A COMPARISON BETWEEN HEAD AND BODY SKIN SECRETION IN CASQUE-HEADED TREE-FROGS AND CLOSELY RELATED SPECIES

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Corythomantis greeningi (Cg), *Aparasphenodon brunoi* (Ab), are casque-headed tree-frogs characterized by a flat and co-ossified head associated with defense against predators and/or dessication. *Itapotihyla langsdorfii* (Il), and *Trachycephalus venulosus* (Tv) are related to these two taxa, but do not have such a modified skull structure. It is known that Cg and Ab present fragmotic behavior, which consists in entering the body backwards into holes and using the head as a lid. Tv, as Cg, when harassed, releases a copious and sticky cutaneous secretion but, similarly to Il, does not present phragmotic behavior. In this work we compared the electrophoretic profiles of body and head secretions of the four species relating results to their natural history and phylogenetic relationships. All species showed differences between head and body secretions, but Ab was most clear in this respect, with several bands appearing only in the body secretion, particularly between 18 and 25 kDa, while Cg showed the larger similarity between body and head secretions, presenting specific 18 and 30kDa bands. Both Il and Tv present similar profiles in head and body with major characteristic bands around 60 kDa in Il and 45 kDa in Tv. A comparison among the electrophoresis profiles showed that the secretions of Cg and Ab share more bands, followed by Pv and, at last, Il. These results seem to be in accordance to the phylogenetic hypothesis of the casque-headed tree-frogs. It is noticeable that the species presenting the largest difference between body and head is the one presenting the most well characterized fragmotic behavior, living inside bromeliads in the sand bar.

KEY WORDS: Amphibia, Hylidae, casque-headed frogs, skin secretion

FINANCIAL SUPPORT: FAPESP, CNPq, FUNDAP

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ISOLATION AND CHARACTERIZATION OF A NOVEL BRADYKININ POTENTIATING PEPTIDE (BPP) FROM THE SKIN SECRETION OF *Phyllomedusa hypochondrialis* (DAUDIN,1802).

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Bradykinin potentiating peptides (BPPs) from *Bothrops jararaca* venom were first described in the middle of 1960s and were the first natural inhibitors of the angiotensin-converting enzyme (ACE). BPPs present a classical motif and can be recognized by their typical pyroglutamyl (Pyr) / proline rich sequences presenting, invariably, a proline residue at the C-terminus. In the present study, we describe the isolation and biological characterization of a novel BPP isolated from the skin secretion of the Brazilian tree-frog *Phyllomedusa hypochondrialis*. This new BPP, named Phypo Xa presents the sequence Pyr-Phe-Arg-Pro-Ser-Tyr-Gln-Ile-Pro-Pro and is able to potentiate Bradykinin activities *in vivo* and *in vitro*, as well as efficiently and competitively inhibit ACE. This is the first canonical BPP (i.e. Pyr-Aaan-Gln-Ile-Pro-Pro) to be found not only in the frog skin but also in any other natural source other than the snake venoms.

KEY WORDS: *Phyllomedusa hypochondrialis*, Bradykinin-Potentiating peptide, mass spectrometry, de novo sequencing, natural peptides, secretion, Bradykinin.

FINANCIAL SUPPORT: CAT/CEPID, FAPESP, CAPES, CNPq.

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EVALUATION OF THE GLANDULAR DISTRIBUTION OF A HIGHLY TOXIC PROTEIC FRACTION FROM *Leptodactylus pentadactylus* SKIN SECRETION, IN THE GRANULAR CUTANEOUS GLANDS OF ANURA

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Leptodactylus pentadactylus skin secretion is very rich on bioactive compounds. Our group had been purified a highly toxic proteic fraction (LP8) from this secretion and pharmacological assays (rats, i.v.) showed that it induces an acute decrease of the blood pressure, together with a inotropic positive effect upon heart, but without interfering on the heart frequency rate. Here we report a imounohystochemical investigation about glandular location of probable storage site of LP8 or precursor molecules, using polyclonal mouse IgG anti-LP8 antibody. Thus specific antibodies were assayed with 7 µm slices of dorsal skin of some anura, such as *Bufo jimmi*, *Scinax* sp. and *L. pentadactylus*, previously processed by hystological routine, after Boiun fixation. Reaction was started, first by incubation the slides with goat IgG anti-mice IgG linked to peroxidase, following by incubation with 3,3'-diaminobenzidine-hydrogen peroxide solution. We just found positive reaction on *L. pentadactylus* glandular tissue. In these preparations, positive reactions were observed on secretory granules located on pericentral zone of serous cutaneous glands, being absence on peripheral synthesis zone of these glands. Glands that released all their secretory products not presented any immunoreation. Discrete immunoreaction were observe near the secretory-contractile interface. Based on these results, we suggested that LP8 originates as a large proteic precursor, being storage on mature secretory granules after maturational processes, migrating to central alveolar zone of serous gland after process, in order to be ready to secretion events. The absence of immunoreaction on the other preparations of anuran skin lead us to believe that this toxin is a specific compound of *L. pentadactylus* serous gland secretion.

KEY WORDS: immunohistochemistry, *Leptodactylus pentadactylus*, anuran serous cutaneous gland

FINANCIAL SUPPORT: FUNCAP, CNPq, PRONEX

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ANTIFUNGAL ACTIVITY OF NEW PEPTIDES FROM SKIN SECRETION OF THE SOUTH AMERICAN FROG *Leptodactylus pentadactylus*

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Several animals like some plants defend themselves against invading pathogenic microorganisms producing cationic antimicrobial peptides. The magainins (*Xenopus laevis*) and dermaseptin (*Phyllomedusa sauvagei*) are exemple of antifungal peptides isolated from skin frog secretions. The aim of the present work was identify antifungal activity of compounds present in gland secretion of *L. pentadactylus* skin. Adult specimens of *L. pentadactylus* were collected in Paraipaba (Ceará, Brazil) and maintained in captivity. The secretion from their cutaneous glands was obtained by mild electrical stimulation (2-7 V), collected, lyophilized and kept at -25ºC. The secretion was purified by HPLC with a C18 column (25X250mm), eluted with a flow of 4,5ml/min and a 0-80% linear gradient of acetonitrile. Fractions were manually collected and lyophilized. The purified fractions were submitted to mass spectrometry analysis using MALDI-TOF technique. To verify antifungal activity, all fractions were tested by incubating with *Candida albicans*, *C. tropicalis* and filamentous fungi *Microsporum canis* and *Trichophyton rubrum* (Nat. Committee for Clinical Lab. Standards, 2002). No fractions exhibited antifungal activity against yeast *C. albicans* and *C. tropicalis*. However, incubations with filamentous fungi showed five fractions capable to inhibit 100% of the *in vitro* growth at millimolar level. The results show active peptides with 1268, 1476, 2066, 2008, 1747 and 1899 Da. The chemical characterization of these compounds is in progress. Understanding the structural design of these new peptides may contribute to the improvement of development of antifungal drugs for future therapeutic uses.

KEY WORDS: *Leptodactylus*, antifungal peptides, antimicrobial peptides

FINANCIAL SUPPORT: FUNCAP, CAPES, CNPq

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PURIFICATION AND PRIMARY STRUCTURE OF TWO ANTIMICROBIAL PEPTIDES FROM SKIN SECRETION OF *Leptodacytlus pentadacylus* (AMPHIBIAN: ANURA)

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The potency of a particular peptide against microorganisms is determined by a complex interaction between factors relating to conformation cationicity, relatively hydrophobic, and have propensity to form an amphipathic α-helice in membrane-mimetic environment. The present study describes the purification and the structural and biological characterization of two peptides with antimicrobial activity from skin secretions of *L. pentactylus*. Adult specimens of *L. pentadctylus* were collected in Paraipaba (Ceará, Brazil) and maintained in captivity. The secretion from their cutaneous glands was obtained by mild electrical stimulation (2-7V), collected in a becker freezing, lyophilized and kept at -25ºC. The antimicrobial peptides were purified by HPLC with a C18 column (25X250mm), eluted with a flow of 4,5ml/min and using a 0-80% linear gradient of acetonitrile. The molecular mass was analyzed by ESI-Q-TOF Ultima API (Micromass). The purification assay resulted in the isolation of two fractions with molecular mass of 2275,34 and 2208,34 Da. The primary structure of new pentdacytins 1 and 2 were established with ESI-Q-TOF as GVLDLLKGAAKDLAGHLASKALD-NH_2 and GVLDLLKGAAKNVVGLASKALD-NH_2. A search using non-redundant database by CLUSTALW revealed 70% of homology to amphibian antimicrobial peptides pentadactylins. Prediction of the secondary structure indicates that these peptides have the propensity to adopt an extended α-helice conformation from residue (3-22).

KEY WORDS: *Leptodactylus*, antimicrobial peptides

FINANCIAL SUPPORT: FUNCAP, CAPES, CNPq

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ISOLATION AND CHARACTERIZATION OF NEW BRADYKININ RELATED PEPTIDES FROM THE SKIN SECRETIONS OF PHYLLOMEDUSA HYPOCHONDRIALIS

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The amphibian skin secretions may provide new molecules with biotechnological applications, which should play different roles, either in the regulation of physiological functions of the skin or in the defense against predators or microorganisms. We targeted the identification of novel peptides with bradykinin-like structure present in the skin of this frog. Glandular secretions of *P. hypochondrialis* specimens were purified by rp-HPLC after solid phase extraction. MS fingerprinting was performed by MALDI-MS. Peptides were de novo sequenced on a ESI-Q-TOF-MS/MS. Peptides were synthesized by employing the Fmoc-strategy. BK activity was assessed by guinea-pig ileum contraction and by intravital microscopy. First, the SPE-processed sample was fractionated into 29 pools. The fractions were monitored by MALDI-ToF mass spectrometry for evaluation of molecular masses and purification grade. These pools were screened for low molecular weight peptides (700 to 1100 Da) with the aid of ESI-Q-TOF mass spectrometry. Several peptides were sequenced and three of them presented sequences that slightly resemble that of Bradykinin. These peptides were sequenced and identified as KPLWRL-NH2 (Phypo 3), RPLSWLPK (Phypo 5) and VPPKGVSM (Phypo 7a). Bradykinin-like peptides were chosen for the direct administration of the crude skin secretion cause several effects on the circulatory system, including inflammation.

KEY WORDS: Amphibian; Bradykinin; *Phyllomedusa hypochondrialis*

SUPPORTED BY: CNPq, CAPES and FAPESP.

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ISOLATION AND CHARACTERIZATION OF A NOVEL BRADYKININ POTENTIATING PEPTIDE FROM THE SKIN SECRETION OF *Phyllomedusa hypochondrialis*

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Bradykinin is often associated to inflammatory processes. Classical studies showed that there existed a factor in the venom of *B. jararaca* which potentiated bradykinin. This factor (BPPs) could inhibit the angiotensin-converting enzyme. We describe the isolation and biochemical characterization of a novel BPP, from the skin of *P. hypochondrialis*. Glandular secretions of *P. hypochondrialis* specimens were purified by rp-HPLC after solid phase extraction. MALDI-MS fingerprinting was performed and peptides were sequenced on a ESI-Q-TOF-MS/MS. BK potentiation was assessed by guinea-pig ileum contraction and by MAP measurements on anesthetized rats. We were able to purify and characterize a novel BPP from the skin secretion of *P. hypochondrialis* that was able to inhibit ACE, lower the blood pressure and potentiate smooth muscle contraction. The skin secretions of the members of the Phyllomedusinae frog family are widely known to be one the richest sources of bioactive peptides. This novel peptide has a typical BPP motif. Moreover, *in vivo* and *in vitro* assays were able to clearly demonstrate that this peptide meets all the functional requirements for a bioactive BPP. And mostly important, physiologically, at the level of the frog skin and its mechanisms of defense, the presence of BPPs can make all the well documented BKs and BRPs extremely powerfull weapons.

KEY WORDS: Amphibian; Bradykinin potentiating peptide; *Phyllomedusa hypochondrialis*

FINANCIAL SUPPORT: FAPESP, CAPES and CNPq.

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PHOSPHOLIPASIC ACTIVITY IN SKIN SECRETION OF AMAZONIAN ANURAN (AMPHIBIA) SPECIES

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Peptides (≤14KDa), alkaloids and steroids show several biological activities with biotechnological applications are principal compounds in anuran skin secretion. Skin secretion enzymes of Amazonian anuran species were not studied yet. The aim of this work was detect phospholipasic activity in skin secretion of amazonian anuran species. Specimens were collected in Manaus region and skin secretions were obtained by low voltage electric stimulation from dorsum skin glands. Skin secretions were lyophilized to be used in experiments. Protein molecular masses profiles of skin secretions was done using SDS-PAGE system. The phospholipasic activity was detected using 3% egg yolk incorporated in 1% agarose plate gel method in buffer PBS 0,10 mM pH 8,1 and 0,09 mM CaCl2. Immunological assays were performed with polyclonal antibodies against elapidae and crotalinae snake venoms. *Bothrops atrox* venom (10 µg) was used as phospholipase activity control. *Phyllomedusa bicolor* and *P. tarsius* showed phospholipasic activity. SDS-PAGE profile of both samples showed proteins bands between 24 kDa and 50 kDa range. Phospholipasic activity of P. tarsius sample (60 µg) was six times less potent that *B. atrox venom* and *P. bicolor* sample (90 µg) showed very low phospholipasic activity. Immunological tests showed no neutralization phospholipasic activity and non ELISA crosses reactions with polyclonal antibodies snake venons. The molecular characterization of proteins with phospholipasic activity is in course. Our data show new compounds in Amazonian anuran skin secretions with possible biotechnological relevance but without possible immunological relationships with elapidae and crotalinae toxins.

KEY WORDS: Amazonia, Anura, Skin Secretion, Phospholipase, Biotechnology.

FINANCIAL SUPPORT: CAPES, FMTAM, FINEP, FAPEAM

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NEW BRADYKININ POTENTIATING PEPTIDES (BPPS) FROM *Phyllomedusa*: STUDY *in vivo* AND *in vitro*

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More than 40 years ago bradykinin potentiating peptides (BPPs) isolated of the venom of the poisonous brazilian pitviper *Bothrops jararaca* were described and characterized as the first natural inhibitors of the angiotensin-converting enzyme (ACE). The BPPs were essentials for the development of the Captopril, the first active site directed inhibitor of the ACE currently used clinically for cardiovascular dysfunction treatment. Now, BPPs have also been isolated from other snakes or other toxins as well. Typically, BPPs can be recognized by their pyroglutamyl proline-rich oligopeptide sequences of 5-14 amino acid and presenting a proline residue at the C-terminus. In this study, two novel BPPs isolated from amphibians were assayed by their ability to potentiate bradykinin (BK) response, on the isolated guinea pig ileum, and on mean arterial blood pressure (MAP) in anesthetized spontaneously hypertensive rats (SHR). BPP isolation was done as follows: 1g of the venom of *Phyllomedusa* was dissolved in 80% ethanol (V/V), centrifuged at 10.000g during 30 min and the supernatant was eluted with HPLC-grade water using a linear gradient of 0-60% acetonitrile on a C18 column and named BPPI and BPPII. BPPs (30 ng/mL) showed to be able to potentiate the contractile activity of the isolated guinea pig ileum due to a single dose of bradykinin. BPP I: 772% ± 27,7%, BPP II: 410% ± 11,0%. Additionally, BPPs potentiated BK effect on MAP of SHR rats. The percentual potentiation of BK-induced (0.5 mg.Kg-1) decrease in MAP after 5 min intravenous bolus admnistration of BPPs (0.3, 3 or 30 mg.Kg-1) were: BPP I - SHR rats: 26,1% ± 14,9%; 38,7% ± 10,8%; 61,5% ± 8,5%, respectively. BPP II – SHR rats: 26,9% ± 8,9%; 38,7% ± 8,1% and 77,3% ± 4,6%, respectively.

KEY WORDS: bradykinin potentiating peptides, hypertension.

FINANCIAL SUPPORT: FUNCAP, CNPq

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INDUCTION OF ANTIMICROBIAL PEPTIDE SYNTHESIS IN *Leptodactylus pentadactylus* (ANURA, AMPHIBIA) SKIN, BY MECHANICAL, CHEMICAL, AND MICROBIAL STIMULUS

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The anuran granular skin glands synthesize and store polypeptides with a broad spectrum of antimicrobial activity that play an important role in the innate immunity and constitute the first-line defense against invading pathogens. The aim of this work was verify the influence of mechanical, chemical and microbial stimuli in the production of antimicrobial peptides in the skin of the anuran *L. pentadactylus*. Thus, adult specimens had remained in captivity under space and nutricional conditions adjusted separately and submitted to different treatments, such as: i) light cuts in the skin, ii) subcutaneous injections of incomplete Freud Adjuvant (200 μl), iii) subcutaneous injections of *Candida* sp. suspensions (200 μl), and iv) subcutaneous injections of *Pseudomonas aeruginosa* (200 μl). After 3, 7 and 14 days the animals were submitted to electric stimulation (2-7V) for extraction of the cutaneous secretions, that were collected, lyophilized, weighed and kept -20ºC. Aliquots of these samples were purified by HPLC using a C18 column (25X250mm), eluted with 0-80% linear gradient of acetonitrile at a flow of 0,8ml/min. The analysis of the chromatographic profiles with all the treatments used shows a significant increment of the peaks at the region correspondent to elution with 30-50% of acetonitrile. The analysis of mass spectrometry of these fractions indicated the presence of peptides with molecular mass of 1.500 to 25.000 Da, presenting antimicrobial activity against *Pseudomonas aeruginosa.* These results suggest that *L. pentadactylus* cutaneous secretory machinary is activate in response to aggressive stimuli by inespecific manner, in agreement with the innate immune pathway on these animals.

KEYWORDS: *Leptodactylus*, peptides, stimulation

FINANCIAL SUPPORT: FUNCAP, CAPES, CNPq

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A NEW GLYCINE-RICH ANTIMICROBIAL PEPTIDE FROM SKIN SECRETION OF THE SOUTH AMERICAN FROG *Leptodacytlus pentadactylus*

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Skin granular glands of anurans species (frog and toad) synthesize and store polypeptides with a broad spectrum of antimicrobial activity that play an important role in the innate immunity and constitute the first-line defense against invading pathogens. This study describes the purification and the partial sequence of a new peptide with bacterial growth inhibitory activity present in high concentration in skin secretion from *L. pentadactylus*. Adult specimens of *L. pentadactylus* were collected in Paraipaba (Ceará, Brazil) and maintained in captivity. The secretion from their skin was obtained by electrical stimulation (2-7V), collected in a beaker freezing and immediately lyophilized. The antimicrobial peptide (Lpg) was purified by HPLC with a C18 column (25X250mm), eluted with a flow of 4,5ml/min and using a 0-80% linear gradient of acetonitrile. The molecular mass was analyzed by ESI-Q-TOF Ultima API (Micromass). In order to verify antimicrobial activity, Lpg was tested against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*. The results showed that Lpg is capable to inhibit the growth bacterial of *Pseudomonas aeruginosa* at 200mM level. The analyzed compound is a 1762 Da peptide with 22 amino acid residues (60% glycine, 36% leucine and 4% of praline). This frog peptide should be classified into category of glycine-rich antimicrobial peptides. Structurally it is similar to acanthoscurrin, a peptide antimicrobial isolation of tarantura spider *Acanthoscurria gomesiana*. The results suggest that LPG may contribute to the improvement of a new antibacterial drug design for future therapeutic uses.

KEY WORDS: *Leptodactylus*, peptide glycine-rich, antimicrobial activity

FINANCIAL SUPPORT: FUNCAP, CAPES, CNPq

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