Peptides 30 (2009) 888–892

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

A glycine-leucine-rich peptide structurally related to the plasticins from skin secretions of the frog *Leptodactylus laticeps* (Leptodactylidae)

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ARTICLE INFO

Article history:
Received 25 November 2008
Accepted 9 January 2009
Available online 22 January 2009

Keywords:
Frog skin
Leptodactylidae
Phyllomedusinae
Plasticin
Ocellatin

ABSTRACT

A glycine-leucine-rich peptide was isolated from norepinephrine-stimulated skin secretions of the Sante Fe frog *Leptodactylus laticeps* (Leptodactylidae) whose primary structure (Gly-Leu-Val-Asn-Gly-Leu-Leu-Ser-Ser-Val-Leu-Gly-Gly-Gly-Gln-Gly-Gly-Gly-Gly-Leu-Leu-Gly-Gly-Ile-Leu) contains the $(GXXXG)_3$ motif found in the plasticins, previously identified only in phyllomedusid frogs (Hylidae). Circular dichroism studies showed that the secondary structure of the peptide, termed plasticin-L1, was markedly solvent-dependent displaying a random coil conformation in water, a β-sheet structure in methanol, and an α-helical conformation in 50% trifluoroethanol–water. A synthetic replicate of the peptide did not inhibit the growth of *Escherichia coli* or *Staphylococcus aureus* or lyse human erythrocytes at concentrations up to 500 μM. At relatively high concentrations (≥1 μM), the peptide produced a significant ($P < 0.05$), although modest (139% of basal rate at 3 μM), increase in the rate of glucose-induced release of insulin from rat clonal BRIN-BD11 β cells without increasing the rate of release of lactate dehydrogenase. A peptide, termed ocellatin-L2 was also identified in the skin secretion that was identical to the previously described ocellatin-L1 except for the substitution $Asn^{23} \rightarrow Asp$. Ocellatin-L2 was devoid of antimicrobial and hemolytic activity but also showed significant activity in stimulating insulin release from BRIN-BD11 cells (181% of basal rate at 3 μM).

1. Introduction

The skins of anurans (frogs and toads) constitute an important source of biologically active peptides with potential for development into therapeutically valuable pharmaceutical agents [6,26]. Cationic α-helical peptides with broad-spectrum antibacterial and antifungal activities are synthesized in the skins of many, but by no means all, anuran species and represent a component of the animal's system of innate immunity [19]. South American frogs belonging to the Hylidae family represent a particularly rich source of such peptides that may be grouped together in families on the basis of limited structural similarity [3]. A recent comprehensive review [25] has identified six such families – dermaseptins, dermatoxins, hyposins phylloseptins, phylloxins, and plasticins – in skin secretions from species belonging to the genera *Agalychnis*, *Hylomantis*, *Pachymedusa,* and *Phyllomedusa*.

According to recently proposed taxonomic recommendations [15], the extensive family Leptodactylidae (at least 95 species) has been divided into four genera: *Hydrolaetare*, *Leptodactylus*, *Paratelmatobius*, and *Scythrophrys* but both morphological [18] and molecular [16] investigations have indicated that the extensive genus *Leptodactylus* (currently 85 species) may not constitute a monophyletic group. The South American Santa Fe frog (also known as the red-spotted burrowing frog) *Leptodactylus laticeps* Boulenger, 1918 is a large, predominantly nocturnal animal that is found in regions of Argentina, Bolivia, and Paraguay. Regrettably, the species is in great demand by the pet trade with the result that numbers in the wild are becoming severely depleted. A previous study [8] has described the isolation from norepinephrine-stimulated skin secretions of *L. laticeps* of a peptide with a selective ability to inhibit the growth of Gram-negative bacteria that was originally termed laticeptin but, following recent recommendations for a systematic nomenclature [7], has been

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0196-9781/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2009.01.008

renamed ocellatin-L1. The current study has re-examined these skin secretions for the presence of additional low molecular mass ($M_r < 4000$) peptides that may possess biological activity. A peptide has been identified that shows structural similarity to members of the plasticin family, previously identified only in phyllomedusid frogs belonging to the Hylidae family [3,25], together with a second peptide with structural similarity to ocellatin-L1.

2. Materials and methods

2.1. Collection of skin secretions

The collection of norepinephrine-stimulated skin secretions from adult specimens of *L. laticeps* ($n = 3$) at Denver Zoo (Denver, CO) and partial purification on Sep-Pak C-18 cartridges have been described in detail previously [8].

2.2. Peptide purification

The lyophilized skin secretions, after partial purification on Sep-Pak cartridges, were redissolved in 0.1% (v/v) trifluoroacetic acid (TFA)/water (2 ml) and injected onto a (1.0-cm × 25-cm) Vydac 218TP510 (C-18) reversed-phase HPLC column (Separations Group, Hesperia, CA) equilibrated with 0.1% (v/v) TFA/water at a flow rate of 2.0 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min and to 63% (v/v) over 60 min using linear gradients. Absorbance was monitored at 214 and 280 nm and fractions (1 min) were collected. Freeze-dried aliquots (50 μl) of the fractions were analyzed by mass spectrometry as described in Section 2.3.

The peptide subsequently shown to be plasticin-L1 (peak 4 in Fig. 1) was purified to near homogeneity by sequential chromatography on (1.0-cm × 25-cm) Vydac 214TP510 (C-4) and (1.0-cm × 25-cm) Vydac 219TP510 (phenyl) columns. The concentration of acetonitrile in the eluting solvent was raised from 21% to 56% over 50 min and the flow rate was 2.0 ml/min. Ocellatin-L2 (peak 3 in Fig. 1) was purified under the same conditions of chromatography.

2.3. Structural characterization

The primary structures of the peptides were determined by automated Edman degradation using a model 494 Procise sequenator (Applied Biosystems, Foster City, CA). MALDI-TOF MS was carried out using a Voyager DE-PRO instrument (Applied Biosystems) that was operated in reflector mode with delayed extraction and the accelerating voltage in the ion source was 20 kV. The instrument was calibrated with peptides of known molecular mass in the 2000–4000 Da range. The accuracy of mass determinations was ±0.02%.

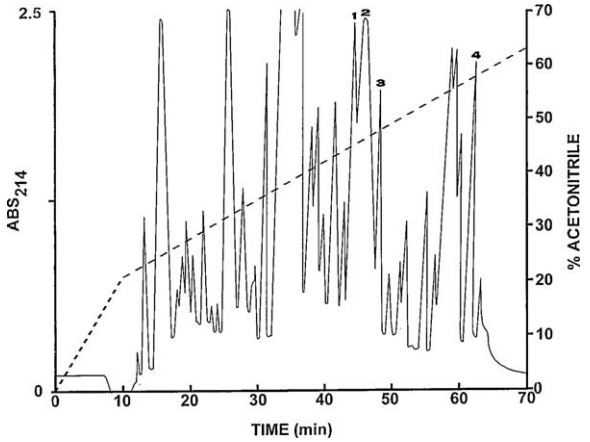

Fig. 1. Reversed-phase HPLC on a semipreparative Vydac C-18 column of skin secretions from *L. laticeps* after partial purification on Sep-Pak cartridges. Peak 4 contained plasticin-L1 and peak 3 contained ocellatin-L2. Peak 1 was previously shown to contain ocellatin-L1-(1-22)-peptide and peak 2 contained ocellatin-L1 [8]. The dashed line shows the concentration of acetonitrile in the eluting solvent.

2.4. Peptide synthesis

Synthetic plasticin-L1 and ocellatin-L2 were supplied in crude form by GL Biochem Ltd. (Shanghai, China) and were purified to near homogeneity by reversed-phase HPLC on a (2.2-cm × 25-cm) Vydac 218TP1022 (C-18) column equilibrated with acetonitrile/water/trifluoroacetic acid (21.0/78.9/0.1, v/v/v) at a flow rate of 6 ml/min. The concentration of acetonitrile was raised to 56% (v/v) over 60 min using a linear gradient. Absorbance was measured at 214 and 280 nm and the major peak in the chromatogram was collected manually. The monoisotopic molecular masses of the peptides determined by electrospray mass spectrometry were consistent with the masses calculated from the proposed structure. The purity of the peptides were >98%.

2.5. Determination of cytolytic activity

Minimum inhibitory concentrations (MIC) of the synthetic peptides against *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25726) and hemolytic activites (LD_{50}) against human erythrocytes from a healthy donor were measured as previously described [8,20,27].

2.6. Determination of insulin-releasing activity

The effect of peptides upon the release of insulin from rat clonal BRIN-BD11 β cells in the presence of 5.6 mM glucose was measured as previously described in detail [1,2]. Incubations ($n = 8$) with synthetic plasticin-L1 (10^{-9} to 3×10^{-6} M) or synthetic ocellatin-L2 (10^{-9} to 3×10^{-6} M) were carried out for 20 min at 37 °C. The effect of the insulin-releasing amino acid alanine (10 mM) was determined as a positive control. In order to determine cytotoxicity, BRIN-BD11 cells were incubated with synthetic peptides (10^{-7} to 3×10^{-6} M) for 20 min at 37 °C and the concentration of lactate dehydrogenase (LDH) in the cell supernatants was measured using a CytoTox96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI) as described [1,2]. Results are expressed as mean ± S.E.M. and values were compared using ANOVA followed by Newman–Keuls post hoc test. Groups of data were considered to be significantly different if $P < 0.05$.

2.7. Circular dichroism studies

The electronic circular dichroism (CD) spectra of the peptides (0.5 mg ml^{-1}) were recorded at 20 °C in (a) water, (b) 100% methanol, and (c) 50% (v/v) trifluoroethanol (TFE)–water using a CD6 spectropolarimeter (Jobin Yvon, Longjumeau, France) as previously described [9,10]. Mean residue ellipticity values [θ] were calculated as described [9].

3. Results

3.1. Purification of the peptide

The skin secretions, after concentration and partial purification on Sep-Pak cartridges, were chromatographed on a Vydac C-18 semipreparative reversed-phase HPLC column (Fig. 1). Analysis of the fractions by mass spectrometry indicated that Peak 4 contained