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Ocellatins: New Antimicrobial Peptides from the Skin Secretion of the South American Frog *Leptodactylus ocellatus* (Anura: Leptodactylidae)

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The emergence, in recent years, of microbial resistance to commonly used antibiotics has aroused a search for new naturally occurring bactericidal and fungicidal agents that may have clinical utility. In the present study, three new antimicrobial peptides were purified from the electrical-stimulated skin secretion of the South American frog *Leptodactylus ocellatus* by reversed-phase chromatographic procedures. Ocellatin 1 (^{1}GVVDILKGAG-KDLLAHLVG ISEKV^{25}-$CONH_2$), ocellatin 2 (^{1}GVVDILKGAGKDLLAHLVGKISEKV^{25}-$CONH_2$) and ocellatin 3 (^{1}GVLDILKNAAKNILAHAAEQI^{21}-$CONH_2$) are structurally related peptides. These peptides present hemolytic activity against human erythrocytes and are also active against *Escherichia coli*. Ocellatins exhibit significant sequence similarity to other amphibian antimicrobial peptides, mainly to brevinin 2ED from *Rana esculenta*.

KEY WORDS: Amphibian; antimicrobial peptides; hemolysis; *Leptodactylus ocellatus*; ocellatins; skin secretion.

1. INTRODUCTION

It has been recognized since a long time ago that the skin secretion from amphibians, especially anurans, is a rich source of bioactive peptides (Barra and Simmaco, 1995; Erspamer, 1994; Nascimento *et al*., 2003). These peptides exhibit an array of biological activities such as cardio-, mio-, or neurotoxic, cholinomimetic, sympathomimetic, vasoconstrictor, hypertensive, hemolytic, hallucinogenic, antinociceptive or antibiotic.

The first report describing an anuran antimicrobial peptide was concerned with the purification of bombinin from *Bombina variegata* (Csordas and Michl, 1969). Scientific interest on this sort of peptides was further consolidated by the isolation of magainins from *Xenopus laevis* (Zasloff, 1987). To date, many potent antimicrobial peptides have been isolated and characterized. However, the majority of the studies focuses on members of the genera *Rana*, *Bombina*, *Phyllomedusa* and *Litoria*. Some examples are:

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Abbreviations: u, atomic mass unit ESIMS, electrospray ionization mass spectrometry LB, Luria-Bertani RP-HPLC, reversed-phase high performance liquid chromatography RT, room temperature TFA, trifluoroacetic acid.

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bombinins and bombinins H, isolated from *Bombina* species (Mangoni *et al.*, 2000; Simmaco *et al.*, 1991); dermaseptins, from *Phyllomedusa* genus (Batista *et al.*, 1999; Mor and Nicolas, 1994a; Mor *et al.*, 1991); brevinins, from the Japanese frog *Rana porsa brevipoda* (Morikawa *et al.*, 1992); ranatuerins, from *R. catesbeiana* (Goraya *et al.*, 1998); temporins, from *R. temporaria* (Simmaco *et al.*, 1996); caerins and caeridins, from the Australian genus *Litoria* (Steinborner *et al.*, 1997; Wong *et al.*, 1997).

In contrast to the mode of action of classic antibiotics, which is based on the inhibition of enzymes and requires a period of days to be accomplished, antimicrobial peptides act promptly, causing the lysis of bacteria, fungi and protozoa. This effectiveness comes from the ability of antimicrobial peptides to interact with microorganism membranes and induce several damages, causing their destabilization (Lohner and Prenner, 1999). Some features shared by most antimicrobial peptides from anurans strongly contribute to their efficient action. The main features are: presence of a large number of hydrophobic and positively charged residues, amphipathic α-helix conformation, positive net charge, high hydrophobic moment and wide apolar face (Oren and Shai, 1998; Simmaco *et al.*, 1998). The amphipathic helical motif displays polar side chains aligned along one side and hydrophobic residues along the opposite side of the helix, thereby favoring interaction between peptides and the amphiphilic structure of biological membranes (Dathe and Wieprecht, 1999). Meanwhile, the cationicity makes the affinity to negatively charged phospholipids, more exposed in bacterial membranes, higher than the affinity to zwitterionic phospholipids, exposed in mammalian cells. Therefore, the action of the peptides is frequently selective (Blondelle *et al.*, 1999; Matsuzaki, 1999). Other outstanding aspects of the antimicrobial activity of amphibian peptides are the remarkable synergism when these molecules are combined with classic antibiotics and with other antimicrobial peptides, and the improbability of resistance induction in pathogens, since the activity is not mediated by receptors or any other chiral elements (Feder *et al.*, 2000; Mignogna *et al.*, 1998).

Considering that only a limited number of frog families have so far been studied, we decided to investigate the presence of antimicrobial peptides in frog species commonly found in the Brazilian fauna. In the present study, we describe the purification and structural characterization of three antibacterial peptides isolated from the skin secretion of the South American frog *Leptodactylus ocellatus* (Anura: Leptodactylidae).

2. MATERIALS AND METHODS

2.1. Chemicals

Solvents for chromatographic procedures were of HPLC grade from several sources. Reagents and solvents for protein sequencing were purchased from Applied Biosystems (USA). Only analytical grade reagents from commercial suppliers were used throughout the experiments, and all solutions were prepared with Milli Q water (Millipore Reagent Water System, USA).

2.2. Specimens Collection and Skin Secretion Preparation

Adult specimens of *L. ocellatus* were collected in Brasilia (Distrito Federal, Brazil) and maintained in captivity at the University of Brasilia. The secretion from their cutaneous glands was obtained by mild electrical stimulation, collected in a beaker by washing the skin surface with Milli Q water, and immediately lyophilized. No animals were sacrificed during these experiments, and they immediately reassumed their normal behavior.

2.3. Purification of Ocellatins

Aliquots of lyophilized skin secretion from *L. ocellatus* (5.0 mg) were dissolved in 200 μl of 0.1% (v/v) TFA/water and injected into a C_8 reversed-phase column (Sephasil Peptide, 4.6 × 250 mm, Pharmacia Biotech, Sweden) equilibrated with 0.1% (v/v) TFA/water. Elution was performed at a flow rate of 0.8 ml/min, with an initial 5-min wash in 0.1% (v/v) TFA/water and then a 0–50% linear gradient of acetonitrile (containing 0.1% (v/v) TFA). The absorbance was monitored at 216 nm. All fractions were manually collected, lyophilized and evaluated for hemolytic activity. Hemolytic fractions were individually rechromatographed using a C_{18} reversed-phase HPLC column (Vydac 218TP54, 4.6 × 250 mm, The Separations

Group, USA) equilibrated with 0.1% (v/v) TFA/water at a flow rate of 0.8 ml/min. The elution was performed using linear acetonitrile gradient and the eluant was monitored by UV absorbance at 216 nm. Fractions were collected and dried *in vacuo* for subsequent analysis.

2.4. Hemolysis Assay

The hemolysis assay was modified from the protocol of Onuma *et al.* (1999). Cells were separated from plasma by sedimentation and a 1% (v/v) suspension of O^+ human red blood cells (washed three times with NaCl 0.15 *M*, Tris–HCl 0.01 *M*, pH 7.4) was prepared. The samples were resuspended in 150 μl of saline-buffer and 50 μl of 1% (v/v) blood cell suspension was added. After incubation at RT for 60 min, the tubes were centrifuged at 3000 rpm for 2 min. A 100 μl portion of each supernatant was transferred to the wells of flat-bottomed microtiter plates (Nunc, Denmark) and measured for absorption at 405 nm with a BioRad microplate reader Model 3550-UV. Reference samples were employed using 1% (v/v) blood cell suspension incubated in 0.1% (v/v) Triton X-100 as a 100% lysis reference and the 1% (v/v) blood suspension alone as the 0% reference.

2.5. Antibacterial Assay

To verify whether ocellatins were active against microorganisms, an *in vitro* inhibition zone assay on LB-agarose plates (Hultmark *et al.*, 1983) was performed. *Escherichia coli* (ATCC 25922) cells were first grown in LB broth at 37°C to an $OD_{590\,nm}$ of 1.0. *E. coli* cells (approximately 2×10^5 bacteria/ml) were added to LB-agarose medium, spread on Petri dishes and 3.0 mm holes were made, after solidification. Each hole received 6 μl of the samples dissolved in 20% (v/v) ethanol filtered through Millipore 0.22 μm Millex filters (USA). The used peptide solutions were: 2 μg/μl of ocellatin 1 (0.8 m*M*); 4 μg/μl of ocellatin 2 (1.8 m*M*) and 4 μg/μl of ocellatin 3 (1.8 m*M*). Inhibition zones were evaluated after overnight incubation at 37°C.

2.6. Mass Spectrometry Analysis

The experimental values of the molecular masses of ocellatins were assessed by μ-ESIMS using a triple-quadrupole mass spectrometer (PE-SCIEX API 300). Samples were dissolved in 10% (v/v) acetic acid in 50% (v/v) acetonitrile and injected into the ion source by automatic syringe injection, at a flow rate of 0.03 ml/h, using a pump (Harvard apparatus, Inc.) as solvent delivery system. The ion spray voltage was set at 5000 V and the orifice voltage was 30 V. Nitrogen gas was used as curtain gas while compressed air was used as a nebulizer gas. The mass spectrometer was scanned from m/z 200 to m/z 2000 in the positive ion mode. Step size was 0.1 u, and dwell time was 1 ms/step. Solutions of polypropylene glycol were used to calibrate the mass scale. The average molecular mass value of the protein was calculated from the m/z peaks in the charge distribution profiles of the multiply charged ions.

2.7. N-terminal Sequencing

The primary structures of native ocellatins were determined by automated Edman degradation using an Applied Biosystems 477A sequencer, modified as described by Fontes *et al.* (1998). Phenylthiohydantoin (PTH) amino acids were identified using an on-line reversed-phase PTH-C_{18} column in an Applied Biosystems 120A HPLC apparatus. The chromatography system was calibrated with PTH amino acid standards prior to each analysis.

2.8. Amidated C-terminus Determination

Amidation at the C-terminal portion of ocellatins was investigated by mass spectrometric analysis of the methylated peptides (Hunt *et al.*, 1986). Aliquots of each ocellatin were dissolved in methanolic HCl reagent and incubated at RT for 30 min. Solvent was removed by lyophilization and the samples (methylated ocellatins) were reconstituted with 30 μl of 10% (v/v) acetic acid in 50% (v/v) acetonitrile for standard infusion into the electrospray mass spectrometer (PE-SCIEX API 300).

2.9. Sequence Analysis

The BLASTP program (Altschul *et al.*, 1997–http://www.ncbi.nlm.nih.gov/BLAST) was used to search through the NCBI non-redundant database for amino acid sequence similarities. Clustal W 1.82 Thompson *et al.*, 1994–http://www.ebi.ac.uk/clustalw)

was used for multiple sequence alignments. Theoretical molecular masses were calculated from the sequences using Compute pI/Mw Tool (http://www.expasy.org/tools/pi_tool.html).

3. RESULTS AND DISCUSSION

The purification of soluble components from the skin secretion of the frog *L. ocellatus* was initially performed by RP-HPLC using a C_8 column, as shown in Fig. 1. At least 30 fractions were obtained by this procedure, and some of the most hydrophobic fractions exhibited hemolytic activity against human erythrocytes. These fractions were further purified by RP-HPLC using a C_{18} column and well-defined peaks were obtained for all of them, indicating the homogeneity of each peptide (Fig. 2I–III). These hemolytic peptides were named ocellatins.

Direct automated Edman degradation of ocellatins permitted unequivocal identification of their primary structures, as shown in Table 1. The sequencing results were confirmed by mass spectrometry analysis of the native peptides using μ-ESIMS. The molecular masses experimentally determined for ocellatins were in good agreement with the masses calculated from the amino acid sequences obtained (Table 1), but indicated that ocellatins could be post-translationally modified due to mass differences close to 1 u.

The most common post-translational modification in amphibian antimicrobial peptides is the occurrence of an amidation at the C-terminus, which consists in the substitution of –OH (17 u) by $–NH_2$ (16 u). Taking this fact into account together with the differences close to 1 u between the theoretical and the experimental molecular masses of the ocellatins, we can infer that these peptides show amidated C-termini.

The three ocellatins were submitted to derivatization in the presence of methanolic HCl reagent and analyzed by mass spectrometry. Upon derivatization, all the three peptides showed one less methylated group than their number of potential methylation sites, suggesting obstructed C-terminus. This experi-

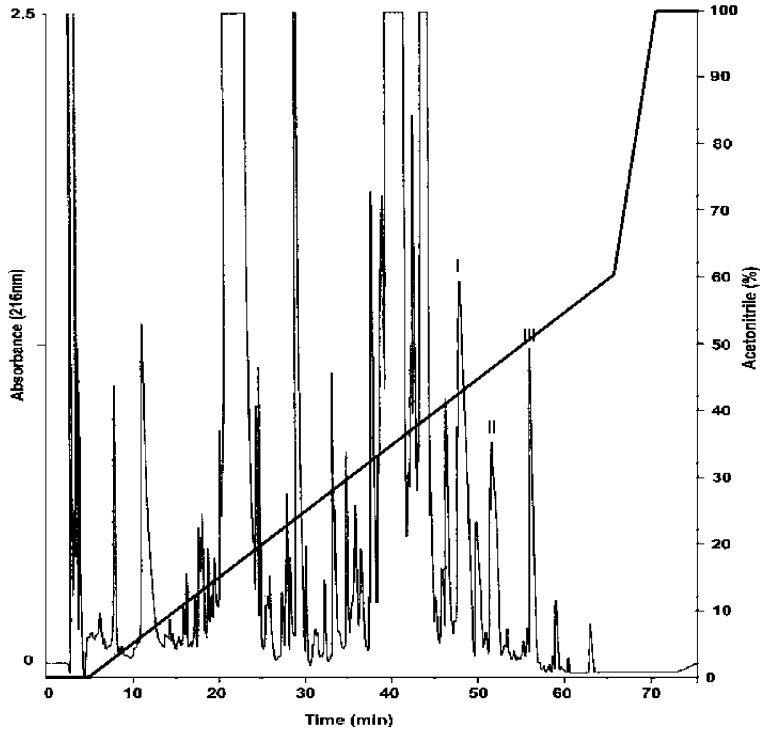

Fig. 1. Isolation of ocellatins from *L. ocellatus* skin secretion. Aliquots of 5.0 mg of dried secretion were loaded into a C_8 reversed-phase column (Sephasil Peptide C_8, 4.6 × 250 mm, Pharmacia Biotech) equilibrated with 0.1% (v/v) TFA/water. The elution was performed with the indicated acetonitrile gradient at a flow rate of 0.8 ml/min. The absorbance was monitored at 216 nm. Fractions were manually collected, lyophilized and tested for hemolytic activity. Fractions (I), (II) and (III) were individually rechromatographed using a C_{18} reversed-phase column.

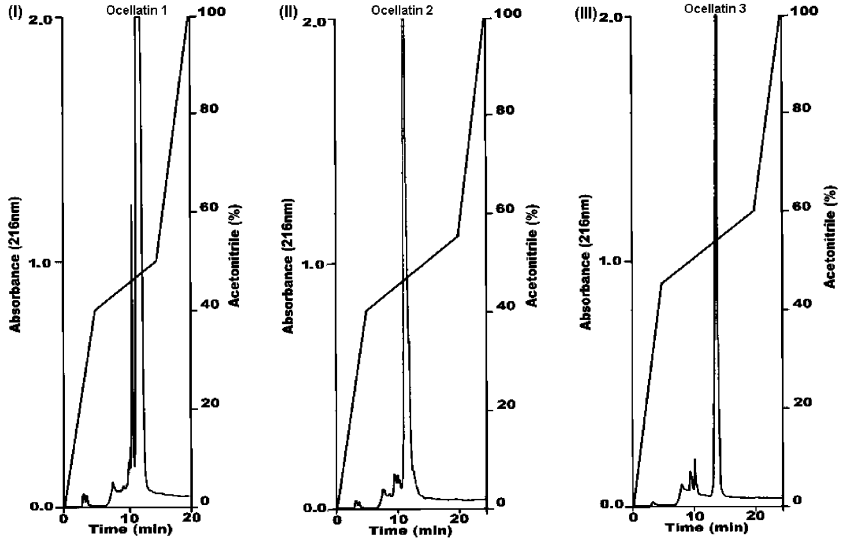

Fig. 2. RP-HPLC purification of ocellatins. Fractions I, II and III obtained after a C_8 reversed-phase chromatography were individually rechromatographed using a C_{18} reversed phase column (Vydac 218TP54, 4.6 × 250 mm, The Separations Group) equilibrated with 0.1% (v/v) TFA/water. The elution was performed with the indicated acetonitrile gradients at a flow rate of 0.8 ml/min. Absorbance was monitored at 216 nm and eluted fractions were manually collected and freeze dried. (I) RP-HPLC elution profile of ocellatin 1; (II) RP-HPLC elution profile of ocellatin 2 and (III) RP-HPLC elution profile of ocellatin 3.

Table 1. Primary structures of ocellatins

Peptide	Primary structure	Molecular mass
Ocellatin 1	^{1}GVVDILKGAGKDLLAHLVGKISEKV^{25}-$CONH_2$	2559.19 (2560.08)
Ocellatin 2	^{1}GVLDIFKDAAKQILAHAAEKQI^{22}-$CONH_2$	2250.34 (2251.65)
Ocellatin 3	^{1}GVLDILKNAAKNILAHAAEQI^{21}-$CONH_2$	2200.89 (2202.58)

The molecular masses were determined by μESIMS and the values in parenthesis are the masses calculated from the proposed amino acid sequences.

ment strengthened the hypothesis that the studied ocellatins might actually have amidated C-termini. The C-terminal amidation often increases the cytolytic activity of peptides, probably because it makes the total positive charge higher, alters the dipole moment and decreases the susceptibility to degradation by carboxypeptidases (Mor and Nicolas, 1994b). Also, in some peptides, amidation can induce C-terminal helix formation or stabilize already existing helical conformations (Shalev *et al.*, 2002).

Pairwise and multiple alignments were performed and revealed a significant level of identity among ocellatins (44–82% of amino acid positional identities). The ocellatins constitute a group of cationic molecules of similar sizes (21–25 residues), with 11 positions occupied by identical residues and 5 positions that present conserved substitutions (Fig. 3a). The search in protein databases pointed out some similarities between the studied peptides and other amphibian antimicrobial peptides, such as brevinins, dermaseptins and uperins (Table 2). Ocellatins, brevinins and uperins have highly similar amino-terminal regions, while the level of sequence similarity between ocellatins and dermaseptins is lower, but also considerable. Although significant levels of sequence similarities were found, in all cases several gaps had to be included to maximize the similarity (Fig. 3b–d), thus ocellatins should constitute a new peptide group.

Due to the short availability of the purified peptides, only preliminary antimicrobial assays have been performed. The three ocellatins are able to inhibit the growth of *E. coli* at millimolar level (Fig. 4). Further experiments must be conducted in order to evaluate the minimal inhibitory concentrations (MICs) of these peptides. The MICs of ocellatins may be much lower than the millimolar

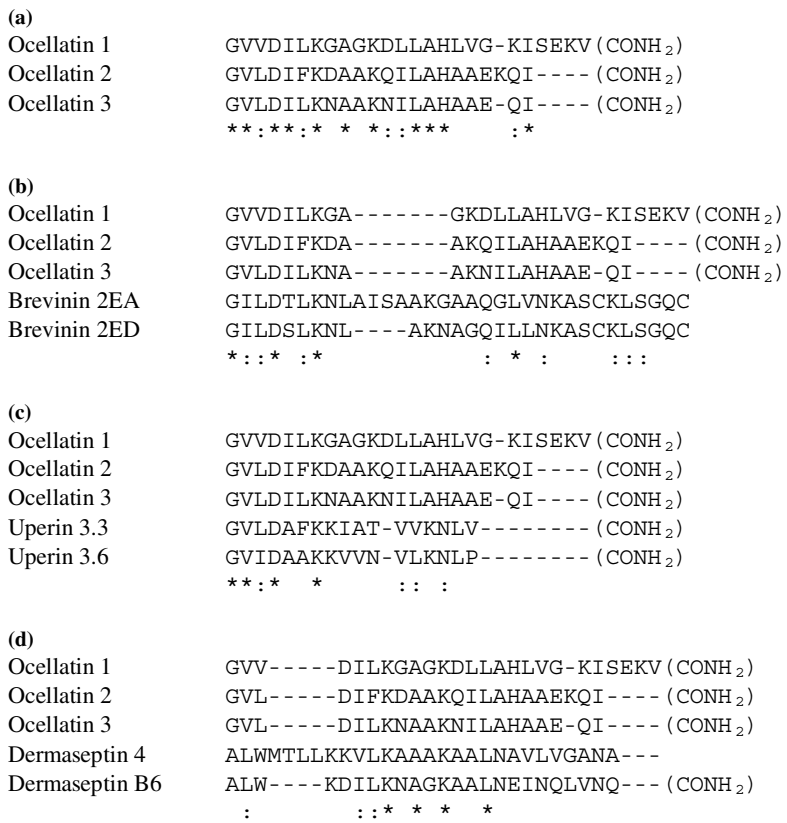

Fig. 3. (a) Amino acid sequence comparison between ocellatins; (b) between ocellatins and brevinins; (c) between ocellatins and uperins; and (d) between ocellatins and dermaseptins. Gaps were introduced to enhance similarities. The symbol "*" denotes identical residues and ":" indicates conserved substitutions.

Table 2. Percentages of identity between ocellatins and other antimicrobial peptides from different frog species

Peptides	Ocellatin 1	Ocellatin 2	Ocellatin 3
Ocellatin 1	100	44	48
Ocellatin 2	44	100	82
Ocellatin 3	48	82	100
Brevinin 2EA	27	24	30
Brevinin 2ED	34	38	41
Dermaseptin B6	31	25	33
Dermaseptin 4	29	21	21
Uperin 3.3	36	32	33
Uperin 3.6	24	18	29

The percentages of identities were obtained by pairwise alignment using Optimal Global alignment (similarity matrix: BLOSUM62).

concentrations used in our initial tests, since the following MICs have been found for other amphibian antimicrobial peptides: ranateurin 1 exhibits a MIC of 2 μM (Goraya *et al.*, 1998), esculentin 1ARa, 2 μM (Ali *et al.*, 2002) and pseudin 1, 4.5 μM (Olson *et al.*, 2001) against *E. coli.*

Antimicrobial peptides are, in general, of remarkable biotechnological interest because of their wide spectrum of action and their numerous potential applications. Currently, the main applications under evaluation concern the development of transgenic plants and animals resistant to pathogens (Hancock and Chapple, 1999) and the use as food preservers, topic antibiotics and antiseptics (Hancock and Lehrer, 1998; Ponti *et al.*, 2002). Furthermore, synthesized D-enantiomers of natural antimicrobial peptides have been shown to remain unaffected by degradation enzymes, while keeping their activity (Wade *et al.*, 1990), so there is the possibility of future systemic therapeutical use.

Though ocellatins are hemolytic, besides having antimicrobial properties, they are still of biotechnological interest. Understanding the characteristics of new peptides contributes to the improvement of antimicrobial drugs design and, most importantly, there is the possibility that substitutions of L-amino acids by D-amino acids can reduce the hemolytic

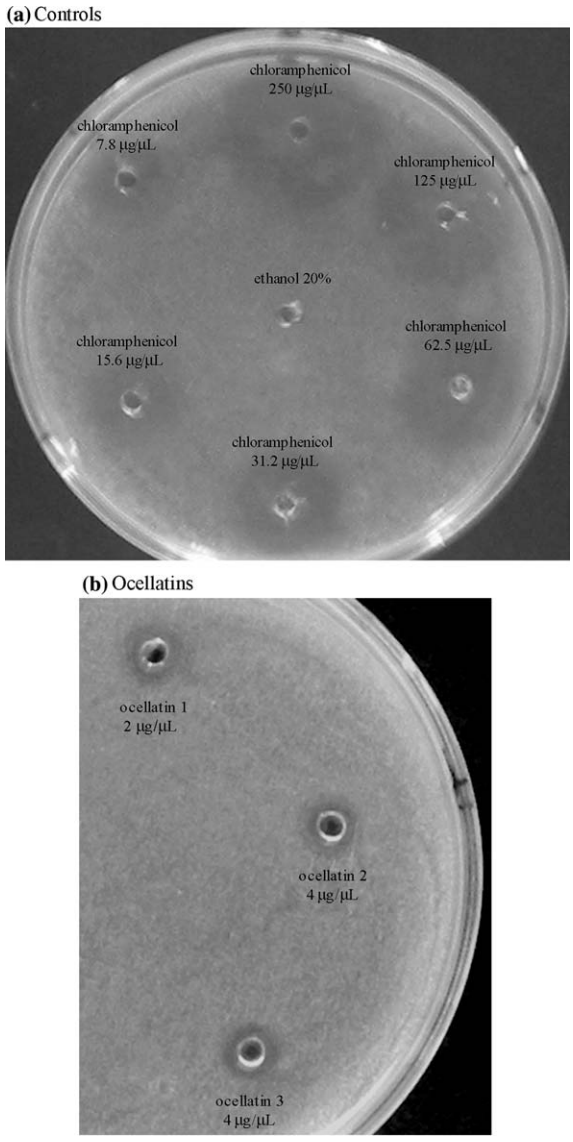

Fig. 4. Antimicrobial activity of ocellatins against *Escherichia coli* (ATCC 25922) using an *in vitro* inhibition zone assay on LB-agarose plates. Inhibition zones were evaluated after overnight incubation at 37°C. (a) Controls: chloramphenicol (250 μg/μl to 7.8 μg/μL) and ethanol 20% (v/v). (b) Ocellatins: 2 μg/μl of ocellatin 1 (0.8 m*M*); 4 μg/μl of ocellatin 2 (1.8 m*M*) and 4 μg/μl of ocellatin 3 (1.8 m*M*).

action while maintaining the microbicidal activity, as already reported for some peptides (Oren *et al.*, 1999).

4. CONCLUSION

This study describes the purification and characterization of three new antimicrobial peptides from the skin secretion of the Brazilian frog *L. ocellatus*, which we named ocellatins. This is the first report on antimicrobial peptides found in frogs of the family Leptodactylidae. The three studied peptides present hemolytic activity against human erythrocytes and also show inhibitory activity against the Gram-negative bacterium *E. coli*. They exhibit sequence similarity to other amphibian antimicrobial peptides already described. However, considering their structural features, ocellatins should be included in a new family. This fact evidences the richness of anuran skin secretion as a source of novel molecules with useful biological activities.

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REFERENCES

Ali, M. F., Lips, K. R., Knoop, F. C., Fritzsch, B., Miller, C., and Conlon, J. M. (2002). *Biochim. Biophys. Acta* **1601**: 55–63.

Altschul, S. F., Maden, T.L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., *et al.* (1997). *Nucleic Acids Res.* **25**: 3389–3402.

Barra, D., and Simmaco, M. (1995). *Trends Biotechnol.* **13**: 205–209.

Batista, C. V., da Silva, L. R., Sebben, A., Scaloni, A., Ferrara, L., Paiva, G. R., *et al.* (1999). *Peptides* **20**: 679–686.

Blondelle, S. E., Lohner, K., and Aguilar, M. I. (1999). *Biochim. Biophys. Acta* **1462**: 89–108.

Csordas, A., and Michl, H. (1969). *Toxicon.* **7**: 103–108.

Dathe, M., and Wieprecht, T. (1999). *Biochim. Biophys. Acta* **1462**: 71–87.

Erspamer, V. (1994). In: Heatwole, E. (ed.),*Amphibian Biology:Bioactive Secretions of the Amphibian Integument,* Surrey Beatty and Sons, NSW, Australia, pp. 190–292.

Feder, R., Dagan, A., and Mor, A. (2000). *J. Biol. Chem.* **275**: 4230–4238.

Fontes, W., Cunha, R. B., Sousa, M. V., and Morhy, L. (1998). *Anal. Biochem.* **258**: 259–267.

Goraya, J., Knoop, F. C., and Conlon, J. M. (1998). *Biochem. Biophys. Res. Commun.* **250**: 589–592.

Hancock, R. E. W., and Chapple, D. (1999). *Agents Chemother*. **43**: 1317–1323.
Hancock, R. E. W., and Lehrer, R. (1998). *Trends Biotechnol*. **16**: 82–88.
Hultmark, D., Engstrom, A., Andersson, K., Steiner, H., Bennich, H., and Boman, H. G. (1983). *EMBO J*. **2**: 571–576.
Hunt, D., Yates, J.R. III, Shabanowitz, J., Winston, S., and Hauer, C. R. (1986). *Proc. Natl. Acad. Sci. USA* **83**: 6233–6237.
Lohner, K., and Prenner, E. (1999). *Biochim. Biophys. Acta* **1462**: 141–156.
Mangoni, M.L., Grovale, N., Giorgi, A., Mignogna, G., Simmaco, M., and Barra, D. (2000). *Peptides* **21**: 1673–1679.
Matsuzaki, K. (1999). *Biochim. Biophys. Acta* **1462**: 1–10.
Mignogna, G., Simmaco, M., and Barra, D. (1998). *EXS* **85**: 29–36.
Mor, A., and Nicolas, P. (1994a). *Eur. J. Biochem*. **219**: 145–154.
Mor, A., and Nicolas, P. (1994b). *J. Biol. Chem*. **269**: 1934–1939.
Mor, A., Nguyen, V. H., Delfour, A., Migliore-Samour, D., and Nicolas, P. (1991). *Biochemistry* **30**: 8824–8830.
Morikawa, N., Hagiwara, K., and Nakajima, T. (1992). *Biochem. Biophys. Res. Commun*. **189**: 184–190.
Nascimento, A. C. C., Fontes, W., Sebben, A., and Castro, M. S. (2003). *Protein Pept. Lett*. **10**: 227–238.
Olson, L. III, Soto, A. M., Knoop, F. C., and Conlon, J. M. (2001). *Biochem. Biophys. Res. Commun*. **288**: 1001–1005.
Onuma, Y., Satake, M., Ukena, T., Roux, J., Chanteau, S., Rasolofonirina, N., *et al*. (1999). *Toxicon*. **37**: 55–65.
Oren, Z., and Shai, Y. (1998). *Biopolymers* **47**: 451–463.
Oren, Z., Hong, J., and Shai, Y. (1999). *Eur. J. Biochem*. **259**: 360–369.
Ponti, D., Mangoni, M. L., Mignogna, G., Simmaco, M., and Barra, D. (2002). *Biochem. J*. **370**: 121–127.
Shalev, D., Mor, A., and Kustanovich, I. (2002). *Biochemistry* **41**: 7312–7317.
Simmaco, M., Barra, D., Chiarini, F., Noviello, L., Melchiorri, P., Kreil, G., *et al*. (1991). *Eur. J. Biochem*. **199**: 217–222.
Simmaco, M., Mignogna, G., and Barra, D. (1998). *Biopolymers* **47**: 435–450.
Simmaco, M., Mignogna, G., Canofeni, S., Miele, R., Mangoni, M. L., and Barra, D. (1996). *Eur. J. Biochem*. **242**: 788–792.
Steinborner, S. T., Waugh, R. J., Bowie, J. H., and Tyler, M. J. (1997). *Rapid Commun. Mass Spectromet*. **11**: 997–1000.
Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). *Nucleic Acids Res*. **22**: 4673–4680.
Wade, D., Boman, A., Wahlin, B., Drain, C. M., Andreu, D., Boman, H. G., *et al*. (1990). *Proc. Natl. Acad. Sci. USA* **87**: 4761–4765.
Wong, H., Bowie, J. H., and Carver, J. A. (1997). *Eur. J. Biochem*. **247**: 545–557.
Zasloff, M. (1987). *Proc. Natl. Acad. Sci. USA* **84**: 5449–5454.