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Jelleines: a family of antimicrobial peptides from the Royal Jelly of honeybees (*Apis mellifera*)

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Abstract

Four antimicrobial peptides were purified from Royal Jelly of honeybees, by using reverse phase-HPLC and sequenced by using Q-Tof-MS/MS: PFKLSLHL-NH_2 (Jelleine-I), TPFKLSLHL-NH_2 (Jelleine-II), EPFKLSLHL-NH_2 (Jelleine-III), and TPFKLSLH-NH_2 (Jelleine-IV). The peptides were synthesized on-solid phase, purified and submitted to different biological assays: antimicrobial activity, mast cell degranulating activity and hemolysis. The Jelleines-I–III presented exclusively antimicrobial activities against yeast, Gram+ and Gram− bacteria; meanwhile, Jelleine-IV was not active in none of the assays performed. These peptides do not present any similarity with the other antimicrobial peptides from the honeybees; they are produced constitutively by the workers and secreted into Royal Jelly.

Keywords: Royal Jelly; Africanized Honeybees; *Apis mellifera*; Mass spectrometry; Antimicrobial peptides

1. Introduction

The serious problem of resistance by microorganisms to the currently used antibiotic drugs, demands searches for new classes of antimicrobial agents against which resistance may not develop. In the last decades molds and bacteria have been investigated as sources of new antibiotics, while the insects which are one of the most diverse and populous group of eukaryotic organisms of the planet only recently became the focus of investigations of their antibacterials [13].

Despite of insects do not have a lymphocyte-based immune system; they can successfully eliminate bacteria [2–4], even acquiring a transient immunity [3,24], suggesting the existence of very potent antimicrobials produced by the insects. This aspect has made the immune system of these animals very interesting targets for the search of attractive candidates for the development of novel antimicrobial drugs.

The first line of defense of the insects against the pathogens and parasites is the presence of a cuticle [23]. However, if this physical defense is ruptured, a complex interaction between the innate humoral and cellular immune systems will take place, in both, tissues and hemocele, resulting in pathogens elimination [2,24]. The most understood aspect of this interaction is the synthesis of antibacterial polypeptides in the fatty body tissue and some blood cells after an infection challenge [2,4]. Protein and peptides presenting simple structures, no complex modifying moieties or rare amino acids are expressed and frequently secreted to the hemolymph, providing a broad-spectrum antibiosis against bacteria and fungi [4].

Several different mechanisms are currently known to explain the mechanism of action of antibacterial peptides: (i) in the barrel-stave model peptide monomers associate and form a bundle of helices embedded in the membrane, forming a transmembrane channel [1–4]; (ii) in the “carpet-like” model the peptides act like detergents, forming eventually toroidal pores [14]. In both models the physico-chemical properties of peptides, such as: length, charge distribution, net charge, volume, amphipaticity, and oligomeric state in solution, play essential roles in peptide interactions with the membrane surface and/or with the membrane core. These peptides can differ significantly in their primary sequences, length and structures; they may be linear, or cyclic due to the existence of disulfide bridges. Some adopt an α-helical,

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β-sheet or even a combination of both structures [4]; despite to this, the most of these peptides seem to adopt an amphipatic arrangement, with the hydrophobic and charged faces, arranged in opposite positions, when in contact with the bacterial membrane.

Antimicrobial peptides may be classified according to their spectrum of action, into three major groups: (i) peptides active against bacteria, but not against normal mammalian cells and fungi; (ii) peptides active both against bacteria and normal mammalian cells, making them potentially active against fungi; (iii) peptides which are active exclusively against fungi, but not against bacteria and normal mammalian cells [21].

In honeybees a series of antimicrobial polypeptides were identified in the hemolymph after workers have been challenged with artificial infections at sub lethal doses of bacteria [5,6]. These peptides were referred to as apidaecins [5], abaecin [6], and hymenoptaecin [7] and showed to inhibit the viability of both Gram-positive and Gram-negative bacteria.

The Royal Jelly (RJ) is a yellowish-white, creamy, acidic secretion, with a slightly pungent odor and taste produced by the honeybees. It is the principal food of the queen honeybees and is produced by the hypopharingeal and mandibular glands of worker bees [10]. RJ is a nutritive secretion produced by the worker bees, rich in proteins, carbohydrates, vitamins and minerals [16,20] which is stored in the nest, under field conditions. RJ is very susceptible to colonization by microorganisms (bacteria and yeasts) associated to bee hive products, such as honey, pollen and propolis. Royalisin, an antibiotic polypeptide (51 amino acid residues) was previously isolated from the Royal Jelly of *Apis mellifera*, providing protection against infection of RJ by Gram-positive bacteria [10]. Here we report on the isolation and characterization of novel antibacterial peptides isolated from the RJ of honey bees—the Jelleines, a series of short peptides presenting a broad-spectrum of activity against Gram-positive and Gram-negative bacteria, and also against yeasts.

2. Methods

2.1. Sample preparation

Royal Jelly (RJ) was harvested from nests of Africanized *A. mellifera* in the apiary of the Bioscience Institute, Rio Claro, SP southeast Brazil. The samples were collected when the larvae of honeybees were 3 days old as described by Palma [20], during the spring season and kept frozen at −20 °C until be used for fractionation.

2.2. Peptides purification

About of 500 mg of RJ were solubilized in 5 mL bidistilled water and centrifuged during 20 min at 4000 × *g* at 4 °C. The supernatant was filtered through Microcon 3 (AMICON) and the soluble material presenting MW lower than 3 kDa was collected, dried by lyophylization and solubilized into 1 mL of 5% (v/v) acetonitrile (containing 0.04% (v/v) trifluoroacetic acid).

This material was submitted to fractionation under reverse phase-HPLC with a C-18 TOSOH-ODS column (150 mm × 4.6 mm; 5 μm), at a flow rate of 600 μL/min, by using a gradient from 5 to 60% (v/v) acetonitrile (containing 0.04% (v/v) trifluoracetic acid), during 45 min at 30 °C. The elution was monitored at 220 nm with a UV-DAD detector (SHIMADZU, mod. SPD-M10A) and each peak eluted was manually collected into plastic vials. The peak of interest (presenting antibiotic activity) was submitted to reversed phase-HPLC in a C-18 TOSOH-ODS column (150 mm × 4.6 mm; 5 μm), at a flow rate of 600 μL/min, under isocratic elution with 55% (v/v) acetonitrile (containing 0.04% (v/v) trifluoracetic acid), during 30 min. at 30 °C. The elution was monitored at 220 nm and each fraction was manually collected into plastic vials.

2.3. ESI mass spectrometry analysis

All the mass spectrometric analysis were performed in a hybrid quadrupole time of flight (Q-Tof^{TM}) mass spectrometer (MICROMASS, mod. Ultima API). Samples were dissolved in 50% (v/v) acetonitrile (containing 0.1% (v/v) trifluoracetic acid) and analyzed by positive electrospray ionization (ESI+) using typical conditions: a capillary voltage of 3 kV, a cone voltage of 30 V and a dessolvation gas temperature of 80 °C. The mass spectrometer was calibrated with intact horse heart myoglobin and its typical cone-voltage induced fragments to operate at resolution 5000. About 0.5 pmol of each sample was injected into electrospray transport solvent by using a micro syringe (50 μL) coupled to a micro infusion pump (KD Scientific) at a flow rate of 4 μL/min. The spectra were obtained in the continuous acquisition mode, scanning from *m/z* 100 to 2500 at a scan time of 5 s.

2.4. Peptide sequencing by Q-Tof-MS/MS

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: a capillary voltage of 3 kV, a cone voltage of 30 V and a dessolvation gas temperature of 80 °C. The doubly charged (di-protonated) precursors were selected in Q1 and subjected to collision-induced dissociation (CID) with argon gas at 50 eV collision energy; the product ions (presenting peak widths 0.16 Da) were detected with the TOF mass analyzer.

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 low energy CID conditions with argon gas for the natural peptides; meanwhile, the distinction between the isobaric residues Q/K was performed by comparative Q-Tof-MS analysis of the peptides acetylated and non-acetyated as described elsewhere [18].

2.5. Peptide synthesis and purification

The peptides were prepared by step-wise manual solid-phase synthesis using *N*-9-fluorophenylmethoxycarbonyl (Fmoc) chemistry with Novasyn TGS resin (NOVABIOCHEM). Side-chain protective groups included *t*-butyl for serine and *t*-butoxycarbonyl for lysine. Cleavages of the peptides-resin complexes were performed by treatment with trifluoroacetic acid/1,2-ethanedithiol/anisole/phenol/water (82.5:2.5:5:5:5 by volume), 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 a centrifugation at 1000 × *g*, during 15 min at room temperature. The crude peptides were solubilized in water and chromatographed under reverse phase-HPLC using a semi-preparative column (SHISEIDO C-18, 250 mm × 10 mm, 5 μm), under isocratic elution with 50% (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 220 nm with a UV-DAD detector (SHIMADZU, mod. SPD-M10A) and each fraction eluted was manually collected into plastic vials. The homogeneity and correct sequence of the synthetic peptides were assessed by analytical HPLC and Q-Tof-MS analysis.

2.6. Biological activities

Mast cell degranulation was determined by measuring the release of β-D-glucosaminidase, which co-localizes with histamine. Mast cells were obtained by peritoneal washing of adult Wistar rats with a solution containing 0.877 g NaCl (MERCK), 0.028 g KCl (MERCK), 0.043 g NaH_2PO_4 (SYNTH), 0.048 g KH_2PO_4 (SYNTH), 0.10 g glucose (SYNTH), 0.10 g BSA (SIGMA), 90 μL $CaCl_2$ (MERCK) 2 M solution, 50 μL liquemine (heparin, ROCHE) in 100 mL water. Mast cells were incubated in the presence of peptides for 15 min at 37 °C. After centrifugation, the supernatants were sampled for β-D-glucosaminidase assay. Briefly, 50 μL of the samples were incubated in 50 μL of the substrate (3 mg of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminidine (SIGMA) dissolved in 10 mL of 0.2 M sodium citrate, pH 4.5 solution), for 6 h. The absorbance of colored product was assessed at 405 nm and the values were expressed as the percentage of total β-D-glucosaminidase, which was determined from lysed mast cells in the presence of 1% (v/v) Triton X-100 (ALDRICH).

Washed rat red blood cells (WRRBC) were used to evaluate the hemolytic activity of the peptides. About 500 μL of Wistar rats red blood cell suspensions were washed three-times with physiological saline solution (NaCl 0.85% and $CaCl_2$ 10 mM) and then suspended in 50 mL of the same solution. Aliquots of WRRBC were then incubated at 37 °C in the presence of each peptide for 120 min, with gentle mixing. Samples were then centrifuged and the absorbances of the supernatants were measured at 540 nm. The absorbance measured from lysed WRRBC in presence of 1% (v/v) Triton X-100 was considered as 100%.

Antibacterial activity of the peptides was examined in sterile 96-well plates (COSTAR microtiter plates) in a final volume of 100 μL as follows. Aliquots (85 μL) of a suspension containing bacteria at a concentration of 10^6 colony-forming units/mL Müller–Hinton medium was added to 15 μL of physiological solution containing individually each peptide in different concentrations. Growth inhibition was determined by measuring the absorbance at 492 nm, following incubation for 18–24 h at 37 °C. Antibacterial activity is expressed as the minimal inhibitory concentration (MIC), the concentration at which 100% inhibition growth was observed after 18–24 h of incubation. The bacteria used were: *Staphylococcus aureus* (ATCC 6535), *Staphylococcus saprophyticus*, *Bacilus subtilis*, *Bacilus cereus*, *Bacilus thuringensis*, *Bacillus pumilus*, *Escherichia coli* (CCT 1371), *Enterbacter cloacae* (ATCC 23355), *Klebsiella pneumoniae* (ATCC 13883), *Proteus mirabilis*, *Pseudomonas aeruginosa* (ATCC 28853); and the yeast *Candida albicans* was also used.

3. Results

The low molecular weight fraction from *A. mellifera* Royal Jelly (MW < 3 kDa) was initially fractionated under reverse phase-HPLC by using a gradient from 5 to 60% (v/v) acetonitrile (containing 0.04% (v/v) trifluoracetic acid). The chromatographic profile (Fig. 1) shows the existence of 10 peaks. However, the antibiosis assays revealed that only the fractions 6 (the major one) and 8 (among the minor ones) presented antimicrobial activity. The ESI-MS spectra of these peaks revealed that the fraction 6 was constituted of a low molecular weight compound of (MW: 186 Da), while the fraction 8 seems to be constituted by at least four different peptide components with MW ranging from 942 to 1082 Da (not shown results). The component of fraction 6 was further characterized by ^{1}H NMR as 10-hydroxydecenoic-2-ene acid (known as queen pheromone) and it is not characterized in the present investigation. Thus, the fraction 8 apparently contained unkwnon antimicrobial peptides; therefore, it was re-fractionated in reverse phase-HPLC by using isocratic elution as described above. Four peaks resulted from this chromatography and were designated by 8.1, 8.2, 8.3, and 8.4 eluted (Fig. 2). All these fractions presented antimicrobial activity, both against

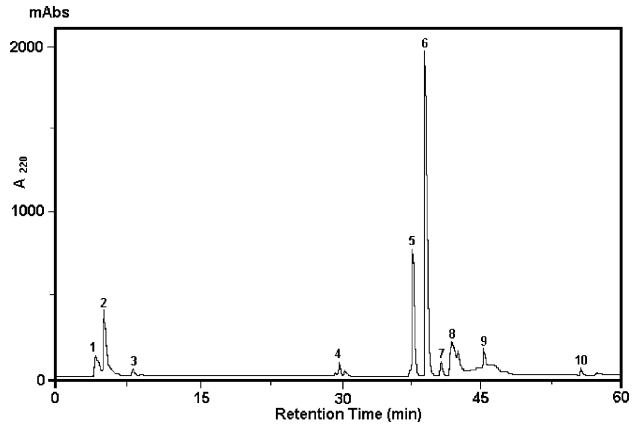

Fig. 1. Chromatographic profile of the low molecular weight fraction (MW < 3 kDa) of the Royal Jelly, under RP-HPLC by using a TOSOH-ODS column (150 mm × 4.6 mm; 5 μm), at a flow rate of 600 μL/min, eluted under gradient from 5 to 60% (v/v) MeCN (containing 0.04% TFA), at 30 °C, during 45 min at 30 °C; the elution was monitored at 220 nm.

Gram-positive and Gram-negative bacteria (not shown results). The ESI-MS spectrum of these fractions revealed the molecular masses 953.24 ± 0.17 Da, 1054.30 ± 0.18 Da, 1082.32 ± 0.19 Da, and 942.13 ± 0.17 Da, for the fractions 8.1, 8.2, 8.3, and 8.4, respectively (not shown results). Considering the origin from the Royal Jelly, Jelleine designated them.

In spite to be pure enough to be submitted to the primary sequencing by Edman degradation, the amounts of each peptide did not permit the recovery of enough material to be used in the automatic peptide sequencer. Thus, the peptides

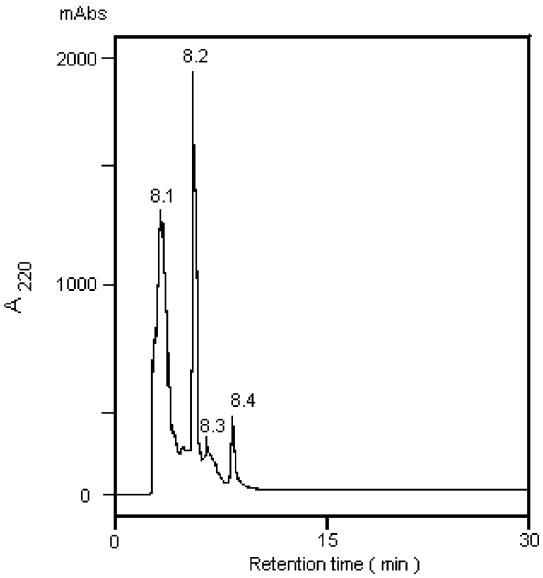

Fig. 2. Chromatographic profile of the re-fractionation of the fraction 8 under RP-HPLC by using a TOSOH-ODS column (150 mm × 4.6 mm; 5 μm), at a flow rate of 600 μL/min, under isocratic elution with 55% (v/v) MeCN (containing 0.04% TFA), during 30 min at 30 °C; the elution was monitored at 220 nm.

from the fractions 8.1, 8.2, 8.3, and 8.4 were submitted to sequencing through tandem mass spectrometry. Q-Tof-MS/MS analysis was applied and the resulting pattern of fragmentation obtained for each peptide (Figs. 3–6) revealed a long series of the complementary y- and b-type ion-fragments, which were very informative about the primary sequence of the peptides.

The doubly charged molecular ion (*m/z* 477.70) of the peptide component from the fraction 8.1 was submitted to Q-Tof-MS/MS analysis and the tandem mass spectrum is showed in Fig. 3. In this spectrum can be observed the molecular ion (*m/z* 954.19) as $[M + H]^+$ and a complete series of y-type ions: *m/z* 857.5 (y_7), *m/z* 710.4 (y_6), *m/z* 582.3 (y_5), *m/z* 469.3 (y_4), *m/z* 382.2 (y_3), *m/z* 269.2 (y_2), and *m/z* 132.1 (y_1). The subtraction of these successive *m/z* values has permitted the assignment of the sequence as showed at the top of Fig. 3.

The doubly charged molecular ion (*m/z* 528.20) of the peptide component from the fraction 8.2 was submitted to Q-Tof-MS/MS analysis and the result is showed in Fig. 4. In this spectrum can be observed the molecular ion (*m/z* 1055.62) as $[M + H]^+$ and a complete series of y-type ions: *m/z* 954.5 (y_8), *m/z* 857.5 (y_7), *m/z* 710.4 (y_6), *m/z* 582.4 (y_5), *m/z* 469.3 (y_4), *m/z* 382.2 (y_3), *m/z* 269.2 (y_2), and *m/z* 132.1 (y_1). The subtraction of these successive *m/z* values has permitted the assignment of the sequence as showed at the top of Fig. 4.

The doubly charged molecular ion (*m/z* 542.10) of the peptide component from the fraction 8.3 was submitted to Q-Tof-MS/MS analysis and the result is showed in Fig. 5. In this spectrum it is possible to observe the molecular ion (*m/z* 1083.55) as $[M + H]^+$ and a complete series of y-type ions: *m/z* 954.5 (y_8), *m/z* 857.5 (y_7), *m/z* 710.4 (y_6), *m/z* 582.3 (y_5), *m/z* 469.3 (y_4), *m/z* 382.1 (y_3), *m/z* 269.1 (y_2), and *m/z* 132.1(y_1). The subtraction of these successive *m/z* values has permitted the assignment of the sequence as showed at the top of Fig. 5.

The doubly charged molecular ion (*m/z* 472.16) of the peptide component from the fraction 8.4 was submitted to Q-Tof-MS/MS analysis and the result is showed in Fig. 6. The molecular ion (*m/z* 943.13) as $[M+H]^+$ can be observed in this spectrum and also a complete series of y-type ions: *m/z* 841.1 (y_7), *m/z* 744.4 (y_6), *m/z* 597.4 (y_5), *m/z* 469.3 (y_4), *m/z* 356.2 (y_3), *m/z* 269.1 (y_2), and *m/z* 156.1 (y_1). The subtraction of these successive *m/z* values has permitted the assignment of the sequence as showed at the top of Fig. 6.

The experimental values of the molecular masses of Jelleines are compatible with their C-terminal residues in the amidated form. Thus, the interpretation of the MS/MS spectra permitted to know the peptide sequences as showed in Table 1.

Since the amount yielded of each natural peptide was not enough to be used in the bioassay characterizations, the peptides were manually synthesized on-solid phase. After purification the identity and homogeneity of the synthetic peptides were assessed by comparing the retention times of

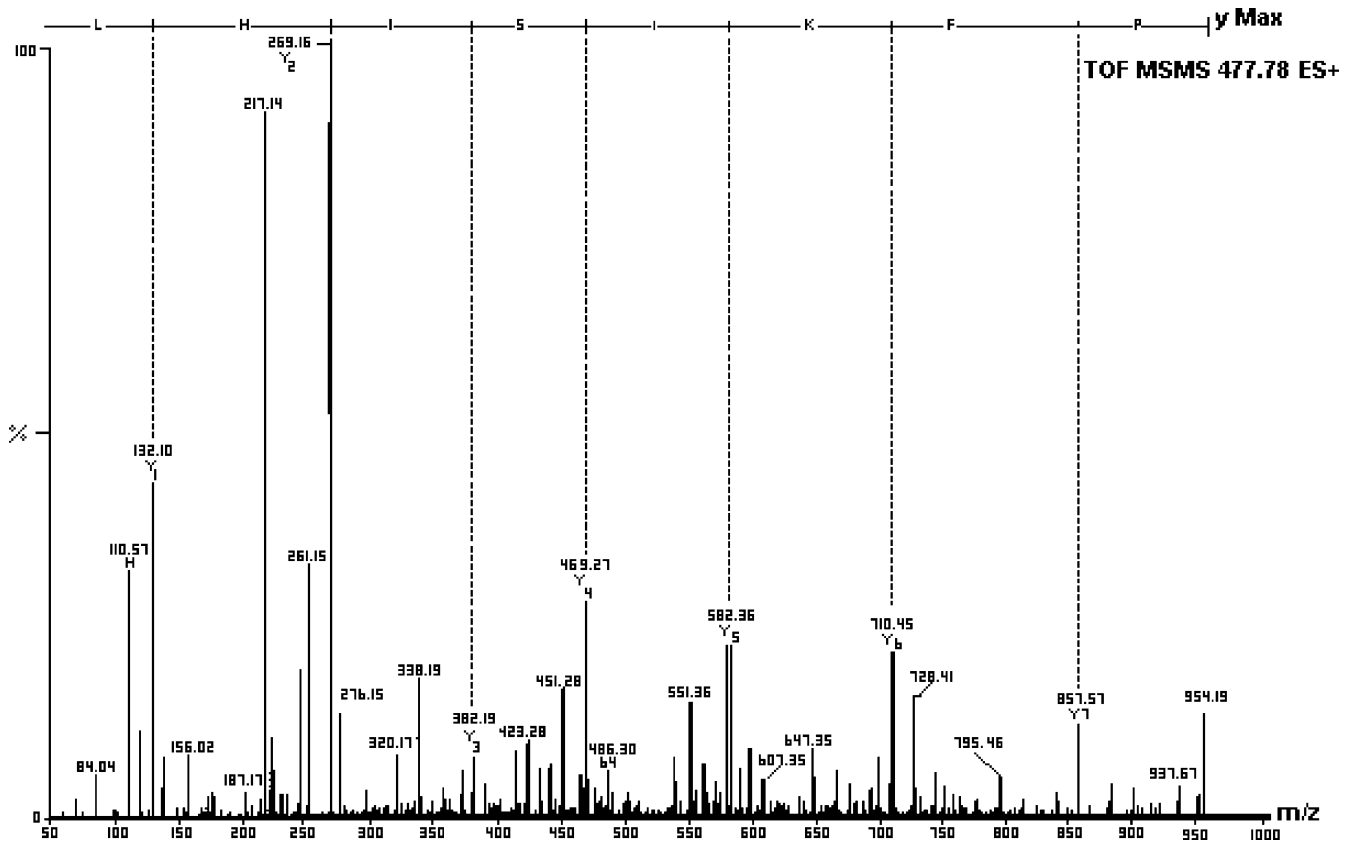

Fig. 3. ESI-Tof-MS/MS spectrum of the $[M + H]^{2+}$ ion (m/z 477.78), from Fr-8.1 acquired at 50 eV collision energy. The mass differences of the consecutive y_n ions and their correspondence to the amino acid sequence are shown.

the synthetic and the natural ones in reverse phase-HPLC eluted under isocratic conditions with 55% (v/v) MeCN (containing 0.04% (v/v) TFA); the molecular masses of the synthetic peptides were also determined by Q-Tof-Ms analysis and compared with the values described above for the natural Jelleines.

The Jelleines were then submitted to antimicrobial assays, against eleven species of bacteria and only one specie of yeast. The results (Table 2) show that the Jelleines-I and -II are active against the most of bacteria species (Gram-positive and Gram-negative) and the yeast assayed, while the Jelleine-III presented a reduced spectrum of antibiosis, when compared to the previous peptides; the Jelleine-IV apparently presented no antimicrobial activity.

In order to extend the biological characterization of these peptides, they were also investigated concerning to their potential for mast cell degranulation and hemolytic activities. These results are represented in Table 3, and they show clearly that the Jelleines present very reduced activity of mast cell degranulation and erythrocytes disruption.

Table 1
Amino sequences of the Jelleines peptides

Fraction	Peptide	Sequence	Molecular masses (Da)
8.1	Jelleine-I	PFKISIHL-NH_2	953.24
8.2	Jelleine-II	TPFKISIHL-NH_2	1054.30
8.3	Jelleine-III	EPFKISIHL-NH_2	1082.32
8.4	Jelleine-IV	TPFKISIH-NH_2	942.13

4. Discussion

Cationic antimicrobial peptides generally present from 12 to 50 amino acids with a net positive charge from 2+ to 7+ owing to the an excess of basic amino acid residues and more than 50% of their amino acid sequences are constituted of hydrophobic residues, which influence directly in their interactions with bacterial membranes [11,12]. The Jelleines present from 8 to 9 amino acid residues, with a hypothetical net charge of 1+ or 2+ (depending on Jelleine sequence, from -I to -IV) and the most of these residues are hydrophobic. Thus, in spite to be a little bit shorter than the most antimicrobial peptides, the Jelleines present the basic structural properties of the antimicrobial peptides.

While honeybees gather pollen and nectar from the nature, they become heavily exposed to and likely to be infected by plant associated microorganisms. Preventing the bees to get infected, a complex interaction of innate and cellular immune reactions exists both in tissues and hemocele, in order

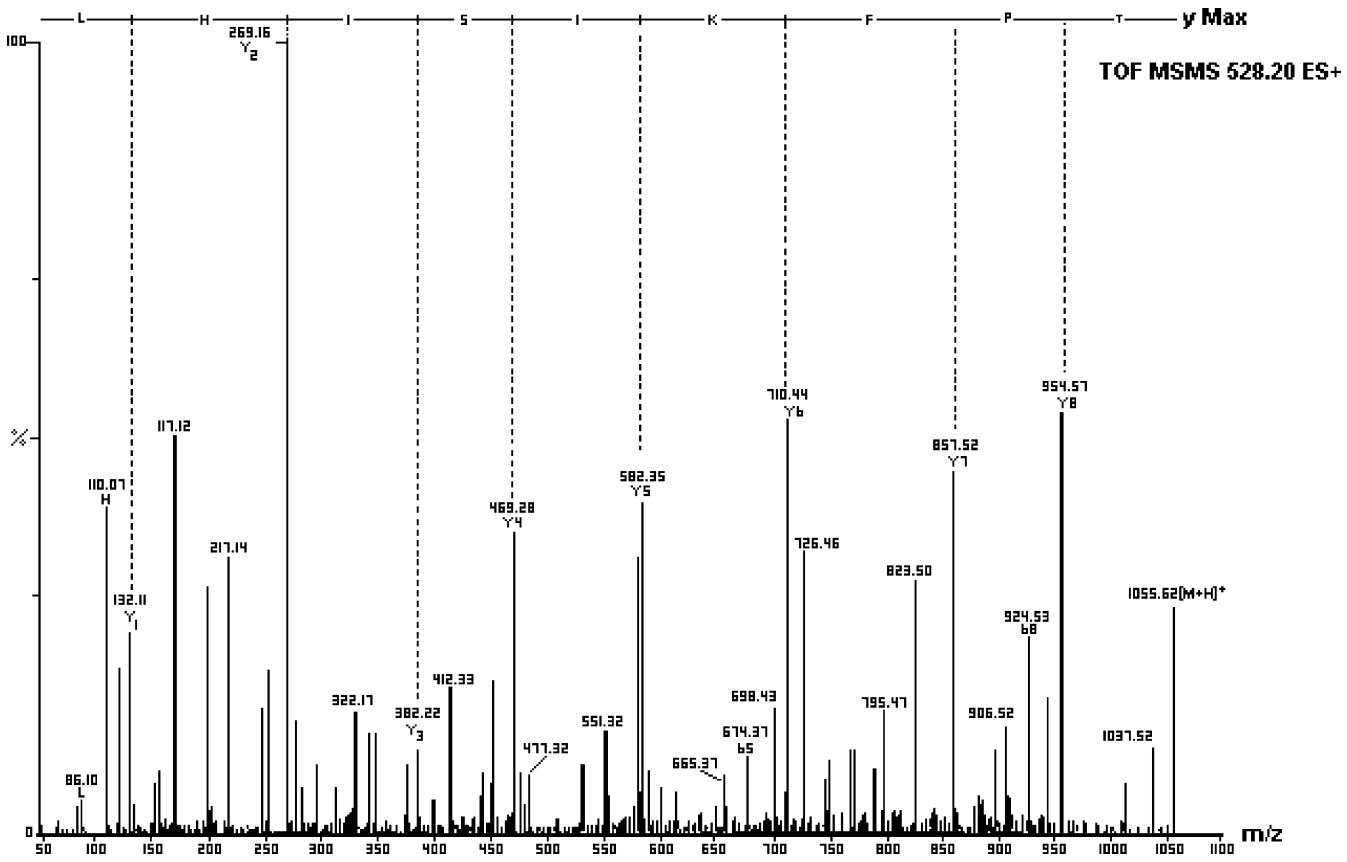

Fig. 4. ESI-Tof-MS/MS spectrum of the $[M + H]^{2+}$ ion (*m*/*z* 528.20), from Fr-8.2 acquired at 50 eV collision energy. The mass differences of the consecutive y_n ions and their correspondence to the amino acid sequence are shown.

to eliminate the microorganisms [23]. Parts of these defensive responses are mediated by a series of cationic antimicrobial peptides and polypeptides occurring in some tissues, blood cells and hemolymph, after injuries and immunological challenges. The cationic antimicrobial peptides involved with some of these defensive responses are: apidaecins, from 2108 to 2122 Da [5]; abaecin, 3863 Da [6]; and hymenoptaecin, 10287 Da [7]. In addition to these peptides, an ubiq-

Table 2
Antimicrobial spectrum of Jelleines showing their values of MIC for each sensitive microorganism

Microorganisms	MIC (μg/mL)			
	Jelleine-I	Jelleine-II	Jelleine-III	Jelleine-IV
Gram-positive bacteria				
S. aureus (ATCC 6535)	10.0	15.0	30.0	R
S. saprophyticus	15.0	10.0	30.0	R
B. subtilis (CCT 2471)	10.0	30.0	R	R
B. cereus	R	R	R	R
Bacillus thuringiensis	R	R	R	R
B. pumilus	R	R	R	R
Gram-negative bacteria				
E. coli (CCT 1371)	2.5	15.0	15.0	R
E. cloacae (ACCT 23355)	10.0	15.0	R	R
K. pneumoniae (ACCT 13883)	10.0	15.0	R	R
P. mirabilis	R	R	R	R
Pseudomonas aeruginsa (ACCT 27853)	10.0	15.0	30.0	R
Yeasts				
C. albicans	2.5	2.5	R	R

R: microorganism resistant the peptide.

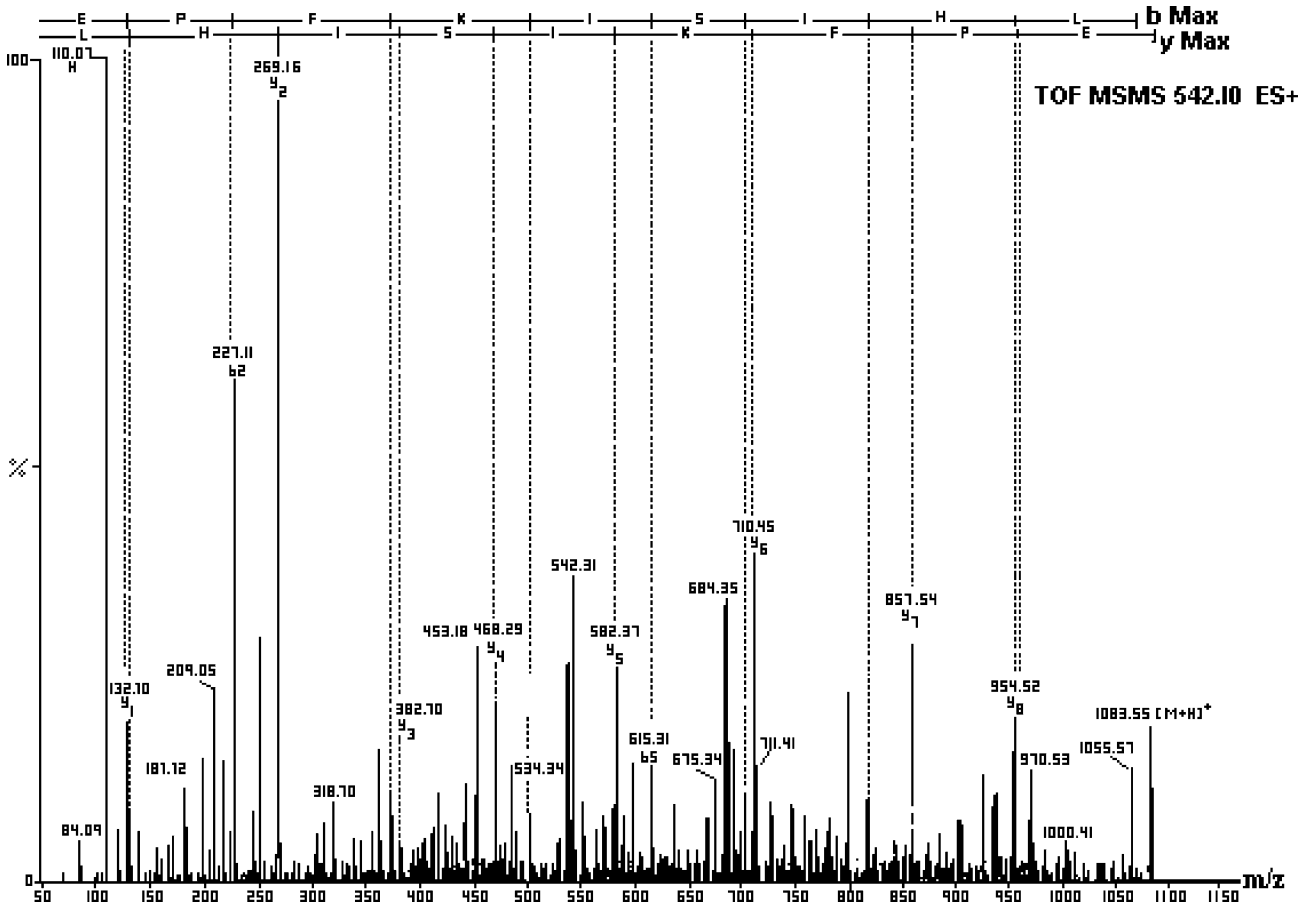

Fig. 5. ESI-Tof-MS/MS spectrum of the $[M + H]^{2+}$ ion (*m/z* 542.10), from Fr-8.3 acquired at 50 eV collision energy. The mass differences of the consecutive y_n ions and their correspondence to the amino acid sequence are shown.

uitous representative of insect defensin family was identified in bee Royal Jelly, the royalisin, 5532 Da [10].

The apidaecins, abaecin and hymenoptaecin are produced by the bees following artificial infections and released in the hemolymph providing a broad-spectrum of activity against Gram-positive and Gram-negative bacteria [5–7].

Royalisin, a defensin-like polypeptide is released into the RJ to prevent infections caused by Gram-positive bacteria [10]. The Jelleines-II and -III are very short antimicrobial peptides naturally found in RJ from Africanized honeybees, presenting broad-spectrum of activity against bacteria (Gram-positive and Gram-negative) and yeast. Apparently the Jelleines do not need any immune challenge of the bee workers to be produced and secreted into the RJ.

A comparison between the MIC values of the Jelleines and the other honeybee antibacterial peptides (Tables 1 and 3) reveals that apidaecins and abaecin are much more active against Gram-negative bacteria than the Jelleines, which in turn are more active against the Gram-positive bacteria than the former peptides. Another aspect to be emphasized from this comparison is the absence of activity of royalisin against Gram-negative bacteria, which in turn are sensitive to Jelleines-I–III. Yeasts are also common microorganisms infecting RJ and the Jelleines-I–III seems to be active to prevent yeast growth. Thus, the Jelleines and royalisin together, by virtue of their complementarities seem to be part of the permanent immunity system of the honeybees, providing a wide-spectrum antimicrobial protection of the RJ (Table 4).

The action of the most antimicrobial peptides is not limited to direct killing of microorganisms; they may be involved with many other aspects of innate host defenses, such as: mast cell degranulation leading to histamine release with the consequent vasodilation; also chemotaxis of neutrophils and T helper cells may be induced, resulting in leukocyte recruitment to the infection site, among other effects [12].

Table 3
Mast cell degranulating and hemolytic activities of Jelleine peptides

Peptide	Hemolysis (%)[a]	Mast cell degranulation (%)[b]
Jelleine-I	5.0 ± 1.3	1.5 ± 1.1
Jelleine-II	11.3 ± 2.8	1.7 ± 0.9
Jelleine-III	9.6 ± 1.9	2.5 ± 1.3
Jelleine-IV	3.1 ± 0.8	0.8 ± 0.6

[a] Measured for washed rat red blood cells.
[b] Measured for rat peritoneal mast cells.

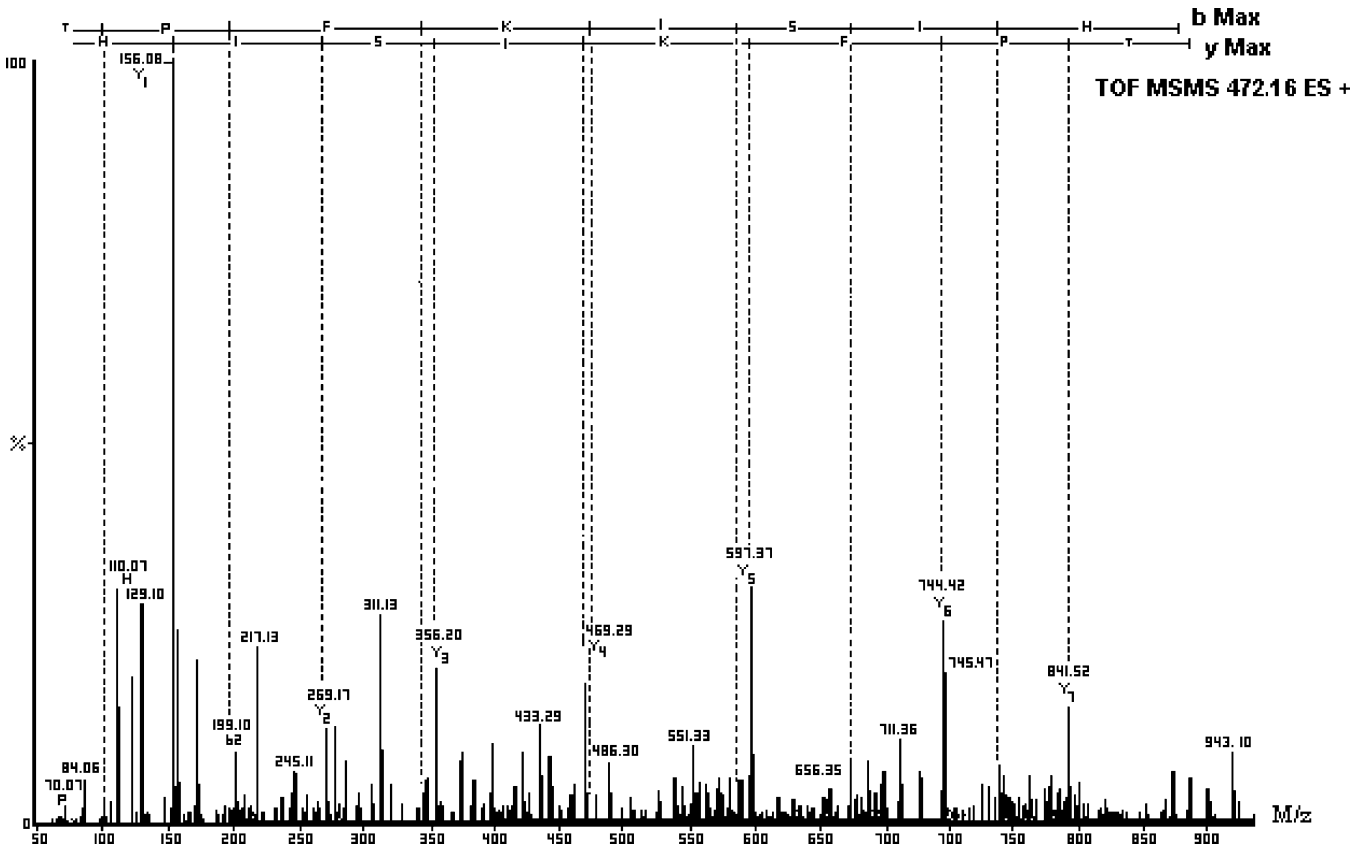

Fig. 6. ESI-Tof-MS/MS spectrum of the $[M + H]^{2+}$ ion (*m/z* 472.16), from Fr-8.4 acquired at 50 eV collision energy. The mass differences of the consecutive y_n ions and their correspondence to the amino acid sequence are shown.

Apparently, all the Jelleines are not cytolytic (since they caused a very reduced hemolysis in rat erythrocytes) or directly involved with inflammatory effects, since they caused no important degranulation of rat peritoneal mast cells (Table 2). Thus, the Jelleines-I–III seem to be exclusively antimicrobial peptides, while Jelleine-IV was not active in any assay performed in the present investigation.

Thus, it is possible to establish some relationships between the antimicrobial activity of the Jelleines and their primary sequence, despite of their primary structure be maintained conserved; small differences were observed in the potency of each peptide. The most active peptides against the bacteria and yeast assayed were the Jelleines-I and -II, followed by Jelleine-III, which was not active

Table 4
Antibacterial spectrum of hymenoptaecin, apidaecins, abaecin and royalisin showing their values of MIC for each sensitive microorganism [6–8]

Microorganisms	MIC (μg/mL)					
	Hymenoptaecin	Apidaecin-Ia	Apidaecin-Ib	Apidaecin-II	Abaecin	Royalisin
Gram-positive						
Bacilus negaterium	5.0	25.0	100.0	50.0	10.0	NT
B. subtilis	R	R	R	R	NT	R
B. thuringensis	NT	R	R	R	NT	NT
Staphylococcus areus	NT	NT	NT	NT	NT	6.0
Streptococcus thermiphylus	NT	NT	NT	NT	NT	6.0
Lactobacilus bulgaricus	NT	NT	NT	NT	NT	6.0
Gram-negative						
E. coli	5.0	0.1	0.1	0.1	25.0	R
Samonela typhimurium	NT	0.1	0.1	0.1	NT	R
Erwinia salicis	NT	<0.1	<0.1	<0.1	25.0	NT
K. pneumoniae	NT	NT	NT	NT	NT	R
Agrobacterium tumefaciens	50.0	NT	NT	NT	NT	NT

against the most microorganisms tested. The only difference between the sequences of the Jelleine-II and -I is the removal of the Thr residue from the N-terminal position of Jelleine-II, which does not seem to be very important for the antibiotic activity of Jelleine-I. However, the replacement of the Thr residue by a Glu residue at the N-terminal position of Jelleine-II caused a significative decrease in antibiotic activity of Jelleine-III. The removal of the Leu residue at the C-terminal position of Jelleine-IV resulted in the complete loss of the antimicrobial action of this peptide. The presence of Leu residue in the amidated form at the C-terminal position of the cationic short-chain peptides both from the Hymenopteran venom toxins [8,15,17,22] and the most of proline-rich peptides from insects in general [4] seem to be a mandatory structural requisite for the antimicrobial activity of these peptides. Apparently, the removal of this residue from the sequence of Jelleine-IV may be an explanation for the absence of antimicrobial activity of this peptide, specially when compared to Jelleine-II.

The mode of action of Jelleines against yeast and bacteria is currently unknown; since yeasts are eucariotic organisms, this result suggests that Jelleines also may be active against fungi. Yeasts are surrounded in addition to the plasma membrane, by an external barrier, which contains mainly polysaccharide compounds. Thus, to be active against yeasts it is necessary to cross this carbohydrate barrier in order to reach and interact with the plasma membrane. A common characteristic of the most antimicrobial peptides is their net positive charge, to make possible to bind the negative charges of phospholipids in the membrane. Non-hemolytic antimicrobial peptides like Jelleines, probably bind strongly and permeate more efficiently the negatively charged phospholipid membranes of bacteria, than the zwitterionic membranes of mammalian cells [9,21,23].

Jelleines do not present any similarity of primary sequences with any other antimicrobial peptides from honeybees (hymenoptaecin, apidaecins, abaecin and royalisin). However, performing database queries with the sequences of Jelleines in the protein engine search Mascot (http://www.matrixscience.com) it was found a high similarity (with significant score for $P < 0.05$) with the last nine amino acid residues from the C-terminal region of the protein MRJP-1, as shown below

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                361                       382
MRJP-1     ......ILNTRCENPD NDRTPFKISIHL
Jelleine-II                    TPFKISIHL
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Considering the presence of an Arg residue at the position 373 of the primary sequence of MRJP-1, neighbor to the Thr residue (position 374) of the last nine amino acid residues, it may be suggested that Jelleine-II may be a product of a tryptic digestion of MRJP-1, which is produced in the hypopharyngeal glands of the worker honeybee and secreted into the RJ; an exoproteinase action either on N- or on C-terminal positions of the tryptic fragment could result in the formation of the Jelleines-I and -IV, respectively. MRJP-1 is the most abundant protein component in the RJ and plays an important role in queen honeybee nutrition and development [19]; and in addition to this, it may be also a precursor form of the Jelleines.

5. Conclusions

Four antimicrobial peptides were purified and sequenced from the Royal Jelly of Africanized honeybees (*A. mellifera*) by using Q-Tof-MS/MS. The peptides were manually synthesized on-solid phase, purified and submitted to different biological assays: antimicrobial activity, mast cell degranulating activity and hemolysis. Three of these peptides, presenting Leu residue in the amidated form at the C-terminal position (Jelleines-I–III) presented exclusively antimicrobial activities against yeast, Gram+ and Gram− bacteria; meanwhile, Jelleine-IV (presenting His residue in the amidated form at the C-terminal position) was not active in none of the assays performed. The Jelleines are very short peptides, presenting hydrophobic sequences; these peptides do not present any similarity with any other known antimicrobial peptides, including those from the honeybees (hymenoptaecin, abaecins, apidaecins, and royalisin). While hymenoptaecin, abaecins, and apidaecins were produced and secreted to the haemolymph after infective challenges of the honeybee workers, the Jelleines and royalisin are produced constitutively by the workers and secreted into RJ, to provide a broad-spectrum protection of this bee hive product against microbial infections.

Peptides representing new types of antimicrobial agents both structurally and functionally are systematically evaluated for use as new therapeutic agents or as model compounds for the rational development of new antibiotics. The short extension of the Jelleines, the poor cytolytic and mast cell degranulating activities of these peptides and the broad-spectrum of antibiosis against bacteria and yeast, with relatively reduced MIC values, make the Jeleines-I–III, attractive candidates/models for future development of new antimicrobial agents.

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