A novel heterodimeric antimicrobial peptide from the tree-frog *Phyllomedusa distincta*

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Abstract We present here the purification and the analysis of the structural and functional properties of distinctin, a 5.4 kDa heterodimeric peptide with antimicrobial activity from the tree-frog *Phyllomedusa distincta*. This peptide was isolated from the crude extract of skin granular glands by different chromatographic steps. Its minimal inhibitory concentration was determined against pathogenic *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* strains. Amino acid sequencing and mass spectrometric investigations demonstrated that distinctin is constituted of two different polypeptide chains connected by an intermolecular disulphide bridge. Circular dichroism and Fourier-transformed infrared spectroscopy studies showed that this molecule adopts, in water, a structure containing a significant percentage of antiparallel β-sheet. A conformational variation was observed under experimental conditions mimicking a membrane-like environment. Database searches did not show sequence similarities with any known antimicrobial peptides. In the light of these results, we can consider distinctin as the first example of a new class of antimicrobial heterodimeric peptides from frog skin.

Key words: Antimicrobial peptide; Disulphide; Heterodimer; Conformation analysis; *Phyllomedusa distincta*

1. Introduction

Antimicrobial peptides, small toxins and venom components have long been identified in a wide range of organisms as parts of complex defence mechanisms as well as invaluable predatory weapons [1–3]. Amphibian peptides represent an important chapter in the vastly investigated area of antimicrobial substances; they have been shown to inhibit the growth of numerous species of bacteria, fungi, protozoa and also some types of tumour cells without showing significant haemolytic activity [4,5]. These molecules range in length from less than 15 to more than 40 amino acid residues with most having a net positive charge and a potential amphipathic α-helical conformation. In addition, they possess a high hydrophobic moment, a broad non-polar face and a narrow polar face. Although their mechanism of action is not totally understood, studies in the presence of lipid bilayers as models of biological membranes demonstrated that these peptides fold into amphipathic α-helices when bound to the lipid membrane. In this process, it is thought that these molecules permeabilise the bilayer either by forming pores consisting of sets of associated peptide helices or by simple disrupting the lipid acyl chain packing in the membrane [6,7].

Among the known antimicrobial peptides from amphibians, magainins (isolated from *Xenopus laevis*), esculentins, brevinins, ranatuerins and ranalexin (from *Rana* sp.), dermaseptins (from *Phyllomedusa* sp.) and bombinins (from *Bombina* sp.) have been shown to act by enhancing the permeability of biological membranes [4,5,8]. These helical peptides have been classified in five different groups by combining sequence homologies, functional similarities and conformational properties. Esculentins, brevinins, ranatuerins and ranalexins are characterised by the presence of an intramolecular S–S bridge at the C-terminus containing a loop of six or seven residues that resembles the cyclic hepta-peptide or the penta- and octa-depsipeptide moieties observed in membrane active components isolated from *Bacillus polymyxa* and *Pseudomonas syringae*, respectively [9].

Other antimicrobial peptides with one or more intramolecular S–S bridges have been isolated from different animals. Only a single disulphide bond is found in a cyclic dodecapeptide from bovine neutrophils [10]. All other molecules have at least two or three S–S bridges; for example defensins isolated from mammalian granulocytes, β-defensins purified from chicken leukocytes and mammalian tissues, protegrins from porcine leukocytes and cysteine-rich peptides from invertebrates [2,3,11]. Similarly, a high disulphide bonds content has been reported in several antibacterial peptides purified from plants [12]. Most of these molecules present a substantial degree of amphipathic β-sheet structure [2,11].

Among a great number of dermaseptin-related components present in skin extracts of *Phyllomedusa distincta* [13], we isolated a new antimicrobial peptide. In this work we describe

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Abbreviations: CD, circular dichroism; ESMS, electrospray mass spectrometry; TFE, 2,2,2-trifluoroethanol; FTIR, Fourier-transformed infrared; LUV, large unilamellar vesicle; MIC, minimal inhibitory concentration

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the purification, the structural characterisation, the conformational analysis and the biological activity of this S–S bridged heterodimer.

2. Materials and methods

2.1. Materials

P. distincta specimens were captured in Itanhaém, São Paulo State, Brazil. All solutions were prepared using water from a Milli-Q apparatus (Millipore). Solvents were high performance liquid chromatography (HPLC) grade. All reagents were analytical grade.

2.2. Peptide purification

Frog skin secretion was obtained by manual compression of the granular glands and collected in distilled water as a crude extract. This material was filtered by gravity in paper filter, frozen in liquid nitrogen and lyophilised. Peptide separation was performed as previously reported [13]. Peptides were manually collected and lyophilised. Independent collections from different individuals at various times were performed.

2.3. Reduction and alkylation reaction

Peptide samples were reduced in 0.25 M Tris–HCl, 1.25 mM EDTA, pH 8.5, by incubation with a 10:1 molar excess of dithiothreitol over the molecule –SH groups, at 37°C for 2 h, under N_2 atmosphere, and alkylated using a 10:1 molar excess of iodoacetamide over the total –SH groups, for 1 h at 25°C, in the dark, under N_2 atmosphere. Carboxyamidomethylated peptides were purified, manually collected and lyophilised.

2.4. Sequence determination

The N-terminal sequence of either native and alkylated samples of peptides was determined using an Applied Biosystems 477A pulsed-liquid protein sequencer. Quantitative amino acid composition of pure peptide was determined with a 6300 E Beckman amino acid analyser (Palo Alto, CA, USA). Samples were hydrolysed in 6 N HCl, containing 0.5% phenol, in sealed and evacuated Pyrex glass tubes for 24 h at 110°C. Peptide mass determination was obtained using a Platform single quadrupole mass spectrometer (Micromass, Manchester, UK) as previously reported [13].

2.5. Antimicrobial tests

The microorganisms used for the antimicrobial assay were purchased from American Collection (Rockville, MD, USA). They were *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. To reactivate the freeze-dried *E. faecalis*, the strain was cultured with shaking at 30°C; the other microorganisms were cultured in stationary culture at 37°C. Bacteria were grown in tryptic soy broth. The bioassays were performed by the microdilution method as previously reported [13].

2.6. Spectroscopy studies

Circular dichroism (CD) spectra were recorded by using a Jasco J-720 spectropolarimeter (Easton, MD, USA) calibrated with D-10-camphorsulphonic acid. The peptides were dissolved in water or water/2,2,2-trifluoroethanol (TFE) solutions (0.250 mg/ml) and analysed in a cell of 0.10 cm path length, at room temperature. CD band intensities are represented as mean residue ellipticity. Percentage of secondary structure elements was calculated using a method based on linear combination of CD spectra obtained from proteins with known secondary structures [14].

Infrared spectroscopic studies were performed with a PROTA™ Fourier-transformed infrared (FTIR) protein analyser (Bomem/Vysis, USA). The instrument is based on Bomem's MB-104 FTIR spectrometer, equipped with a DTGS detector, non-hygroscopic ZnSe beamsplitter and Arid-Zone purge system. This system configuration is optimised for measurement of protein spectra at low protein concentrations in aqueous solutions and to minimise water vapour interference. Spectra were collected in the transmission mode at a resolution of 4 cm^{-1}, accumulating 500 scans obtained with a scan speed of 20 scan/min. The peptide was dissolved in water at a concentration of 1.0 mg/ml. A demountable cell with a 6 μ mylar spacer (Specac Ltd., UK) was used, although the cell was not taken apart during the experiment in order to keep the path length constant. A water blank was subtracted using an automated algorithm present in the instrument software, that uses linear regression to minimise the combination band of water at ~2125 cm^{-1}. Second derivative and Fourier self-deconvolution spectra were calculated to enhance the spectral features using Spectrum Square Associates (Ithaca, NY, USA) software available within the instrument. Lorentzian bandshape was assumed for both calculations, with full width at half-height equal to 14 cm^{-1} for second derivative and 16 cm^{-1} for deconvolution, with additional parameter k equal to 1.8 (where k is defined as original width divided by new width). Quantitative secondary structure calculations were performed using Factor Analysis algorithm available with the instrument software [15].

2.7. Secondary structure prediction and model construction

Secondary structure prediction was carried out using PHD [16]. A model of the chain 2 was constructed using O [17], assuming a helical orientation parallel to the lipid chains [8]. Symmetrical helical bundles were constructed with the helical axis orientation chosen to maximise the exposure of hydrophilic residues to the central channel. Side chains were modelled as the most common rotamers [18]. In order to sample conformational space more effectively we used MODELLER [19], with a 4 Å randomisation step prior to restraint refinement, to 'breed' more structures from the original model. Strong restraints were applied to enforce exact 6-fold symmetry. We used PROCHECK [20] for geometrical analysis of the final model.

3. Results

Initial fractionation of *P. distincta* crude gland extracts separated more than 30 components [13]. Species were further purified on an analytical reversed-phase column. This procedure allowed obtaining different peptides not yet known in a homogeneous form, as judged by HPLC profile and electrospray mass spectrometry (ESMS) analysis. A general screening of all species isolated was performed to find lytic activity against synthetic membranes using large unilamellar vesicles (LUVs) constructed with phosphatidylcholine. Previous studies on other antimicrobial peptides demonstrated that the ability to disrupt synthetic membranes like liposomes could be related to their antimicrobial activity [8]. Among a number of dermaseptin-related species [13], a strong lytic activity against LUVs was associated also to peptide N.

Although ESMS analysis of this component revealed only the occurrence of a single species with a molecular mass of 5478.4 ± 0.6 Da, primary structure investigations by automated Edman degradation yielded a simultaneous double sequence, corresponding to two independent peptides consisting of more than 20 amino acid residues each. Indeed, reversed-phase HPLC analysis of peptide N, following reduction and alkylation reactions, yielded two different species, as reported in Fig. 1A. Their molecular masses were measured by ESMS (Fig. 1B,C). Automated Edman degradation of each peak yielded unequivocal results allowing the reconstruction of the primary structure of each peptide. The theoretical molecular masses calculated from the sequences for the carboxyamidomethylated peptides (2584.0 Da and 3010.5 Da, respectively) were in excellent agreement with the values experimentally determined. These results, together with unsuccessful attempts to alkylate and resolve peptide N without prior treatment with reductive agents, clearly demonstrated that the cysteine residues present in each peptide chain were linked by a disulphide bridge to yield a heterodimeric species whose theoretical molecular mass (5478.6 Da) was in excellent agreement with the value experimentally determined for peptide N. The molecular structure determined for this lytic com-

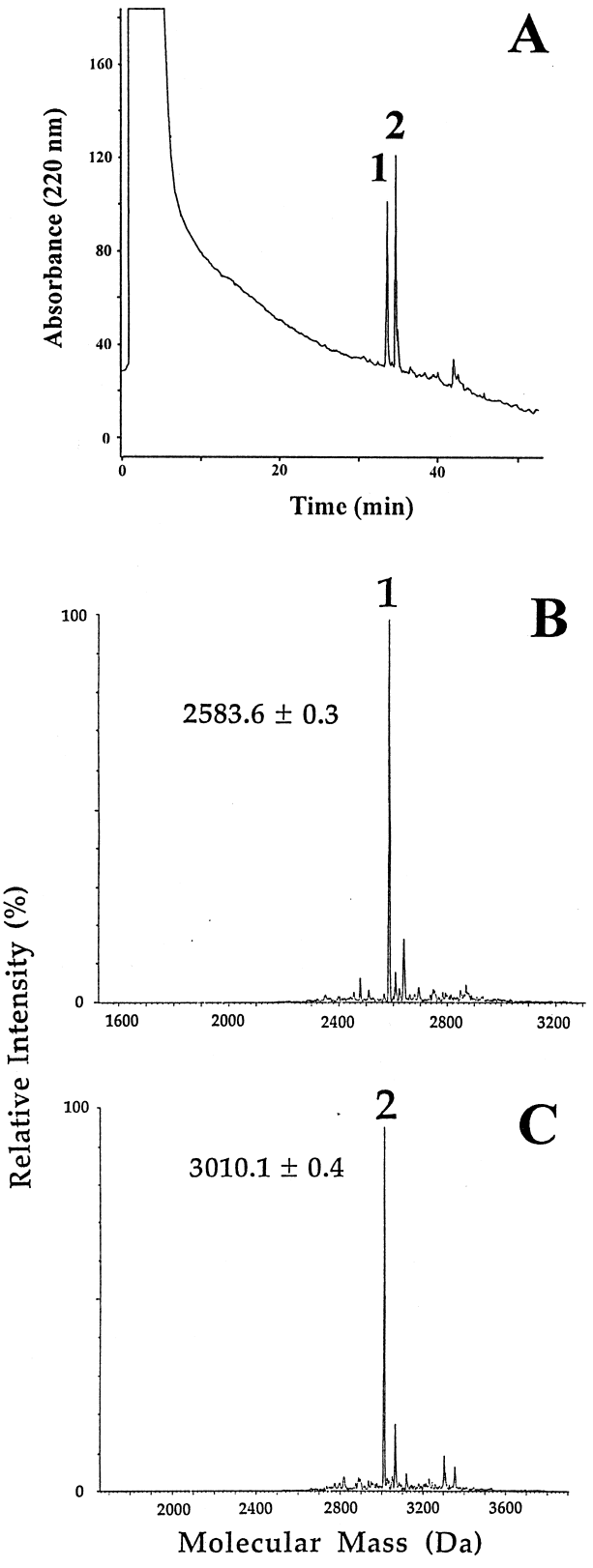

Fig. 1. Reversed-phase HPLC analysis of distinctin following reduction and alkylation (A). Transformed ESMS spectrum of the peptide corresponding to peak 1 (B) and peak 2 (C).

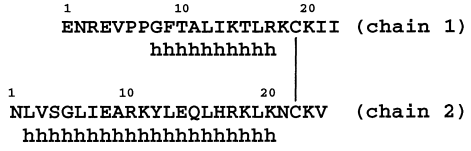

Fig. 2. Molecular structure of distinctin. Secondary structure prediction is shown.

ponent, here named distinctin on the basis of the amphibian species name, is reported in Fig. 2. Minor amounts of a truncated species missing three amino acid residues at the N-terminus of chain 1 were also identified in some preparations. Due to its poor relative abundance, this species was not considered for biological assays. A search in different protein databases did not show similarity of distinctin to other known antimicrobial peptides, strongly suggesting that this molecule could be considered as the first example of a new class of lytic peptides isolated from frog skin.

Distinctin was tested for antimicrobial activity against the pathogenic bacteria species *E. faecalis*, *P. aeruginosa*, *S. aureus* and *E. coli*, respectively. Minimal inhibitory concentration (MIC), defined as the smallest amount of peptide capable of inhibiting bacteria growth, was determined for the peptide using ampicillin, chloramphenicol, neomycin and tetracycline for comparison purposes. The results are shown in Table 1. The peptide concentration corresponding to MIC was added to a culture of solid tryptic soy medium for 24 h at 37°C and no colony was grown in all cases. These experiments demonstrated that distinctin showed a marked antimicrobial activity against both Gram-positive and Gram-negative bacteria, comparable to conventional antibiotics. Given the increasing incidence of multiply resistant bacterial infections, peptides such as distinctin may present an alternative route for the development of therapeutic compounds.

In order to get information regarding the conformation of distinctin in aqueous solution, the peptide was analysed by two complementary approaches: CD and FTIR spectroscopies. CD studies clearly demonstrated that this peptide presented in water a structured conformation. Fig. 3 shows the CD spectrum profile obtained; the single minimum observed at 195–200 nm for native distinctin was characteristic of polypeptides containing β-sheet and β-turn secondary structure with some random coil [14]. The calculated content of secondary structure elements pointed out that distinctin presents an organised structure with predominant anti-parallel β-sheet conformation: 41.7% β-sheet (anti-parallel), 4.1% β-sheet (parallel), 9.8% α-helix, 18.3% β-turn and 26.0% random coil.

The FTIR absorbance spectrum of distinctin is shown in Fig. 4A. The spectrum obtained is typical for polypeptides with a high β-sheet content, although mixed with other structural elements, as demonstrated by the dominance of 1624 cm^{-1} and 1650 cm^{-1} bands [21]. Fig. 4B shows second derivative (top) and Fourier self-deconvolution (bottom) spectra for an expanded region of the amide I band. These spectra emphasised the dominance of β-sheet content (main band at 1624 cm^{-1} with a smaller band at 1683 cm^{-1}) with respect to helices and turns. By using the Factor Analysis program a content of 38% β-sheet, 25% α-helix, 17% turns and 11% bend was calculated.

The results of the antibacterial assays and conformational analysis suggested that distinctin, commonly to other pep-

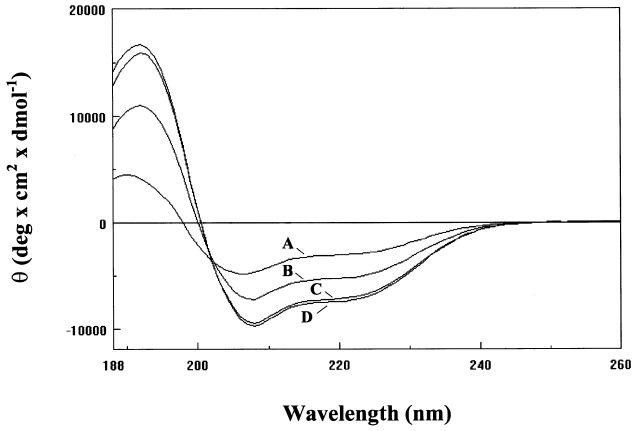

Fig. 3. CD analysis of distinctin in the far-ultraviolet region. The spectra were performed in water (A), 10% TFE (B), 20% TFE (C) and 40% TFE (D), respectively.

tides, might form pores in lipid bilayers by adopting a typical transmembrane β-structure observed also in other classes of pore forming peptides and proteins [22,23]. However, a careful examination of its sequence showed a clear absence of the alternating hydrophobic/hydrophilic amphipathic pattern characteristic of membrane-spanning β-strands [23]. In contrast, secondary structure prediction revealed a significant propensity for chain 2 to form an α-helix in the region 2–21 (Fig. 2). Helical wheel diagrams showed that this portion would form a highly amphipathic helix (data not shown). This result suggested that distinctin, like many other peptides, might alternatively form a transmembrane channel through the parallel association of amphipathic α-helices. To investigate this possibility we performed CD analysis under experimental conditions mimicking a membrane-like environment. Fig. 3 reports the CD spectra obtained at increasing TFE concentrations. A significant conformational change was observed, associated with a strong increase in helical content and a parallel decrease in anti-parallel β-sheet content. In fact, the calculated percentage of secondary structure elements for distinctin in 40% TFE resulted: 21.4% β-sheet (anti-parallel), 7.4% β-sheet (parallel), 25.0% α-helix, 19.3% β-turn and 27.0% random coil, respectively.

In order to investigate on the possible distinctin folding in membranes, we carried out structural modelling of chain 2 (data not shown). We excluded the possibility that chain 1 might also span membranes because of its much shorter extension compared with the accepted minimum for transmembrane helices [24,25]. The largely hydrophilic face of the putative chain 2 helix would favour a large number of associating helices [26,27]. Indeed, four and six helical bundle models were generated and analysed for their structure. The six helical bundle model presented a more favourable packing, with all hydrophobic residues positioned to allow interaction

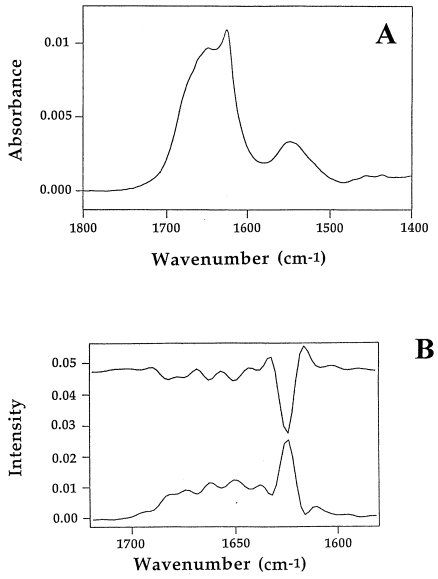

Fig. 4. FTIR absorbance spectrum of distinctin (A). The spectrum was obtained in water as described in Section 2. Calculated second derivative (top) and deconvoluted (bottom) spectra (B).

with lipid chains or participation in inter-helical packing and all hydrophilic residues positioned where they would contact the water-filled channel. According to this model, the C-terminal part of both chains would be hypothesised as located near the membrane surface, with their basic residues concentrated in the vicinity of the disulphide bond and interacting with the negatively charged phospholipid head-groups.

4. Discussion

Among a great number of antimicrobial peptides isolated from the frog skin of *P. distincta* [13], a new heterodimeric peptide, distinctin, was isolated and structurally characterised. Heterodimeric structures connected by a single intermolecular S–S bond were reported only for invertebrates neurotoxins inhibiting neurotransmitter release, imperatoxin I and β-bungarotoxins [28]. The occurrence of an antimicrobial heterodimeric polypeptide has not been described so far, although the isolation of a homodimeric antibacterial component from the guinea pig neutrophils has been reported [29]. These findings lead us to consider distinctin as the first example of a new class of bioactive peptides from frog skin.

Antimicrobial peptides interacting with membranes have been proposed to fall into two distinct structural groups: those containing amphipathic α-helices forming a channel in membranes and those with β-structure oligomerising to generate a transmembrane β-barrel [22]. Distinctin extends the range of known lytic molecules further since, while being

Table 1
MIC of distinctin compared to other conventional antibiotics

Bacterium	Distinctin	Ampicillin	Chloramphenicol	Neomycin	Tetracycline
E. coli	14.5	50.0	50.0	100.0	50.0
S. aureus	28.0	< 0.7	100.0	25.0	6.2
P. aeruginosa	28.0	> 100.0	> 100.0	> 100.0	100.0
E. fecalis	14.5	N.d.	N.d.	N.d.	N.d.

The concentration is expressed in μM. N.d.: not determined.

rich in β-structure, it seems to lack the potential to form amphipathic β-strands. In fact, CD measurements and secondary structure prediction suggested that one of its chains should be capable to form an amphipathic transmembrane helix as a result of a conformational adjustment on moving from water to a lipid environment. Some bacterial toxins, such as colicin [30] and aerolysin [31], exhibit only tertiary structure rearrangement on insertion into the membrane. Similarly, a growing number of peptides are known to undergo changes in secondary structural content, involving an increase in helical structure, on membrane insertion. Among these are cytolysins and equinatoxin II, both obtained from sea anemone [32]. It seems that distinctin falls within this group. However, it has to be mentioned that the model here proposed is not supported by any direct experimental observation. Such issues will be definitively addressed by nuclear magnetic resonance spectroscopic studies in water and in micelles, now in progress. Additional investigations on its haemolytic activity will clarify its possible use as therapeutic compound.

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