Multiple bradykinin-related peptides from the capture web of the spider Nephila clavipes (Araneae, Tetragnatidae)

Evelyn C.F.R. Volsi, Maria Anita Mendes, Maurício Ribeiro Marques, Lucilene Delazari dos Santos, Keity Souza Santos, Bibiana Monson de Souza, Eduardo Feltran Babieri, Mario Sergio Palma

1. Introduction

Nowadays, there is keen attention from scientists to the toxins existing in the animal venoms, since they constitute a rich source of bioactive substances, in particular of neurotoxins. A number of toxins isolated from Arthropod venoms have been reported to act at level of the nervous systems and some of them are being extensively used as research tools in the investigation of neural functions, such as the characterization of the mechanisms of action of ion channels, while other toxins are used in the investigations of neurological disorders of clinical interest. Solitary wasps, scorpions and spiders can be included among those Arthropods since they inject their venoms into their preys to kill/paralyze them, and hence, the venoms may contain a variety of neurotoxins.

In 1954 Jacques and Schachter already had determined that venom of the wasp Vespa vulgaris contains high amounts of a component that exhibited physicochemical properties similar to those of purified bradykinin (BK). A series of different BK-related peptides were identified in the venoms of different wasp species. The striking action of these peptides both on vascular permeability and on the nociceptive afferent neurons attributed to these kinins the role of responsible for the inflammatory and algesic effects caused by the wasps venoms. However, in addition to kinins, a number of other components were also reported to be present in wasp venoms, which could contribute to the observed effects, either by their own or by...
releasing secondary mediators from the injured tissue. Among these factors are histamine, 5-hydroxytryptamine (5-HT) or acetylcholine [14,15,23]. Kinin-related peptides have been isolated from the venoms of snakes, wasps, frogs, bees and spiders [3,9,12,24,26].

Neurotoxic kinins have been reported in the venom of solitary wasps [17,18], such as the threonine-bradykinin (Thr6-BK) and megascoliakinin (Thr6-BK-Lys-Ala) isolated from the venom of the European scolid wasp Megascolia flavifrons [27]; Thr6-BK was also reported in the venom of the wasp Colpa interrupta [19]. An analytical survey with the venom extracts of 27 species of solitary wasps from the families Pompilidae, Specidae, Eumenidae and Scoliidae, by using MALDI-TOF-MS as the experimental tool, demonstrated that BK-related peptides were present in four of these species, where Thr6-BK was the major component in the venom of Megacompsomeris surpristica [8]. The wasp kinins are known to block the synaptic transmission of the nicotinic acetylcholine receptor in the insect central nervous system [5,17,18,20].

The function of the kinin-related peptides in animal venom is not clear; however, taking into account the wide spectrum of actions already reported when these peptides were assayed, such as constriction/relaxation of muscles [14], activation of leukocytes followed by the releasing of cytokines (prostaglandines and leukotrienes) [1] and the blockage of the cholinerger transmission in insect central nervous system (CNS) [20], it seems that the kinin-related peptides have been used as venom toxins for defense or predation, depending on the type of animal venom under consideration.

Recently, it was reported that the orb-web-spider Nephila clavipes produces low molecular mass non-peptide toxins, which are applied by the spider on the capture web, to be used as tools for prey capture [10,11]. In the present manuscript, we are describing the identification and the biochemical and pharmacological characterization of three novel bradykinin-related peptides produced by the spider on very reduced concentrations and applied in the oily droplets of the spiral strand of its web, apparently to be used as toxins for prey capture. These peptides were isolated from aqueous extracts of the web by chromatographic techniques; their amino acid sequences were determined by ESI-MS/MS and some of their biological actions were investigated.

2. **Experimental**

2.1. **Sample preparation**

2.1.1. Biological material and extract preparation

*N. clavipes* spider’s webs were collected from a wooded area in Rio Claro, SP, Southeast of Brazil. The toxins were extracted from the webs by soaking about 5 g of webs into 30 ml water during 4 h at room temperature. The resulting extract was centrifuged at 13,000 rpm/min during 60 min; the supernatant was collected, lyophilized and maintained at −20 °C until be used. This material was designated here as washed-web-extract (WWE).

2.1.2. WWE fractionation

The WWE was solubilized into 10 mM ammonium acetate, pH 6.8 and applied in an AKTA-FPLC system using a Sephacryl S-100 column (16 cm × 60 cm) (Amersham Biosciences, Bucks, UK) equilibrated with the same solution of WWE solubilization. The elution was performed at the flow rate of 30 ml/h using the equilibrium solution, at 8 °C; fractions of 1 ml were collected and monitored at 280 nm.

AKTA-FPLC system coupled to a Hi-Trap Sephadex G-25 Superfine column (1.6 cm × 2.5 cm) (Amersham Biosciences, Bucks, UK) equilibrated with a solution of 10 mM ammonium acetate, pH 6.8 was used to purify the peak containing the peptides fraction. The elution was performed at a flow rate of 30 ml/h using the same solution of equilibrium at 8 °C; fractions of 1 ml were collected and monitored at 280 nm. The acquisition and treatment of the chromatographic data were carried out using UNICORN software.

2.2. **ESI mass spectrometry analysis**

All the mass spectrometric analysis were performed in a triple quadrupole mass spectrometer (MICROMASS, mod Quatro II). The experimental protocol was based in details described in a previous publication [13] and adapted for the present investigation. The mass spectrometer was outfitted with a standard electrospray probe (ESI – Micromass, Altrincham). The samples were injected into electrospray transport solvent by using a micro syringe (500 μl) coupled to a micro infusion pump (KD Scientific) at a flow rate of 4 μl/min.

The mass spectrometer was calibrated with intact horse heart myoglobin and its typical cone-voltage induced fragments to operate at resolution 4000. The samples were dissolved in 50% (v/v) acetonitrile [containing 0.1% (v/v) formic acid] to be analyzed by positive electrospray ionization (ESI+) using typical conditions: capillary voltage of 3.5 kV, cone voltage of 30 V, desolvation gas temperature of 80 °C, flow of nebulizer gas (nitrogen) of 15 l/h and of drying gas (nitrogen) 200 l/h. The spectra were obtained in the continuous acquisition mode, scanning from m/z 100 to 2500 at a scan time of 5 s. The acquisition and treatment of data were performed with MassLynx software.

2.3. **ESI tandem mass spectrometry (MS/MS) analysis and peptide sequencing**

All the tandem mass spectrometry experiments were performed by positive electrospray ionization in a quadrupole time of flight in orthogonal time-of-flight configuration (Q-ToF™) mass spectrometer (MICROMASS, mod. Ultima API), using peptide solutions in water acetonitrile (1:1) acidified with formic acid 0.1% (v/v). Typical conditions were: capillary voltage of 3 kV, cone voltage of 30 V and desolvation gas temperature of 80 °C. The doubly charged (di-protonated) ions were subjected to collision-induced dissociation (CID) with argon as collision gas in the 50 eV collision energy range. The isobaric I/L residues was performed by searching for the presence of d- and u-type fragment-ions obtained under high energy CID conditions with argon gas for the natural peptides; as described elsewhere [13].
2.4. Peptide synthesis and purification

The peptides were prepared by step-wise manual solid-phase synthesis using N-9-fluorophenylmethoxy-carbonyl (Fmoc) chemistry with Novasyn TGS resin (NovaBiochem). Side-chain protective groups included t-butyl for serine and t-butoxycarbonyl for lysine. Cleavage of the peptide–resin complexes was achieved by treatment with trifluoroacetic acid/1,2-ethanedithiol/anisole/phenol/water (82.5:2.5:5:5:5, v/v), using 10 ml/g of complex at room temperature during 2 h. After filtering to remove the resin, ethyl ether at 4°C was added to the soluble material causing precipitation of the crude peptides, which were collected as a pellet after centrifugation at 1000 × g, during 15 min at room temperature. The crude peptides were solubilized in water and chromatographed under RP-HPLC conditions using a semi-preparative column (Shiseido C18, 250 mm × 10 mm, 5 μm), under isocratic elution with 40% (v/v) acetonitrile in water [containing 0.1% (v/v) trifluoroacetic acid] at a flow rate of 2 ml/min. The elution was monitored at 215 nm with a UV-DAD detector (Shimadzu, model SPD-M10A) and each fraction eluted was manually collected in 2 ml plastic vials.

2.5. Biological assay on isolated rat ileum and duodenum

The synthetic peptides were assayed both on isolated ileum and duodenum muscle from young Wistar rats (200–220 g), by using strips of these muscles (1–2 cm) suspended in a 5 ml Tyrode solution, at 37°C. The isotonic contractions and the muscle relaxations were recorded by means of a kymograph. The peptides were individually assayed and the activity was measured on log–dose curve obtained with an established dose of standard bradykinin (10⁻⁵ M/assay). Each experiment was repeated five times and the results were expressed as mean values.

The compounds HOE-140 (p-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸] and [Des-Arg¹⁰] HOE-140 (acquired from RBI/Sigma Chem. Co.) were used as antagonists of bradykinin B₂ and B₁ receptors, respectively.

2.5.1. Insecticide activity

Different doses of each synthetic peptide (from 2 to 150 pmoles/mg of insect) dissolved in physiological solution (0.9% (m/v) NaCl) were injected in a final volume of 1 μl into the pronotum of honeybees (Africanized Apis mellifera), by using a Hamilton microsyringe (10 μl). The insects were maintained within a Petri dish up to 24 h in presence of candy (food) and water supply. During this period of time the toxicity effects and/or the lethal action were observed; injection of physiological solution into the insect pronotum performed control experiments. The toxicity was assayed by using different concentrations of the peptides and compared against a control (N = 10, per concentration). The number of dead insects was determined after 24 h of peptides application. Toxicity levels were calculated according to Probit method [2] and expressed as lethality (LD₅₀).

2.6. Statistics

Statistic treatment of the data was performed with the non-parametric tests, Mann–Whitney U-test or Kruskal–Wallis H-test, followed by a Tukey–Kramer post-test as needed, [22] the value of P < 0.05 was considered to be significant. The results are presented as means ± standard error (S.E.).

3. Results

The WWE from the spider N. clavipes was initially fractioned in an AKTA-FPLC system using a Sephacryl S-100 column (60 cm × 1.6 cm) equilibrated and eluted with a solution of 10 mM ammonium acetate, pH 6.8 at 8°C. The elution was monitored at 280 nm at a flow rate of 30 ml/h and fractions of 1 ml were collected.

The compounds HOE-140 (p-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸] and [Des-Arg¹⁰] HOE-140 (acquired from RBI/Sigma Chem. Co.) were used as antagonists of bradykinin B₂ and B₁ receptors, respectively.

The synthetic peptides were assayed both on isolated ileum and duodenum muscle from young Wistar rats (200–220 g), by using strips of these muscles (1–2 cm) suspended in a 5 ml Tyrode solution, at 37°C. The isotonic contractions and the muscle relaxations were recorded by means of a kymograph. The peptides were individually assayed and the activity was measured on log–dose curve obtained with an established dose of standard bradykinin (10⁻⁵ M/assay). Each experiment was repeated five times and the results were expressed as mean values.

The compounds HOE-140 (p-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸] and [Des-Arg¹⁰] HOE-140 (acquired from RBI/Sigma Chem. Co.) were used as antagonists of bradykinin B₂ and B₁ receptors, respectively.

The compounds HOE-140 (p-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸] and [Des-Arg¹⁰] HOE-140 (acquired from RBI/Sigma Chem. Co.) were used as antagonists of bradykinin B₂ and B₁ receptors, respectively.

2.5. Biological assay on isolated rat ileum and duodenum

The synthetic peptides were assayed both on isolated ileum and duodenum muscle from young Wistar rats (200–220 g), by using strips of these muscles (1–2 cm) suspended in a 5 ml Tyrode solution, at 37°C. The isotonic contractions and the muscle relaxations were recorded by means of a kymograph. The peptides were individually assayed and the activity was measured on log–dose curve obtained with an established dose of standard bradykinin (10⁻⁵ M/assay). Each experiment was repeated five times and the results were expressed as mean values.

The compounds HOE-140 (p-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸] and [Des-Arg¹⁰] HOE-140 (acquired from RBI/Sigma Chem. Co.) were used as antagonists of bradykinin B₂ and B₁ receptors, respectively.

2.5.1. Insecticide activity

Different doses of each synthetic peptide (from 2 to 150 pmoles/mg of insect) dissolved in physiological solution (0.9% (m/v) NaCl) were injected in a final volume of 1 μl into the pronotum of honeybees (Africanized Apis mellifera), by using a Hamilton microsyringe (10 μl). The insects were maintained within a Petri dish up to 24 h in presence of candy (food) and water supply. During this period of time the toxicity effects and/or the lethal action were observed; injection of physiological solution into the insect pronotum performed control experiments. The toxicity was assayed by using different concentrations of the peptides and compared against a control (N = 10, per concentration). The number of dead insects was determined after 24 h of peptides application. Toxicity levels were calculated according to Probit method [2] and expressed as lethality (LD₅₀).

2.6. Statistics

Statistic treatment of the data was performed with the non-parametric tests, Mann–Whitney U-test or Kruskal–Wallis H-test, followed by a Tukey–Kramer post-test as needed, [22] the value of P < 0.05 was considered to be significant. The results are presented as means ± standard error (S.E.).

3. Results

The WWE from the spider N. clavipes was initially fractioned in an AKTA-FPLC system using a Sephacryl S-100 column (60 cm × 1.6 cm) equilibrated and eluted with a solution of 10 mM ammonium acetate, pH 6.8 at 8°C. The elution was monitored at 280 nm at a flow rate of 30 ml/h and fractions of 1 ml were collected.

The compounds HOE-140 (p-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸] and [Des-Arg¹⁰] HOE-140 (acquired from RBI/Sigma Chem. Co.) were used as antagonists of bradykinin B₂ and B₁ receptors, respectively.

2.5. Biological assay on isolated rat ileum and duodenum

The synthetic peptides were assayed both on isolated ileum and duodenum muscle from young Wistar rats (200–220 g), by using strips of these muscles (1–2 cm) suspended in a 5 ml Tyrode solution, at 37°C. The isotonic contractions and the muscle relaxations were recorded by means of a kymograph. The peptides were individually assayed and the activity was measured on log–dose curve obtained with an established dose of standard bradykinin (10⁻⁵ M/assay). Each experiment was repeated five times and the results were expressed as mean values.

The compounds HOE-140 (p-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸] and [Des-Arg¹⁰] HOE-140 (acquired from RBI/Sigma Chem. Co.) were used as antagonists of bradykinin B₂ and B₁ receptors, respectively.

2.5.1. Insecticide activity

Different doses of each synthetic peptide (from 2 to 150 pmoles/mg of insect) dissolved in physiological solution (0.9% (m/v) NaCl) were injected in a final volume of 1 μl into the pronotum of honeybees (Africanized Apis mellifera), by using a Hamilton microsyringe (10 μl). The insects were maintained within a Petri dish up to 24 h in presence of candy (food) and water supply. During this period of time the toxicity effects and/or the lethal action were observed; injection of physiological solution into the insect pronotum performed control experiments. The toxicity was assayed by using different concentrations of the peptides and compared against a control (N = 10, per concentration). The number of dead insects was determined after 24 h of peptides application. Toxicity levels were calculated according to Probit method [2] and expressed as lethality (LD₅₀).

2.6. Statistics

Statistic treatment of the data was performed with the non-parametric tests, Mann–Whitney U-test or Kruskal–Wallis H-test, followed by a Tukey–Kramer post-test as needed, [22] the value of P < 0.05 was considered to be significant. The results are presented as means ± standard error (S.E.).

3. Results

The WWE from the spider N. clavipes was initially fractioned in an AKTA-FPLC system using a Sephacryl S-100 column (60 cm × 1.6 cm) equilibrated and eluted with a solution of 10 mM ammonium acetate, pH 6.8 at 8°C. The elution was monitored at 280 nm at a flow rate of 30 ml/h and fractions of 1 ml were collected.
Fig. 3 – TOF-MS/MS spectrum of the [M + 2H]^+ ion, acquired at 50 eV collision energy: (a) (m/z 538.23) from Fr.III-1; (b) (m/z 552.76) from Fr.III-2; (c) (m/z 544.78) from Fr.III-3 and (d) (m/z 559.77), from Fr.III-4. The mass differences of the consecutive y_n ions and their correspondence to the amino acid sequence are shown.
monitored at 280 nm and the flow rate was 0.5 ml/min. The chromatogram profile (Fig. 2) revealed the presence of five distinct peaks, which were named as: Fr.III-1, Fr.III-2, Fr.III-3, Fr.III-4 and Fr.III-5.

In spite to be pure, the amounts of each peptide was not enough to be submitted to the primary sequencing by Edman degradation chemistry. Thus, the peptides from the fraction Fr.III-1 to Fr.III-5 had their primary sequences determined by degradation chemistry. Thus, the peptides from the fraction Fr.III-1, Fr.III-2, Fr.III-3, Fr.III-4 and Fr.III-5, respectively (Figs.3a–d and4). The low mass region (<200) of such MS/MS spectra often contains ions that are indicative of the presence of specific amino acid residues in the peptides. These immonium ions (H2N = CHR)+, where “R” is a side chain group, arise from the fragmentation of two internal bonds. The low-mass regions of the spectra represented in the Figs. 3a–d and 4 contain ions at m/z 60.3, 70.3, 86.1, 87.1, 102.1, 120.5 and 129.3, suggesting the presence of the amino acid residues Ser, Pro, Ile/Leu, Asn, Glu, Phe and Arg, respectively.

By searching for mass differences corresponding to the masses of the consecutive peaks of –NH-CHR-CO– residue of residues Ser, Pro, Ile/Leu, Asn, Glu, Phe and Arg, respectively. 120.5 and 129.3, suggesting the presence of the amino acid residues Ser, Pro, Ile/Leu, Asn, Glu, Phe and Arg, respectively.

In order to establish the structural identities of these peptides, the CID mass spectra of the [M + 2H]+ ions at m/z 538.23, 552.76, 559.77 and 530.78 were generated from the peptide component of Fr.III-1, Fr.III-2, Fr.III-3, Fr.III-4 and Fr.III-5, respectively (Figs. 3a–d and 4). The low mass region (m/z < 200) of such MS/MS spectra often contains ions that are indicative of the presence of specific amino acid residues in the peptides. These immonium ions (H2N = CHR)+, where “R” is a side chain group, arise from the fragmentation of two internal bonds. The low-mass regions of the spectra represented in the Figs. 3a–d and 4 contain ions at m/z 60.3, 70.3, 86.1, 87.1, 102.1, 120.5 and 129.3, suggesting the presence of the amino acid residues Ser, Pro, Ile/Leu, Asn, Glu, Phe and Arg, respectively.

The MS/MS spectra of Fr.III-1, Fr.III-2, Fr.III-3, Fr.III-4 and Fr.III-5 (Figs. 3b–d and 4) were submitted to the same analysis as the major ones. The major peak with m/z 1074 was assigned to the molecular ion (M + H)+. Loss of ammonia (17 Da) from the (M + H)+ ion will result in the ion at m/z 1057. A series of y-ions can be observed in this spectrum: m/z 1017.5 (y9), m/z 920.5 (y8), m/z 806.4 (y7), m/z 709.4 (y6), m/z 652.3 (y5), m/z 505.3 (y4), 418.3 (y3), 321.2 (y2), m/z 174.1 (y1). These permitted the assignment of the sequence of the peptide Fr.III-1, as shown in Fig. 3a: G-P-N-P-G-F-S-P-F-R-NH2.

The subtraction of the m/z values between consecutive y-ions permitted the assignment of the amino acid sequence of the peptides present in fractions Fr.III-1, Fr.III-2, Fr.III-3, Fr.III-4 and Fr.III-5, as shown below:

Fr.III-1 G-P-N-P-G-F-S-P-P-R-NH2
Fr.III-2 E-A-P-P-G-F-S-P-P-R-NH2
Fr.III-3 P-S-P-G-F-S-P-P-R-NH2
Fr.III-4 L-T-P-E-T-A-S-P-P-R-NH2
Fr.III-5 R-P-P-G-F-S-P-P-R-NH2

The MS/MS spectra of peptides Fr.III-2, Fr.III-3, Fr.III-4 and Fr.III-5 (Figs. 3b–d and 4) were submitted to the same analysis and they also show two ion series, again with a few b-type ions and y-type ions as the major ones. These series of ions are summarized in Table 1.

The subtraction of the m/z values between consecutive y-ions permitted the assignment of the amino acid sequence of the peptides present in fractions Fr.III-1, Fr.III-2, Fr.III-3, Fr.III-4 and Fr.III-5, as shown below:

Fr.III-1 G-P-N-P-G-F-S-P-P-R-NH2
Fr.III-2 E-A-P-P-G-F-S-P-P-R-NH2
Fr.III-3 P-S-P-G-F-S-P-P-R-NH2
Fr.III-4 L-T-P-E-T-A-S-P-P-R-NH2
Fr.III-5 R-P-P-G-F-S-P-P-R-NH2

Table 1 – m/z Values observed for the molecular ions [M + H]+ and for all the series of y-type fragment-ions observed in the TOF-MS/MS spectra of the peptides from Fr.III-1 to Fr.III-5

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Fragment ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fr.III-1</td>
<td>1017.5 920.4 806.4 709.3 652.3 505.3 416.2 321.2 174.1</td>
</tr>
<tr>
<td>Fr.III-2</td>
<td>974.5 903.5 806.4 709.3 652.3 505.3 418.8 321.8 174.1</td>
</tr>
<tr>
<td>Fr.III-3</td>
<td>991.5 904.4 807.4 710.3 653.3 506.3 419.3 322.4 175.1</td>
</tr>
<tr>
<td>Fr.III-4</td>
<td>1005.6 904.5 807.4 710.3 653.3 506.3 419.2 322.2 175.1</td>
</tr>
<tr>
<td>Fr.III-5</td>
<td>1117.2 1005.6 904.5 807.4 710.3 653.3 506.4 419.2 272.1 175.1</td>
</tr>
</tbody>
</table>
The complete identification of these sequences was corroborated by the presence of some a/b pairs, by the identification of some internal fragment ions and also by the presence of peaks corresponding to simple neutral losses (NH$_3$ or H$_2$O) from the main peaks. The sequences above just fit to the experimental values of the respective molecular masses if the C-terminal residues were considered in the amidated form.

A search in the literature clearly shows that the peptide of Fr.III-5 has exactly the same sequence of the bradykinin; thus, the peptide of Fr.-III-5 will be also referred as bradykinin itself in the present manuscript, to avoid any mistaken nomenclature. Comparing the other four peptides to the bradykinin sequence it is possible to observe great similarity between each other and also with other bradykinin-related peptides from literature (Table 2). The peptides from Fr.III-1, Fr.III-2 and Fr.III-3 will be referred as: nephilakinin-I, -II and -III, respectively, since they were extracted from the web of the spider N. clavipes. On the other hand, the peptide of Fr.III-4 does not present a sequence similarity with any known peptide, thus, it will be referred just as Fr.III-4.

Since the amount yielded of each natural peptide in WWE was not enough to be used in the bioassay characterizations, the peptides were manually synthesized on-solid phase. The identity and homogeneity of the synthetic peptides after purification were performed by mass spectrometry (as described above).

These peptides were functionally characterized concerning to their action on mammals muscles and insect lethality. The dose–response curves are represented in Figs. 5 and 6. The nephilakinins produced constriction of rat ileum and relaxation of rat duodenum. These effects were calculated as the relative constriction (Fig. 5) and relaxation (Fig. 6) caused by each peptide, when compared to BK. The results were expressed as percentages of the maximal responses to 10 μM BK and the log–dose curves obtained were used to calculate the ED$_{50}$ value for each peptide in each different situation, as shown in Table 3. The EC$_{50}$ value is the concentration of each peptide causing 50% of the contractile/relaxation response compared with the reference peptide (BK). To characterize the receptor-type mediating the responses of the smooth muscles to nephilakinins, specific antagonists of B$_2$ (HOE-140) and B$_1$ ([Des-Arg$_{10}$]HOE-140) receptors were tested. HOE-140 inhibited the contractile/relaxation responses induced by bradykinin and nephilakinins when assayed at concentration of 100 μM; on the other hand, the specific B$_2$ receptor antagonist up to 100 μM had no effect (not shown results), indicating that the B$_2$ receptor was responsible for the contractile effect induced by nephilakinins in these preparations.

The nephilakinins and BK were toxic to insects, causing paralysis in honeybees 60 min after their applications; the most of honeybees defecated a few minutes after the toxins injection, followed by some intoxication symptoms such as: irregular walking, trembling of legs, difficulties to sustain the body on the legs making the insect to crawl instead of walk. The insects start to die 18 h after the toxins injection. The LD$_{50}$ values were 98, 72, 70 and 54 pmoles/mg of honeybee, for the nephilakinin-I, -II, -III and BK, respectively (Table 3). Apparently, the fraction III-4 was not toxic to insects.

### 4. Discussion

The compounds extracted from fractions III-1, III-2 and III-3 are peptides presenting BK-related sequences, thus were named nephilakinin-I, -II and -III, respectively. In addition,
Taking into account a comparison between the primary reveals that fraction III-4 was not a smooth muscle agonist. The data of Figs. 5 and 6 concretion/relaxation (Table 3), it may be observed that the effect produced by these peptides.

The sequences of nephilakinins were quite similar to BK, wasp kinins and frog skin kinins as shown in Table 2; these peptides generally are a little bit longer than BK, conserving the most of the canonical sequence of this peptide (RPPGFSPF), with a range of minor N- and C-terminally extended forms. A structural modification generally observed in wasps and frog kinins are the replacement of Ser residue in the position 6 by Thr, resulting in the (Thr6)-bradykinin (Table 2).

The observation that HOE-140 suppresses the contraction effects caused BK and nephilakinins, while [Des-Arg10] HOE-140 is inactive concerning to the actions of these peptides, confirm that nephilakinins are B2-type agonists of ileal and duodenal smooth muscles. Despite of the sequence similarity of these peptides to bradykinin, it must be emphasized that contraction/relaxation of smooth muscles also could have received some contributions from the interactions of nephilakinins with other receptor sub-types, in addition to the B2-type as confirmed above. The incomplete S-shape of the dose-response curves observed in the Figs. 5 and 6, suggest that at high concentrations the nephilakinins may have caused some contractions of the duodenum and relaxation of the ileum. This probably starts at concentrations higher than those where the classical B2-type agonist effects are observed for each muscle, so that, at high concentrations of nephilakinins, may exist some relaxation—contraction competition due to interactions of the peptides with other, still unidentified receptor sub-types. Thus, there is a distinct possibility that the nephilakinins are partial agonists at the B2-type receptor rather than full agonists like BK.

A careful analysis comparison between the ED50 data shown in Table 3 reveals that the nephilakinins are lesser potent muscle agonists than BK. The data of Figs. 5 and 6 reveals that fraction III-4 was not a smooth muscle agonist. Taking into account a comparison between the primary sequences of these peptides and the values of ED50 for muscle concretion/relaxation (Table 3), it may be observed that the higher similarity of the primary sequences of nephilakinins in relation to bradykinin, the higher is the constricting/relaxing effect produced by these peptides.

The toxicity assay of the spider web peptides (Table 3) revealed that bradykinin and the nephilakinins are moderately toxic to the model prey insect (honeybee). Apparently, the nephilakinins-I, -II and -III present the same level of lethality to honeybees, when compared to each other; their LD50 values (from 70 to 98 pmoles/mg honeybee) are comparable to the hwentoxins isolated from the venom of the spider Selenocosmia huwena (LD50 of ~50 pmoles/mg insect) [6]. The peptide from Fr.III-4 presented very low lethality to the model prey insect.

Kinin-related peptides have been observed in a series of arthropod venoms, with activities on guinea-pig ileum and rat uterus [3,24,25]. In the insect ganglion, the kinin-related peptides irreversibly block the nicotinic transmission, causing insect paralysis/death [21]. Thus, if some of these actions could occur simultaneously, the kinin-related peptides could be used as toxins both for defensive and for predation use.

The results of the present investigation suggest that the nephilakinins present similar pharmacological properties to other kinin-related peptides isolated from Arthropod venoms [20]. In addition to this, BK and the nephilakinins also caused death to insects when these peptides were bioassayed under laboratory conditions. Thus, BK and the nephilakinins may be used by the orb-web-spider N. clavipes as prey capture tools, when these peptides are applied into the viscous droplets of the capture strand of their webs.

### Acknowledgements

This work was supported by a grant from the São Paulo State Research Foundation (FAPESP). Maria Anita Mendes is Post-doctoral fellow from FAPESP (Proc. 01/05060-4), B.M.S. and M.R.M. are Doctoral student fellows from FAPESP, L.D.S. is Doctoral student from CAPES the Brazilian Council for Scientific a Technological Development (CNPq, 300377/2003-5).

### References


