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

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ABSTRACT

Three bradykinin-related peptides (nephilakinins-I to -III) and bradykinin itself were isolated from the aqueous washing extract of the capture web of the spider Nephila clavipes by gel permeation chromatography on a Sephacryl S-100 column, followed by chromatography in a Hi-Trap Sephadex-G25 Superfine column. The novel peptides occurred in low concentrations and were sequenced through ESI-MS/MS analysis: nephilakinin-I (G-P-N-P-G-F-S-P-F-R-NH$_2$), nephilakinin-II (E-A-P-P-G-F-S-P-F-R-NH$_2$) and nephilakinin-III (P-S-P-P-G-F-S-P-F-R-NH$_2$). Synthetic peptides replicated the novel bradykinin-related peptides, which were submitted to biological characterizations. Nephilakinins were shown to cause constriction on isolated rat ileum preparations and relaxation on rat duodenum muscle preparations at amounts higher than bradykinin; apparently these peptides constitute B$_2$-type agonists of ileal and duodenal smooth muscles. All peptides including the bradykinin were moderately lethal to honeybees. These bradykinin peptides may be related to the predation of insects by the webs of N. clavipes.

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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 neuro-toxins. 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 character-ization of the mechanisms of action of ion channels, while other toxins are used in the investigations of neurological disorders of clinical interest [4]. 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 [16].

In 1954 Jacques and Schachter [7] 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 [25]. 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 *Megacampsomeris prismatica* [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 cholinergic 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, Altrinchan). 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, dessolvation 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^{TM}) 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 dessolvation 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. Peptide sequences were determined manually from the MS/MS product ion mass spectra with the help of the PepSeq software (MICROMASS, UK). The distinction between the isobaric I/L residues was performed by searching for the presence of d- and w-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 $1000 \times g$, 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 (D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8] and [Des-Arg10] HOE-140 (acquired from RBI/Sigma Chem. Co.) were used as antagonists of bradykinin B$_2$ and B$_1$ 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 (LD$_{50}$).

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]
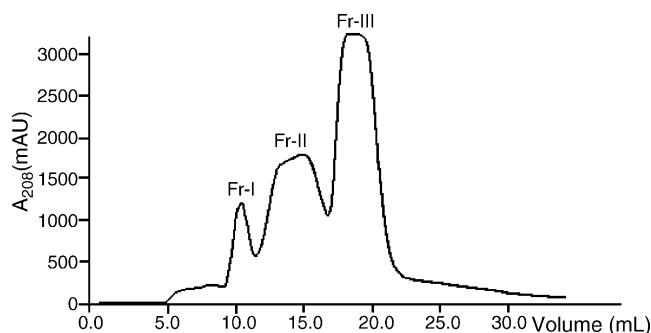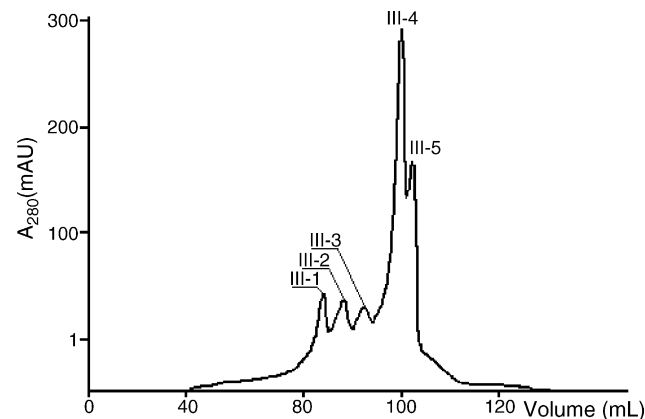

Fig. 1 – Gel permeation chromatographic profile for the aqueous washing extract of *Nephila clavipes* web obtained in an AKTA-FPLC system using a Sephacryl S-100 column (60 cm × 1.6 cm) equilibrated and eluted with a solution of 10 mM ammonium acetate, pH 6.8 at 8 °C. The elution was monitored at 280 nm at a flow rate of 30 ml/h and fractions of 1 ml were collected.

the 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 (16 cm × 60 cm) equilibrated and eluted with 10 mM ammonium acetate solution, pH 6.8. The chromatographic profile presented three distinct fractions and it is represented in Fig. 1. Proteins, constituted fractions-I and -II with molecular masses changing from 50 to 100 kDa, while a mixture of aggregated peptides constituted the fraction Fr.-III. Subsequently, the fraction Fr.-III was re-fractionated in a AKTA-FPLC system coupled to a Hi-Trap Sephadex G-25 Superfine column (1.6 cm × 2.5 cm) equilibrated and eluted with 10 mM ammonium acetate solution, pH 6.8 at 8 °C; the elution was

Fig. 2 – Rechromatographic profile of the Fr.III (obtained from gel permeation chromatography in Sephacryl S-100) in an AKTA-FPLC system using a Hi-Trap Sephadex G-25 Superfine 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.
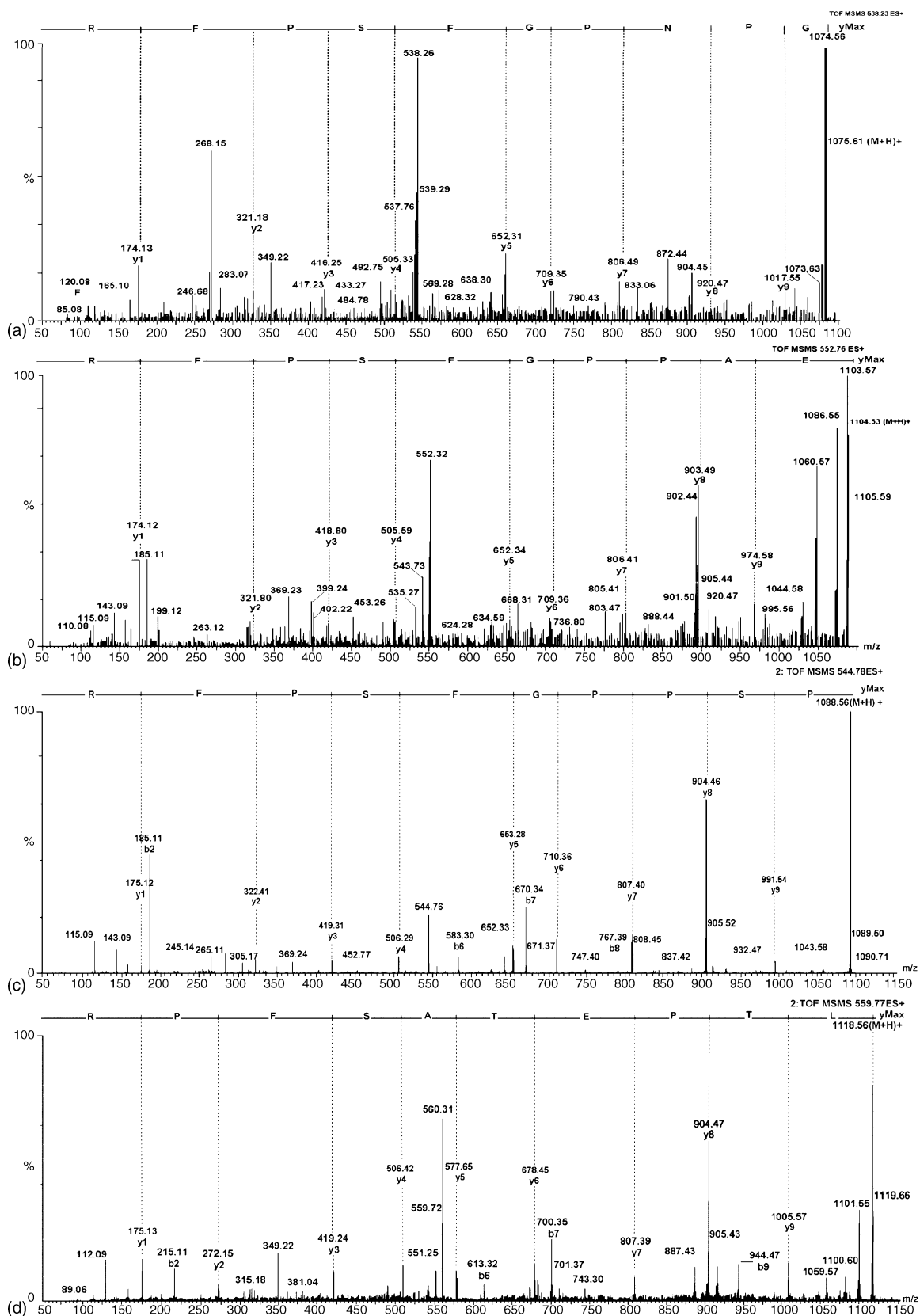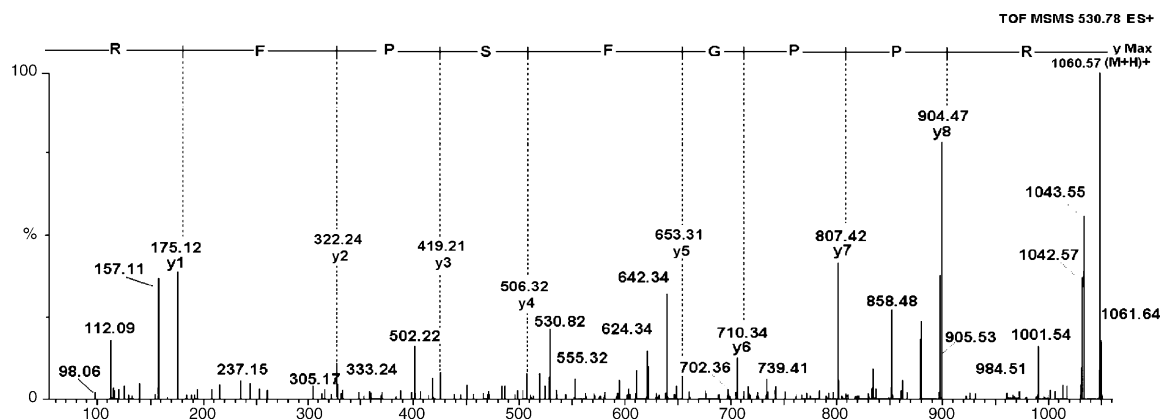
Fig. 3 – TOF-MS/MS spectrum of the [M + 2H]⁺ ion, acquired at 50 eV collision energy: (a) (*m/z* 538.23) from Fr.III-1; (b) (*m/z* 552.76) from Fr.III-2; (c) (*m/z* 544.78) from Fr.III-3 and (d) (*m/z* 559.77), from Fr.III-4. The mass differences of the consecutive y_n ions and their correspondence to the amino acid sequence are shown.

Fig. 4 – TOF-MS/MS spectrum of the [M + 2H]⁺ ion, (m/z 530.78) from Fr.III-5, acquired at 50 eV collision energy. 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 tandem mass spectrometry. When these fractions were analyzed by Q-Tof-MS, peptides with molecular masses 1074.52, 1102.64, 1087.25, 1118.60 and 1059.24 Da were identified in Fr.III-1, Fr.III-2, Fr.III-3, Fr.III-4 and Fr.III-5, respectively (not shown results).

In order to establish the structural identities of these peptides, the CID mass spectra of the [M + 2H]²⁺ ions at m/z 538.23, 552.76, 544.78, 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.

By searching for mass differences corresponding to the masses of the consecutive peaks of –NH-CHR-CO– residue of natural amino acids, it is possible recognize one or more series of ion-fragments. In Figs. 3a–d and 4 these peaks were

assigned as a series of y-type ion-fragments for all peptides. As discussed below, the complete sequence identification of the peptides is corroborated by the presence of some b-type ions, and also by the presence of peaks corresponding to neutral losses (NH₃ or H₂O) from the main peaks.

The MS/MS spectrum of peptide present in Fr.III-1 (Fig. 3a) shows two ions series, i.e., a few b-type ions with y-type ions 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 (y_9), m/z 920.5 (y_8), m/z 806.4 (y_7), m/z 709.4 (y_6), m/z 652.3 (y_5), m/z 505.3 (y_4), 418.3 (y_3), 321.2 (y_2), m/z 174.1 (y_1). 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 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 shown 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-F-R-NH₂
Fr.III-2	E-A-P-P-G-F-S-P-F-R-NH₂
Fr.III-3	P-S-P-P-G-F-S-P-F-R-NH₂
Fr.III-4	L-T-P-E-T-A-S-F-P-R-NH₂
Fr.III-5	R-P-P-G-F-S-P-F-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

Peptide	Fragment ion (m/z)									
	[M + H]⁺	y_9	y_8	y_7	y_6	y_5	y_4	y_3	y_2	y_1
Fr.III-1	1074.2	1017.5	920.4	806.4	709.3	652.3	505.3	416.2	321.2	174.1
Fr.III-2	1103.2	974.5	903.5	806.4	709.3	652.3	505.5	418.8	321.8	174.1
Fr.III-3	1087.5	991.5	904.4	807.4	710.3	653.3	506.3	419.3	322.4	175.1
Fr.III-4	1509.2	–	904.4	807.4	710.3	653.3	506.3	419.2	322.2	175.1
Fr.III-5	1117.2	1005.6	904.5	807.4	678.4	577.6	506.4	419.2	272.1	175.1

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_2O) 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-Arg10]HOE-140) receptors were tested. HOE-140 inhibited the contractile/
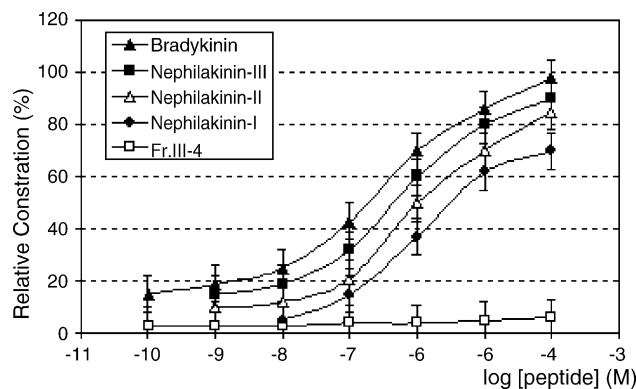
Fig. 5 – Dose–response curves of the constrictions caused by bradykinin, nephilakinins and Fr.III-4 on rat ileum preparations. Each data point represents the mean ± S.E.M. of five replicates.

relaxation responses induced by bradykinin and nephilakinins when assayed at concentration of 100 μM; on the other hand, the specific B_1 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,

Table 2 – Amino acid sequence alignments of bradykinin, nephilakinins, wasp kinins and amphibian skin bradykinins	
Peptides	Primary sequence
Bradykinin	**RPPGFSPFR**
Nephilakinin-III	PS**PPGFSPFR**
Nephilakinin-II	EA**PPGFSPFR**
Nephilakinin-I	G**PNPGFSPFR**
(Thr-6)-Bradykinin (*Megascoiia flavifrons*)	**RPPGF**T**PFR**
Wasp kinin VSK-A (*Vespa analis*)	G**RPPGFSPFR**VI
Vespulakinin 1 (*Vespula maculifrons*)	TATTRRRG**RPPGFSPFR**
Frog skin peptide (*Bombina variegata*)	**RP**AG**FTPFR**
Frog skin peptide (*Bombina variegata*)	V**P**TG**FTPFR**
Frog skin peptide (*Rana nigrornaculata*)	V**PPGF**T**PFR**
Conserved residues are assigned in bold.	
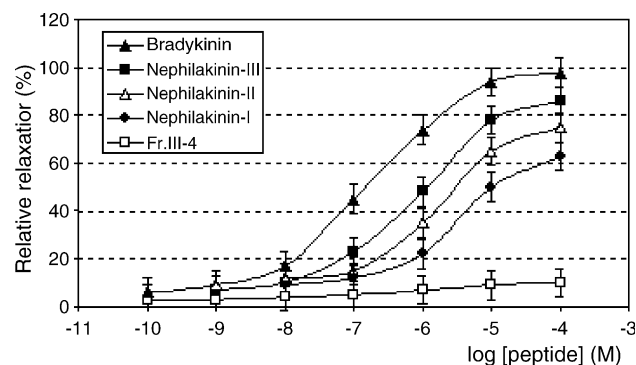
Fig. 6 – Dose–response curves of the relaxations caused by bradykinin, nephilakinins and Fr.III-4 on rat duodenum preparations. Each data point represents the mean ± S.E.M. of replicates.

Table 3 – Bioassay parameters obtained for the nephilakinins-I to -III and bradykinin: ED_{50} values obtained for the constriction of rat ileum and relaxation of rat duodenum by using bradykinin as reference; LD_{50} values obtained for insect toxicity by using honeybees as model-insects

Peptides	Constriction (ED_{50}) (μM)	Relaxation (ED_{50}) (μM)	Insect toxicity (LD_{50}) (pmoles/mg honeybee)
Nephilakinin-I	7.0[*]	10.0[*]	98 ± 22
Nephlakinin-II	1.0[*]	3.2[*]	72 ± 18
Nephilakinin-III	0.7[*]	1.0[*]	70 ± 17
Bradykinin	0.3[*]	0.2[*]	54 ± 13

[*] Significant at level of $P < 0.05$.

fraction III-5 is the BK itself and the peptide of fraction III-4 presents no similar sequence with any other known peptide (Table 2). The comparison between these sequences and that of BK reveals the presence of eight conserved amino acid residues on the C-terminal of nephilakinin-II and -III, while seven-conserved amino acid residues were observed on the C-terminal side for nephilakinin-I.

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 supresses 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 B_2-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 B_2-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 B_2-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 B_2-type receptor rather than full agonists like BK.

A careful analysis comparison between the ED_{50} 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 ED_{50} for muscle constriction/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 moder-ately 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 LD_{50} values (from 70 to 98 pmoles/mg honeybee) are comparable to the hwentoxins isolated from the venom of the spider *Selenocosmia huwena* (LD_{50} of $\sim$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.

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REFERENCES

[1] Bockmann S, Paegelow I. Kinins and kinin receptors: importance for the activation of leukocytes. J Leukoc Biol 2000;68:587–92.
[2] Chou T, Chou C. Dose-effect analysis with microcomputers. Cambridge: Biosoft, 1987. p. 93.
[3] Ferreira LAF, Lucas S, Alves EW, Hermann VV, Reichl A, Habermehl G. Isolation, characterization and biological properties of two-kinin-like peptides (peptide-S and peptide-R) from *Scaptocosa raptoria* venom. Toxicon 1998;36:31–9.

[4] Harvey AL, Anderson AJ, Rowan EG. Toxins affecting ion channels. In: Harvey AL, editor. Natural and synthetic neurotoxins. London: Academic Press; 1993. p. 129–85.

[5] Hue B, Piek T. Irrversible presynaptic aditivation-induced block of transmission in the insect CNS by hemicholinium-3 and threonine-6-btadykinin. Comp Biochem Physiol 1989;93C:87–9.

[6] Huang RH, Liu ZH, Liang SP. Purification and characterization of a neurotoxic peptide huwentoxin-III and a natural inactive mutant from the venom of the spider *Selenocosmia huwena* wang (*Ornithoctonus huwena* Wang). Acta Biochim Biophys Sinica 2003;35:976–80.

[7] Jacques R, Schachter M. The presence of histamine, 5-hydroxytryptamine and a potent, slow-contracting substance in wasp venom. Br J Pharmacol 1954;9:53–8.

[8] Konno K, Palma MS, Hitara IY, Juliano MA, Juliano L, Yasuhara T. Identification of bradykinins in solitary wasp venoms. Toxicon 2002;40:309–12.

[9] Marangoni S, Borges NCC, Marangoni E, Antunes E, Vieira A, Novello JC, et al. Biochemical characterization of a vascular smooth muscle contracting polypeptide purified from *Phenutria nigriventris* (armed spider) venom. Toxicon 1993;31:377–84.

[10] Marques MR, Mendes MA, Tormena CF, Souza BM, Ribeiro SP, Rittner R, et al. Structure determination of an organometallic 1-(Diazenylaryl) ethanol: a novel toxin subclass from the web of the spider Nephila clavipes. Chem Biodiv 2004;1:830–8.

[11] Marques MR, Mendes MA, Tormena CF, Cesar LMM, Rittner R, Palma MS. Structure determination of a Tetrahydro-b-Carboline of arthropod origin: a novel alkaloid toxin subclass from the web of spider *Nephila clavipes*. Chem Biodiv 2005;2:525–34.

[12] Mathias AP, Schachter M. The chromatographic behavior of wasp venomkinin, kallidin and bradykinin. Br J Pharmacol 1958;13:326–9.

[13] Mendes MA, Souza BM, Santos LD, Palma MS. Structural characterization of novel chemotactic and mastoparan peptides from the venom of the social wasp *Agelaia pallipes* by HPLC-ESI-MS/MS. Rapid Commun Mass Spectrom 2004;18:636–42.

[14] Piek T, Buitenhuis A, Simonthomas RT, Ufkes JGR, Mantel P. Smooth muscle contracting compounds in the venom of *Megascolia flaÍifrons* (Hym: scoliidae) with notes on the stinging behaviour. Comp Biochem Physiol 1983;75C:145–52.

[15] Piek T, Buitenhuis A, Veldsema-Currie RD, Mantel P. Smooth muscle contracting compounds in venoms of sphecid wasps (Hym: Sphecidae). Comp Biochem Physiol 1983;75C:153–9.

[16] Piek T, Spanjer W. Chemistry and pharmacology of solitary wasp venoms. In: Piek T, editor. Venoms of the hymenoptera: biochemical, pharmacological and behavioural aspects. London: Academic Press; 1986. p. 161–307.

[17] Piek T, Hue B, Pelhate M, Mony L. The venom of the wasp *Campsomeris sexmaculata* (F.) block synaptic transmission in insect CNS. Comp Biochem Physiol 1987;87C:283–6.

[18] Piek T, Hue B, Mony L, Nakajima T, Pelhate M, Yasuhara T. Block of synaptic transmission in insect CNS by toxins from the venom of the wasp *Megascolia flavifrons* (FAB.). Comp Biochem Physiol 1987;87C:287–95.

[19] Piek T, Hue S, Mantel P, Nakajima T, Pelhate M, Yasuhara T. Threonine-6-bradykinin in the venom of the wasp *Colpa interrupta* (F.) presynaptically blocks nicotinic synaptic transmission in the insect CNS. Comp Biochem Physiol 1990;96C:157–62.

[20] Piek T. Neurotoxic kinins from wasp and ant venoms. Toxicon 1991;29:139–49.

[21] Piek T, Rocchi R, Hue B. Wasp kinin analogues—a lead to new drugs and pesticides. Toxicon 1997; 35:476–7.

[22] Sachs L. Angewandte Statistik. Berlin: Springer, 1992.

[23] Sarangi B, Kar PK, Datta A, Gomes A, Lahiri SC. Isolation, partial purification and pharmacodynamics of a slow contractile substance in the venom sac extract of the wasp *Vespa cincta* Fabr. Indian J Exp Biol 1990;28:557–61.

[24] Schachter M, Thain EM. Chemical and pharmacological properties of the potent, slow-contracting substance (kinin) in wasp venom. Br J Pharmacol 1954;9:352–9.

[25] Schachter M. Introduction to kinins: a group of vasoactive kinins. Fed Proc 1968;27:49–51.

[26] Yasuhara T, Hira M, Nakajima T, Yanaihara N, Hashimoto T, Sakura N, et al. Active peptides on smooth muscle in the skin *Bombina orientalis* Boulenger and characterization of a new bradykinin analogue. Chem Pharm Bull 1973;21:1388–94.

[27] Yasuhara T, Mantel P, Nakajima T, Piek T. Two kinins isolated from an extract of the venom reservoirs of the solitary wasp *Megascolia flavifrons*. Toxicon 1987; 25:527–35.