these venoms. It is worth mentioning that Reisfeld's method is capable of discriminating small differences (charge groups) in scorpion toxins. If sodium dodecyl sulfate gel electrophoresis is used according to the technique of Laemmli[19], most of the toxins comigrate in a single broad band. Thus, we conclude that the beta-alanine-acetate-urea gel system is better suited for verification of scorpion toxin purity, and is a good criterion for judging homogeneity of sample. Futhermore, immunoelectrophoresis is an adequate technique for verification of heterogeneity of samples; at least, it can discriminate mixtures of antigens or possible immunodominant antigens. This was the case found (Fig. 2B) when comparing venom from another related species of scorpion *(Tityus discrepans)* collected in Venezuela. Finally, the chromatographic separation by Sephadex G-50 and CM-Cellulose shows that these venoms are very similar in composition (data not shown).

The separation procedure described here for the isolation of mammalian toxins from the venom of *T. serrulatus* allows the identification of 10 sub-fractions toxic to mice. However, fraction II-11 (originally denominated toxin gamma[27] and III-10 are identical components, as demonstrated by amino acid analysis (Table I) and by amino acid sequence (Table II).

Fractions II and III from Sephadex G50 column (Fig. 3A) are not well separated, hence the subsequent ion exchange chromatography of both fractions in CM-Cellulose columns at pH 4.7 (Fig. 3B and 3C) provided two chromatographic peaks corresponding to the same toxin. The major toxic components in the venom of *T. serrulatus* are toxin gamma, III-8 and IV-5. They have in the order of 60-62 amino acid residues (Table I), with a calculated molecular weight of 6,500 to 7,000. Toxic sub-fraction III-7 is also a major component of the venom, but it is heavily contaminated (data not shown), and still further purification is required. Among the minor components are toxins II-9, II-10, III-9, IV-6 and IV-8. Toxin II-10 has an amino acid sequence identical to toxin gamma up to residue 46 (Table II), already sequenced. Similarly, component II-9B is equivalent to III-8 up to residue 30 (Table II). Chemical differences between these two pairs of components possibly are at the C-terminal region of the molecule.

Minor components III-9 and IV-8 were not studied, and component IV-6 is identical to IV-5 up to residue number 30 (Table II). Moreover, the difference in chromatographic behaviour may be due to a deamination occuring at an Asn (or Gln). Component II-9A is a minor component, initially found by direct sequence[30], with sequence homology to Noxiustoxin, a K^+ channel blocking peptide isolated from *Centruroides noxius* scorpion venom[9]. Rechromatography of component II-9, produced toxin 1, which blocks K^+ channels of squid giant axons[8].

Other authors have studied venom from *T. serrulatus* and found similar toxins named with different nomenclature as the one we have proposed. The first toxin purified was Tityustoxin by the group of Prof. Diniz[15] in Brazil. This toxin is similar in amino acid composition to our component IV-5 (see review[10]). The purification procedure of Toledo and Neves[37] confirms the presence of more than one toxin in the venom of *T. serrulatus*, but presented no amino acid sequence data. Based on amino acid composition and N-terminal amino acid (lysine) we have surmised that toxin TsTx-I[37] is the toxin gamma of our work, while toxin TsTx-II is a new toxin in this venom[37]. It does not correspond with any of the toxins we are describing or to the ones purified by Sampaio *et al.*[35].

The group of Prof. Rochat in France has sequenced a toxin[6] almost identical to gamma toxin[31], which they have called toxin VII. The only difference is at the cysteine at position 61, which they report to be amidated. The publication by Sampaio *et al*[36] reports the purification of five toxins from *T. serrulatus*. Considering the amino acid sequence reported[35], toxin T1VIII is similar to toxin gamma[27], toxin T2III1 is possibly the same as IV-5 of our work. Toxins T1VI and T1IV[35] seem to be toxins previously unreported in this venom.

Concerning the pharmacological effects of venom or purified toxins from *T. serrulatus*, the reviews by Diniz[10] and recently by Freire-Maia and Campos[14] summarize the main results. It is clear that most of the action of the toxic peptides is through impairment of ion channel (sodium and potassium) funcion. Among the most significant works thus far published are the effects of gamma toxin and Tityustoxin on neuroblastoma cells (NIE 115), rat brain synaptosome membranes, chick heart sarcolemma and other membrane preparations (see review by Lazdunski *et al.*[20]). Our group was able to show that gamma toxin affects the peak permeabillyty of Na^+ in cardiac cells, like the beta scorpion toxins[41], while toxin IV-5 modifies Na^+ channel gating like the North African alpha scorpion toxins[18]. Squid giant axon potassium channels are blocked by toxin II-9A (toxin 1) of *T. serrulatus* venom[8]. Experimental pancreatitis in dogs caused by *T. serrulatus* was reported by Machado *et al.*[22], and *T. trinitatis* induced pancreatitis in humans by Bartholomew[5]. Pioneer work with purified scorpion toxin from *T. serrulatus* (tityustoxin) on the pancreatic secretion of the rat was published by Novaes *et al.*[25].

We have demonstrated the effects of whole venom from *T. serrulatus* and purified peptides (III-8, III-10, and IV-5) in rodents (mouse, rat and guinea pig) *in vitro* and *in vivo*[12]. Morphological alterations due to secretagogue effects of whole venom (Fig 4) and its peptide components are apparent by both light and electron microscopy of pancreatic acinar cells. Biochemical measurements of pancreatic secretion were carried out by both radioisotopic pulse-chase experiments as well as determinations of enzymatic activity discharged[12]. These studies indicate that the venom from *T. serrulatus* causes extensive discharge of secretory proteins *in vivo* and *in vitro*.

Humans stung by scorpions of genera *Tityus* and *Centruroides* in North and South America develop potentially lethal symptoms from action of the venom on

peripheral nerves and vital organs[10,14,20,25,41]. Included in a myriad of distress factors is the epigastric pain of acute pancreatitis[5,12,22,25,28]. This pain is accompanied be elevated serum amylase associated with clinical signs of acute pancreatitis[12,25,28]. There are occasions when the pancreatitis alone is severely debilitating and represents a complication that can extend the recovery of these patients considerably. When the individuals are not treated promptly with appropriate antiserum the clinical course is always aggravated and prolonged, particularly in children under age 5[12,22].

The only non toxic protein isolated from *T. serrulatus* venom, in pure form, is the enzyme hyaluronidase that we have described here. This enzyme is known to be ubiquitous in most scorpion venoms[1,27,39], and was initially reported to be present also in *T. serrulatus* venom[27].

The enzyme is of high molecular weight (approx. 42,000) and is likely to be a glycoprotein, since it binds concanavalin A. We have originally described a similar enzyme in the venom of *Centruroides limpidus limpidus*[1].

CONCLUSIONS

1. Venom from the scorpion *T. serrulatus* collected in several distinct geographical localities of Brazil does not show detectable differences in biochemical and pharmacological properties.
2. The venom from Butantan Institute, in our hands, has proved to provide reproducible results over a period of 18 years.
3. The venom contains two classes of mammalian peptide toxins, long-chain neurotoxins with 60 to 62 amino acid residues, which block sodium channel of excitable membranes (variety of tissues and sources), and short chain neurotoxins (approx. 40 amino acids) which block potassium permeability (squid giant axons and rabbit skeletal muscle).
4. The diversity of toxic peptides in the venom of *T. serrulatus* venom confirms similar finding with *Centruroides* species of the North American continent, and *Androctonus, Leiurus* and *Buthus* species of Africa and Asia.
5. We confirm data of a potent secretory effect of venom from *T. serrulatus* in pancreas.

RESUMO: O veneno de escorpiões *Tityus serrulatus* coletados em quatro localidades geográficas distintas do Brasil foi estudado por eletroforese em gel, imunoeletroforese, filtração em Sephadex G-50 e cromatografia de intercâmbio iônico. Também se conduziram experiências de letalidade em camundongos. Nenhum destes métodos revelou diferenças significativas entre as amostras de veneno. O veneno de *T. serrulatus* do Instituto Butantan tem demonstrado propriedades bioquímicas e farmacológicas constantes e reproduzíveis em nossos laboratórios por cerca de 18 anos. O veneno possui vários polipeptídeos tóxicos ao camundongo. Entre os componentes tóxicos que aparecem em maior concentração no veneno estão toxina gama, III-8 e IV-5, as quais modificam a permeabilidade de sódio em canais iônicos. O peso molecular destas toxinas é da ordem de 7,000. Um dos componentes que aparecem em menor concentração no veneno é a toxina II-9A, um pequeno peptídeo (aproximadamente de 4,200 de peso molecular) que modifica a permeabilidade do potássio em axons. Pelo menos outros 4 componentes que aparecem em baixa quantidade no veneno também foram purificados com seqüência de aminoácidos muito semelhantes aos com-

ponentes gama, III-8 e IV-5. O veneno de *Tityus serrulatus* exerce um efeito secretagôgo importante em pâncreas de cobaias.

UNITERMOS: Toxinas de escorpião, *Tityus serrulatus*, pancreatite, seqüência de aminoácidos.

ACKNOWLEDGEMENTS

Dr. Jorge Villegas is acknowledged for providing facilities for two of us (L.D.P. and P.L.F.) during collection of scorpions *Tityus discrepans* of Caracas (Venezuela); and Prof. M.A. Gonzalez-Sponga for identification of the species collected. Technical assistance of Mr. Pedro Sauceda Ramirez and Mercedes Sifuentes Valenzuela during preparation of the art work is recognized.

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TABLE 1
Amino Acid Composition of Toxins from the Venom of *Tityus serrulatus**

Toxin	II-11	III-10	III-8	IV-5
Amino Acid	Residues per Mole	Residues per Mole	Residues per Mole	Residues per Mole
ASP	3.7 (4)	3.8 (4)	4.9 (5)	9.5 (10)
THR	0.9 (1)	1.1 (1)	1.9 (2)	1.9 (2)
SER	3.8 (4)	3.9 (4)	2.8 (3)	2.8 (3)
GLU	3.1 (3)	3.3 (3)	2.2 (2)	2.0 (2)
PRO	3.0 (3)	2.8 (3)	2.8 (3)	2.8 (3)
GLY	8.0 (8)	8.0 (8)	6.0 (6)	4.0 (4)
ALA	3.0 (3)	3.0 (3)	5.6 (6)	2.9 (3)
1/2CYS	8.0 (8)	6.3 (6-8)	6.5 (6-8)	7.0 (6-8)
VAL	1.8 (2)	2.0 (2)	1.9 (2)	1.8 (2)
MET	0.9 (1)	0.9 (1)	0.9 (1)	0.0 (0)
ILE	1.9 (2)	2.0 (2)	1.8 (2)	1.8 (2)
LEU	3.0 (3)	3.1 (3)	1.3 (1)	3.0 (3)
TYR	4.5 (5)	4.7 (5)	5.3 (5)	7.7(8)
PHE	1.0 (1)	1.1 (1)	2.9 (30)	0.0 (0)
HIS	1.0 (1)	1.0 (1)	2.7 (3)	1.0 (1)
LYS	5.5 (5)	6.2 (6)	6.9 (7)	6.7 (7)
ARG	2.6 (3)	2.8 (3)	1.0 (1)	0.0 (0)
TRP	3.0 (3)	3.0 (3)	2.0 (2)	2.0 (2)
Residues	61	61	62	60
Mol. Wt.	6686	6686	6957	6918

*Taken from Possani *et al.*[28]

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TABLE 2
AMINO ACID SEQUENCRE OF T. serrulatus SCORPION TOXINS

TOXIN	AMINO ACID SEQUENCE	REFERENCE
T.s. (II-11)	KEGYLMDHEGCKLSCFIRPSGYCGRECGIKKGSSGYCAWPACYCYGLPNWVKVWDRATNKC	31
T.s. III-10	KEGYLMDHEGCKLSCFIRPSGYCGRECGIKKGSSGYCAWPACYCYGLPNWVKVWDRATNKC	31
T.s. II-10	KEGYMDHEGCKLSCFIRPSGYCGRECGIKKGSSGYCAWPACYCYG...	26
T.s. III-8	KEGYAMDHEGCKFSCFIRPAGFCDGYCKTHLKASSGYCAWPACYCYGVPDHIKVWDYATNKC	28
T.s. IV-5	KKDGYPVEYDNACYICWNYDNAYCDKLCKDKKADSGYCYWV...	26
T.s. IV-6	KKDGYPVEYDNACYICWNYD...	26
T.s. II-9A	VFINAKCRGSPECLPKCKEAXGKAAGKCXN...	30
T.s. II-9B	KEGYAMDHEGCKFSCFIRPAGFCDGYCKTH...	26
T_1 VIII	KZGYLMBHZGCK...	35
T_1 III_1	KKBGYPVCCS...	35
T_1 IV	KZGYXL...	35
T_1 V_1	GHFGK...	35