Amino Acids (2011) 40:113–122
DOI 10.1007/s00726-010-0564-9

ORIGINAL ARTICLE

Peptidomic analysis of the skin secretions of the frog *Pachymedusa dacnicolor*

Erika P. Meneses · Oscar Villa-Hernández · Lorena Hernández-Orihuela · Ruben Castro-Franco · Victoria Pando · Manuel B. Aguilar · Cesar Vicente Ferreira Batista

Received: 15 January 2010 / Accepted: 10 March 2010 / Published online: 30 March 2010

Abstract High-resolution mass spectrometry-based peptidomics has been used to characterize several components in electro-stimulated skin secretions of the endemic Mexican frog *Pachymedusa dacnicolor*. Peptide mass screening performed in an Orbitrap-XL mass spectrometer showed that *P. dacnicolor* skin secretions possess 194 different components with molecular masses ranging mainly from 500 to 6,000 Da. Dozens of molecules were partially sequenced including two novel protease inhibitors. Additionally, one posttranslationally modified bradykinin and two novel dermaseptin-like antimicrobial peptides were fully sequenced. The novel peptide named here DMS-DA5 was fully characterized and showed potent antibacterial activity against various bacteria such as *Escherichia coli*, *Bacillus subtilis*, *Salmonella enterica* serovar *typhimurium*, and *Pseudomonas aeruginosa* with minimal inhibitory concentrations from 3.10 to 25.0 μM.

Keywords Frog skin · Antibacterial peptides · Peptidomics · Mass spectrometry

E. P. Meneses · O. Villa-Hernández · L. Hernández-Orihuela · C. V. F. Batista (✉)
Unidad de Proteómica-Instituto de Biotecnología, Universidad Nacional Autónoma de México (UNAM), Av. Universidad, 2001 Col. Chamilpa, CP 62210 Cuernavaca, Morelos, Mexico
e-mail: fbatista@ibt.unam.mx

R. Castro-Franco
Lab. Herpetología, Centro de Investigaciones Biológicas, UAEM, Av. Universidad 1001, Col. Chamilpa, CP 62209 Cuernavaca, Morelos, Mexico

V. Pando
Centro de Investigación Sobre Enfermedades Infecciosas, Instituto Nacional de Salud Pública, Av. Universidad No. 655. Col. Santa María Ahuacatitlán, Cerrada Los Pinos y Caminera, CP 62100 Cuernavaca, Morelos, Mexico

M. B. Aguilar
Laboratorio de Neurofarmacología Marina, Departamento de Neurobiologia Celular y Molecular, Instituto de Neurobiología, UNAM, Campus Juriquilla, Boulevar Juriquilla 3001, 76230 Juriquilla, Qro., Mexico

Introduction

Multi-nucleated cells of amphibian skin synthesize a wide range of bioactive molecules with potential for development of pharmaceutical agents (Conlon 2004). The most commonly found molecules in the skin secretions of frogs are amphipathic peptides that form part of the animals' innate immune system [Conlon et al. 2009]. More than half of the 900 different eukaryotic gene-encoded antimicrobial peptides described to date have been isolated from frog skin (Nicolas and El Amri 2009). These peptides possess diverse structures that differ in amino acids, the presence of disulfide bridges, sequence extensions and consequently in molecular masses. Secondary structures of antimicrobial peptides (AMP) are classified into four major groups: α-helical, β-sheet, looped, and extended peptides (Hancock and Lehrer 1998). Permeabilization of negative-charged bacterial membranes by cationic AMPs results from pore formation by means of the widely accepted mechanistic models of action such as carpet, barrelstave, toroidal pore, and detergent-type membrane lytic mechanisms, all of which involve conformational changes and oligomerization (Shai 1999; Gottler and Ramamoorthy 2009; Mahalka and Kinnunen 2009). *Pachymedusa dacnicolor* is an orphan species of frog belonging to the Phyllomedusinae sub-family that comprises 59 species of the 869 species in the Hylidae family (Frost 2009). Consistent nomenclature for peptides isolated

from the Phyllomedusinae sub-family was proposed recently by Amiche et al. (2008) based on 80 different peptide sequences, which were aligned and classified into seven distinct families: dermaseptin, phylloseptin, plasticin, dermatoxin, phylloxin, hyposin, and orphan peptides. AMPs from the skin secretions of the frog *P. dacnicolor* have been reported previously. To date, primary structures of six dermaseptin-like peptides (Wechselberger 1998; Nicolas and Rosenstein 2009) were determined as well as two tryptophyllins [Chen et al. 2004; Wang et al. 2009] and one dermorphin (Wechselberger 1998). AMPs belonging to the dermaseptin family have a broad spectrum of activity against mollicutes, bacteria, fungi, protozoa, yeast, and enveloped viruses (Nicolas and El Amri 2009). Although a number of studies performed with frog skin secretions exist in the current literature, little information is available regarding the exact number and molecular mass of all components in each species. Here we present the first complete molecular mass screening of the electro-stimulated skin secretion components of the frog *P. dacnicolor*. Molecular masses were determined by high-resolution mass spectrometry using a hybrid Orbitrap-XL mass spectrometer with experimental errors lower than 5 ppm for molecular weights typical of dermaseptin-like peptides. In addition, many fractions were separated by HPLC, digested with trypsin, and subjected directly to fragmentation using collision-induced dissociation (CID) and high energy collision dissociation (HCD), allowing the partial characterization of dozens of components. Also, five peptides were purified to homogeneity and fully sequenced by means automatic Edman degradation and by the mass spectrometry dissociation methods previously described. Finally, the characterized peptides were tested in antimicrobial assays against various bacteria, and the peptide DMS-DA5 showed potent activity against all bacteria tested.

Materials and methods

Collection of skin secretions

Adults of the frog species *P. dacnicolor* were collected in the state of Morelos, Mexico, and the extraction was performed in situ by electro-stimulation. After extraction the animals were released to the field in a healthy state. The secretions were pooled, acidified by addition of trifluoroacetic acid (TFA) and immediately frozen for shipment to the UNAM laboratory, where they were maintained at −20°C until processing.

Peptide purification

One milligram of the lyophilized skin secretions was dissolved in 1 mL of TFA/water 0.1% (v/v) and centrifuged at 15,000 rpm for 10 min. Only the soluble part of the secretion was analyzed, assuming that the minor amount of precipitable material usually corresponds to cellular debris from the electro-stimulation procedure. The supernatant (100 µl) was loaded onto an analytical Vydac C18 RP column (Hisperia, CA, USA) using a linear gradient from 0% buffer A (0.1% TFA/water) to 60% buffer B (0.1% TFA/acetonitrile) run for 60 min at a flow rate of 1.0 ml/min. Elution of the peptides was monitored by UV detection at 230 nm. All fractions were collected manually and evaporated using a Speed Vac Savant from ThermoFisher Co (San Jose, CA, USA). Some components were repurified as described above except using shallower HPLC gradients.

Molecular mass screening

All fractions separated by HPLC were submitted to molecular mass determination using a hybrid Orbitrap-XL mass spectrometer from ThermoFisher Co (San Jose, CA, USA) with a nano-electrospray ionization source. Automatic and manual deconvolutions were performed to determine the isotopic and average molecular mass composition of the components. Automatic deconvolutions were performed by the X-tract tool, part of the Xcalibur software. Concomitant with the molecular mass determinations, different dissociation methods were used to obtain structural information as described below.

Enzymatic digestion

HPLC fractions were digested in solution (50 mM ammoniun biocarbonate) with trypsin from Promega (Fitchburg, MA, USA). Although dermaseptin-like peptides contain no cysteine residues, samples were previously reduced with (di-thiothreitol) DTT and alkylated with iodoacetamide. The mixture of tryptic peptides was desalted with ZipTip C18 from Millipore (Billerica, MA, USA) and analyzed by mass spectrometry.

Structural characterization

The primary structures of the peptides were determined by automatic Edman degradation using a 491 Procise Sequenator from Applied Biosystems (Foster City, CA, USA). In parallel, CID and HCD, performed in an Orbitrap-XL mass spectrometer from ThermoFisher Co, were used to sequence the samples. The FTMS full scan MS spectra (from 350 to 2,000 *m/z*) were acquired with a resolution of $r = 30{,}000$. CID experiments were performed manually by the TunePlus program into the linear ion trap, and the product ion signals were recorded after Fourier transformation of the image currents generated by *m/z*

oscillatory frequencies in the Orbitrap mass analyzer. The basic parameter settings were: normalized collision energy 25–35% of arbitrary units, activation Q 0.25, electrospray voltage 1.7 kV, capillary temperature 160°C, and isolation width 2.00. HCD experiments were performed into the octapole electrode by using specific fragmentation energy of 25 arbitrary units, and the product ions were analyzed by the Orbitrap mass analyzer, as described above for CID. All fragmentation spectra were analyzed manually.

Antimicrobial assays

The antimicrobial activity of the homogeneous peptides was determined by the micro-dilution method (NCCL 1997). Minimal inhibitory concentrations (MICs), defined as the maximum dilution of the product that inhibited the growth of a test microorganism, were determined by serial dilutions of the peptides in LB bacterial growth media deposited in sterile, 96-well plates. Briefly, aliquots (100 µl) of a bacterial suspension at 2×10^6 colony-forming units (CFU)/ml were added to 100 µl of the peptide solution (serial twofold dilutions) and incubated overnight. Bacteria tested were: *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli* DHSα, and *Salmonella enterica* serovar *typhimurium* STM14028.

Results

Chromatographic isolation and peptide mass fingerprinting of the *P. dacnicolor* skin secretion components

Several 1-mg aliquots of total skin secretions of the endemic Mexican frog *P. dacnicolor* were independently fractionated by RP-HPLC into 45 detectable fractions, as shown in the Fig. 1. Some components were further purified prior to complete sequencing. All fractions were subjected to high-resolution molecular mass determination and concomitant fragmentation using a nano-spray Orbitrap mass spectrometer in order to obtain initial structural information. Table 1 shows the retention times of the HPLC fractions (left column) and the corresponding 194 unique molecular masses (right column) determined. Molecular masses with values below 2.0 kDa are reported as monoisotopic masses and values higher than 2.0 kDa as average molecular masses. The number and values of molecular masses found are represented in the histogram shown in the Fig. 2. Components with molecular masses from 2.0 to 3.5 kDa were the most abundant in the skin secretion of the frog *P. dacnicolor*, suggesting a high occurrence of dermaseptin-like peptides.

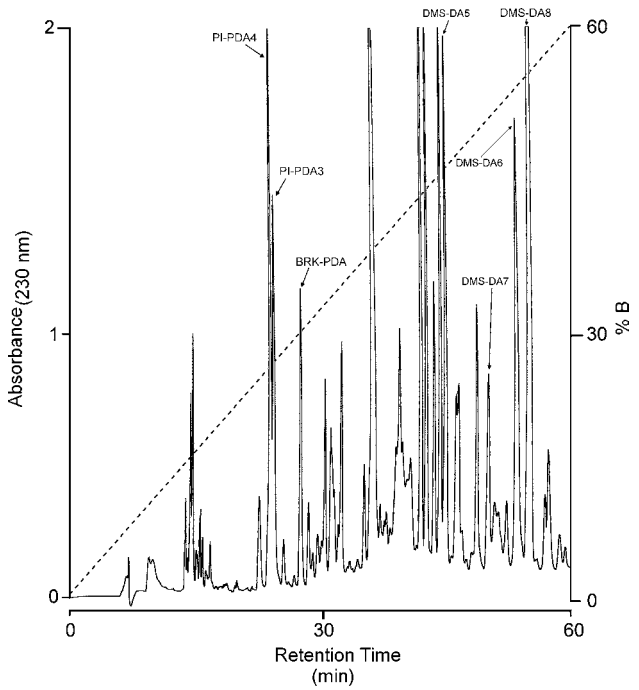

Fig. 1 RP HPLC purification of crude skin secretions of the frog *P. dacnicolor*. HPLC separation of soluble secretions from *P. dacnicolor* in a C18 RP column eluted at a flow rate of 1 mL/min for 60 min with a linear gradient of solvent A (0.12% TFA in water) to 60% of solvent B (0.10% TFA in acetonitrile). Components labeled *PI-PDA4* and *PI-PDA3* correspond to partially sequenced protease inhibitors. *BRK-PDA* (bradykinin) and *DMS-DA5* to *DMA-DA8* are fully sequenced dermaseptins

Structural characterization

A component eluting with a retention time (RT) of 42.50 min (Fig. 1) and possessing average and monoisotopic molecular masses of 2,872.41 and 2,870.58 Da, respectively, was selected for chemical characterization due to its primary structure similarity with other known dermaseptins. This component was named DMS-DA5 following the suggestion made by Amiche et al. (2008): DMS for dermaseptin, DA for dacnicolor, and 5 for being the fifth dermaseptin characterized from this species. Partial sequence was generated during the molecular mass screening when the entire molecule was subjected to CID dissociation. The fraction containing the peptide of interest was further purified by analytical HPLC (gradient from 15 to 60% solvent B over the course of 60 min) using the same system settings as that of Fig. 1, and it was fully sequenced by automatic Edman degradation and MS dissociation methods. The first 24 amino acid residues were determined by direct Edman degradation. CID and HCD were used to sequence the C-terminal portion of DMS-DA5, which was obtained from the tryptic peptide (AALNAVSEAX, 957.5 Da) whose C-terminus is amidated. Similarly, the tryptic peptides GMWGK, 578.3 Da;

Table 1 Peptide mass fingerprinting of the components from the skin secretion of the frog *P. dacnicolor*

Retention time (min)	Molecular masses (Da)	Retention time (min)	Molecular masses (Da)
7.49	1,350.70; 1,474.24; 1,825.07; 1,873.04; 2,126.13	35.90	1,242.74; 1,610.88; 1,983.05; 2,037.03; 2,540.19; 2,682.44
11.55	316.31	36.08	1,345.65; 2,131.1
11.74	1,234.34; 1,875.46; 3,489.69	36.78	955.60; 1,473.74; 2,115.12
14.88	ND	37.76	1,195.68; 1,358.71
15.54	1,842.67; 2,119.77; 2,248.81; 2,464.60; 2,542.63	39.63	3,110.71
18.79	638.30	40.90	2,729.33; 3,168.69; 3,699.89; 3,860.07; 4,088.17; 4,189.22
20.60	686.46	41.70	3,127.69; 3,162.7; 3,573.77; 3,815.78; 3,973.16; 4,171.21; 4,301.33
22.75	669.34; 883.41; **5,953.79**[a]; 6,051.75	42.50	**2,872.41**; 3,080.71; 3,209.75; 3,337.80; 5,953.23; 6,082.26; 6,210.32
23.58	**5,799.59**[a]; 6,065.73; 6,331.89	42.70	2,098.17; 2,246.22; 2,919.50; 3,205.74; 3,275.58
24.40	808.44; 1,605.02; 1,891.17	43.70	2,712.32; 3,018.57; 3,108.75; 3,366.85; 3,464.81; 3,708.96
25.82	792.46; 818.35	43.98	3,375.89; 3,504.93; 3,633.97; 3,771.07
27.34	802.35; **1,089.56**; 1,379.87; 1,441.87	44.30	2,374.32; 2,730.32; 2,745.33; 2,930.52; 3,237.78
28.08	1,154.65; 1,182.65	46.10	938.52; 2,026.99; 2,126.14; 2,310.35; 2,327.37; 2,536.36; 2,672.32; 3,105.62; 3,513.81; 3,548.79
28.94	933.46; 1,136.65; 1,553.97; 5,842.66; 5,889.66	46.70	1,621.93; 1,965.04; 2,259.22; 2,515.43; 3,232.70; 3,545.87; 3,559.88; 3,573.90; 3,587.91; 3,637.76
29.84	1,164.66; 1,237.66	47.64	838.46; 1,050.62; 1,969.05; 2,042.01; 2,200.12; 2,441.3; 2,461.38; 2,554.32; **2,698.16**; 3,005.64; 3,187.74
30.16	715.42; 917.46; 5,953.53	48.90	2,358.32
31.90	1,353.66; 1,868.79	49.07	1,625.02; 3,251.04; 3,518.18
32.46	1,445.65; 2,892.30	50.80	3,472.92; 3,504.91; 3,620.96; 3,843.05
33.00	662.43; 1,202.63; 1,367.71; 1,396.76	52.24	2,477.35; **2,692.18**
33.41	790.47; 1,192.5; 1,429.66; 1,957.01; 2,072.9; 2,649.54; 2,736.56; 2,861.28; 2,910.36	54.00	2,189.26; 2,399.35; 2,515.44; 2,702.48; **2,730.19**
33.79	2,599.40	55.12	1,694.97; 1,825.09; 3,205.76; 3,343.7; 3,652.16; 5,032.83; 6,413.51; 6,527.5
34.64	2,075.08; 2,558.36; 2,922.65	59.80	2,126.14; 2,150.1; 2,210.11; 2,596.41; 2,680.39; 2,729.34; 3,824.10; 4,252.28; 4,276.24; 4,722.54
35.36	1,051.67; 1,130.62; 1,200.71; 1,419.64; 1,553.96; 1,607.96; 2,000.05; 2,295.18; 2,556.2; 2,980.82; 3,168.68		

Molecular mass values in bold represent the components totally or partially sequenced

ND not determined

[a] N-terminal sequenced by Edman degradation

EAAK, 418.2 Da; STAK, 406.23 Da; AAGK, 346.2 Da; and IK, 260.2 Da were also observed, fragmented by the methods previously described, giving an unequivocal sequence as shown in Fig. 3. Theoretical (2,872.41 Da) and experimental average masses (2,872.41 Da) are in perfect agreement. It is important to point out that the last amidated amino acid can be L or I.

DMS-DA6 was obtained from the HPLC effluent fraction eluted with a RT of 52.24 min in almost homogeneous form. The fraction containing the peptide was repurified by HPLC (using a gradient from 15 to 50% of solvent B over 60 min) to homogeneity and analyzed by mass spectrometry, showing an average molecular mass of 2,692.18 Da. Sequencing by direct Edman degradation allowed determination of the N-terminal sequence up to the histidine residue at the position 19. The C-terminal sequence was established by CID and HCD using the tryptic peptide VLGNILPHVFSSNQS-NH_2 (1,610.84 Da) that yielded a perfect sequential overlap with the N-terminal sequence shown in Fig. 3. Additional tryptic peptides observed in the MS full scan mode (GVWGIAK-730.42 Da and IAGK-388.25 Da) matched the monoisotopic molecular masses

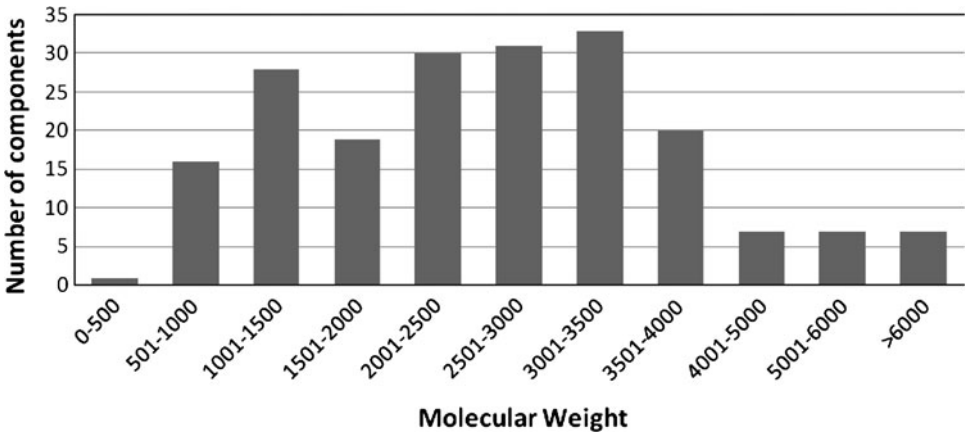

Fig. 2 Molecular mass distribution in the skin secretion of the amphibian *P. dacnicolor*. The histogram shows the distribution of the different MWs (Da) present in the secretions from *P. dacnicolor*. Peptides and proteins having MWs from 2.0 to 3.5 kDa are the most abundant components whereas components with MWs above 4.0 kDa and below 0.5 kDa constitute a minority of the crude secretions

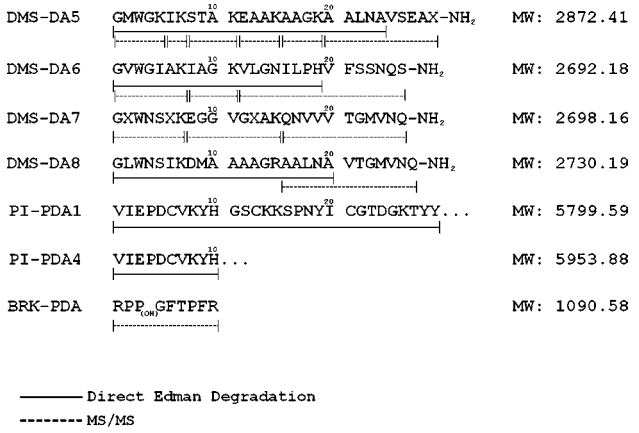

Fig. 3 Amino acid sequences of the peptides isolated from the secretions of the frog *P. dacnicolor*. The letter *X* in the sequences of DMS-DA5 and DMS-DA7 can be either a leucine or an isoleucine residue. The corresponding molecular masses of each peptide are listed on the *right side* of the figure and expressed in Da. *Continuous underlines* mean sequencing by direct Edman degradation, and *dashed underlines* are sequences obtained by CID and/or HCD dissociation methods

expected from the theoretical fragmentation performed in silico and from the merged de novo sequencing. The C-terminus of DMS-DA6 is amidated, and the theoretical (2,692.19 Da) and experimental average molecular masses (2,692.18) are in good agreement.

The DMS-DA7 peptide was isolated from the HPLC fraction eluted at 47.64 min and further purified to homogeneity. This component was not subjected to automatic Edman degradation, and the full sequence was obtained by CID and HCD from the tryptic peptides. The three amino acids labeled as X at positions 2, 5, and 13 were not determined since the isobaric molecular mass of 113.084 Da corresponding to the leucine and isoleucine amino acid residues, which are not easily distinguished by low energy collision dissociation mass spectrometry. Nevertheless, the corresponding tryptic peptides QNVVV TGMVNQ (1,187.60), GXWNSXK (817.4566), and EGGVGXAK (730.4093) were fragmented, and the main ion series (*b* and *y*) were fully assigned. The experimental (2,698.16) and theoretical (2,698.16) molecular masses agree (Fig. 3).

Similar to the other three dermaseptins described above, DMS-DA8 was isolated from the HPLC fraction eluted at a RT of 54.00 min, repurified to homogeneity by analytical HPLC, and analyzed by mass spectrometry, which gave a molecular mass of 2,730.19 Da. The first 20 amino acid residues of DMS-DA8 were established by direct Edman degradation. The sequence of the amidated C-terminal peptide AALNAVTGMVNQ-NH_2 was obtained by CID and HCD dissociation methods; it showed an overlap of five amino acids with the N-terminal segment, completing the sequence presented in the Fig. 3.

Two components that eluted in the early HPLC fractions at 22.75 and 23.58 min were repurified and sequenced by automatic Edman degradation. The analytical strategy allowed the unequivocal assignment of the first 10 and 29 amino acid residues of the components with average molecular masses of 5,953.79 Da (22.75 min RT) and 5,799.59 (23.58 min RT), respectively. A sequence search comparison against public protein data banks revealed 59% identity with the known protease inhibitor PSKP-1 (sp|P83578.1) previously isolated from the frog *Phyllomedusa sauvagii* (Fig. 3). Based on this sequence similarity, the components with 5,799.59 and 5,953.79 Da were named here PI-PDA3 and PI-PDA4, respectively. The letters PI refer the protease inhibition activity, PDA the amphibian species (*P. dacnicolor*) from which the components were isolated, and the numbers 3 and 4 give the chronological sequence of discovery of protease inhibitors characterized from species belonging to the Phyllomedusinae sub-family.

Finally, a component present in the HPLC fraction eluted at 27.34 min was structurally characterized during the molecular mass screening. The HCD spectrum shown in the Fig. 4 was obtained using the doubly charged ion at

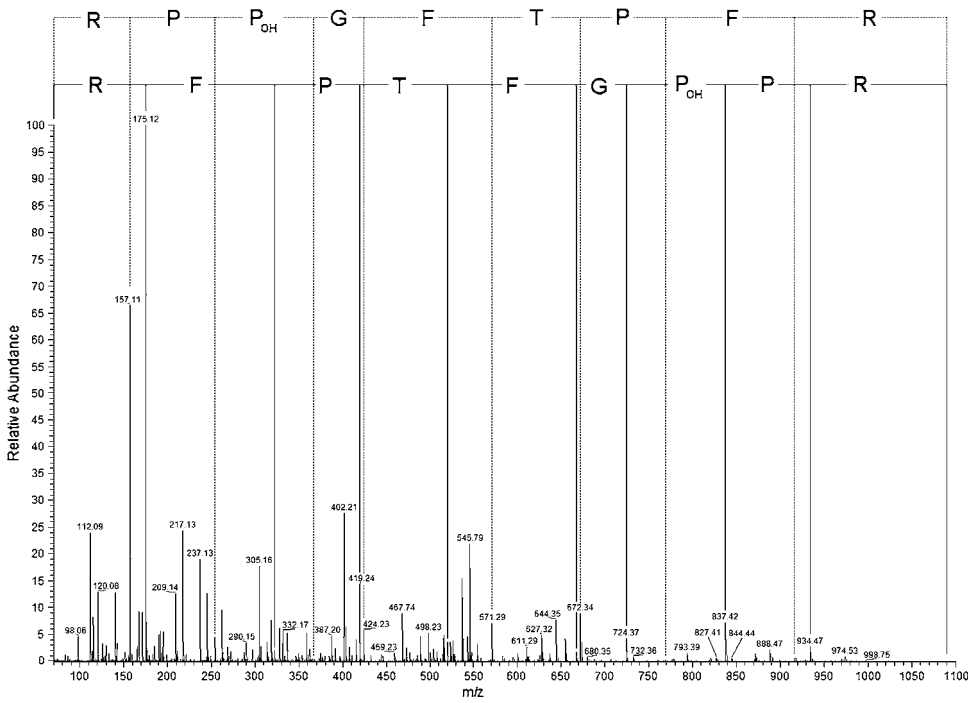

Fig. 4 High energy collision dissociation (HCD) spectrum of the component BRK-PDA. The HCD spectrum was obtained from the doubly charged ion at *m/z* 545.79 in an Orbitrap-XL mass spectrometer. Values for *b* and *y* ion series are labeled with *vertical continuous* and *dashed lines*, respectively. Hydroxyproline is clearly determined in both ion series, giving an experimental mass of 113.05

m/z 545.79, which allowed the elucidation of the primary structure of the component with an experimental monoisotopic molecular mass of 1,089.56 Da; it was named BRK-PDA (Fig. 3). A sequence similarity search against public data banks showed that the peptide has exactly the same sequence as that of a bradykinin previously characterized from the skin secretion of the frog *Phyllomedusa hypochondrialis*.

Antimicrobial assays

This study examined the antimicrobial activity of the three dermaseptins fully sequenced in their native forms. The peptide DMS-DA5 was the most active against the four bacteria tested, showing MICs of 3.1 μM (*E. coli*, *Bacillus subtilis* AIA, and *Salmonella* STM 14,028) and 25 μM (*Pseudomonas aeruginosa*). DMS-DA6 showed activity only against *E. coli* with a MIC of 100 μM and at the highest concentration used in this experiment (100 μM), DMS-DA7 had no activity against any of the four bacteria.

Homology search

The four novel dermaseptins (DMS-DA5-8) that were fully characterized were independently submitted for a sequence similarity search against the SwissProt protein data bank by using the FASTA sequence comparison engine. The four best scores obtained from each distinct search were selected, grouped in Fasta format and aligned by ClustalW2 (Fig. 5a). All novel sequences are dermaseptin-related peptides presenting high percentage of identity. Sequences with high similarity scores corresponding simultaneously to more than one from any of the four query sequences previously searched against the database were deleted to permit the correct ClustalW alignment. Figure 5b shows the primary structural identity (59%) of the novel protease inhibitor PI-PDA3 with respect to PSKP-1 characterized from the frog *Phyllomedusa sauvagii*.

General peptidomic analysis

Peptidomic analysis of crude skin secretions of the frog *P. dacnicolor* was performed using 22 main HPLC fractions similar to those shown in Fig. 1. All fractions were digested in solution with trypsin and analyzed by direct injection into an Orbitrap mass spectrometer. Forty-four peptides were manually sequenced and showed a wide range of sequence similarity to known amphibian molecules previously reported (Table 2).

Discussion

In this study we performed a general peptidomic analysis of the skin secretions of the amphibian *P. dacnicolor* that revealed an impressive molecular biodiversity. Although reports describing the skin secretion contents of this species have been focused only on tryptophilins and dermaseptin-like peptides (Chen et al. 2004), here we demonstrate that this specific secretion is much more

(A)

```
DMS-DA8                          GLWNSIKDM---AAAA-----GRAALN-AVTGMVNQ--- 27
gi|82592821|sp|P83914.1|DLP_SC   DLWNSIKDM---AAAA-----GRAALN-AVTGMVNQ--- 27
gi|13959332|sp|O93223.1|DMS3_A   SLWSKIKEM---AATA-----GKAALN-AVTGMVNQGEQ 30
DMS-DA7                          GLWNSXKE-----GGV-----GXAKQNVVVTGMVNQ--- 26
gi|115502150|sp|P84937.1|DMS2_   GLWSKIKDV---AAAA-----GKAALG-AVNEALGEQ-- 28
gi|114149281|sp|Q17UY8.1|DMS3_   GLWSTIKNV---AAAA-----GKAALG-----ALGEQ-- 24
gi|114149282|sp|P84599.1|DMS4_   GLWSTIKQKGKEAAIAAAKAAGKAVLN-AASEAL----- 33
gi|114149287|sp|P84880.1|DMS7_   GLWSTIKQKGKEAAIAAAKAAGQAALN-AASEAL----- 33
DMS-DA5                          GMWGKIKSTAKEAAKA----AGKAALN-AVSEAL----- 29
gi|13959338|sp|O93453.1|DMS3_P   GMWSKIKNAGKAAAKASKKAAGKAALG-AVSEALGEQ-- 36
gi|41016984|sp|P83638.1|DDSK_P   GLWSKIKAAGKEAAKAAAKAAGKAALN-AVSEAV----- 33
gi|238064941|sp|P84921.1|DMS1    GLWSKIKETGKEAAKA----AGKAALN-KIAEAV----- 29
gi|585056|sp|P31107.2|DMS2_PHY   GLWSKIKEVGKEAAKAAAKAAGKAALG-AVSEAVGEQ-- 36
gi|3913493|sp|P81487.1|DMS5_PH   GLWNKIKEA---ASKA----AGKAALG-FVNEMV----- 26
gi|114149279|sp|P84597.1|DMS2_   GLWKSLLKN---VGVA----AGKAALN-AVTDMVNQ--- 28
gi|41016985|sp|P83639.1|DDSL_P   ALWKTLLKN---VGKA----AGKAALN-AVTDMVNQ--- 28
DMS-DA6                          GVWG-IAKI---AGKV----LGNILPH-VFSSNQS---- 26
                                  :*          . .     *
```

(B)

```
PI-PDA3      VIEPDCVKYHGSCKKSP---NYICGTDGKTYY  29
             VIEP C KY G  KK P   N +CGTD +TYY
sp|P83578.1  VIEPKCYKYEG--KKCPPDINPVCGTDKRTYY  30
```

Fig. 5 CLUSTAL 2.0.12 multiple sequence alignment and sequence search comparison. **a** The four novel sequences (DMS-DA 5–8) are aligned by CLUSTAL 2.0.12 using the (2–4) best scores obtained from a sequence similarity search of the SwissProt protein databank by the FASTA sequence comparison algorithm at the University of Virginia. Signs in the last row mean: *dot* conserved, *colon* highly conserved, *asterisk* fully conserved. **b** Sequence search comparison using the partial primary structure of PI-PDA3 as the query sequence and the proteinase inhibitor PSKP-1 (sp|P83578.1) isolated from the frog *Phyllomedusa sauvagii* as the subject sequence (59% identity)

complex, and it contain several different molecular families of bioactive peptides. Commonly, studies conducted to discover novel bioactive proteins and peptides from natural sources are limited to one single type of component (or group of them, Auvynet et al. 2009; Wang et al. 2010). In contrast, the analytical strategy adopted in this work was designed to include the maximum number of components in a high-throughput fashion. In order to detect all components, the crude secretion of the amphibian was fractionated by HPLC, and all fractions were individually analyzed by high-resolution mass spectrometry. Previous proteomic reports on toxinology, including general analysis of scorpion (Batista et al. 2006) and snake venoms (Olamendi-Portugal et al. 2008), have demonstrated that peptide and protein mass fingerprinting analysis involves complex details to extract reliable data obtained by mass spectrometry, especially when electrospray ionization source is used for this purpose. Successive direct injections of a mixture of component in ESI-mass spectrometers, particularly when the amount and ionization capacity of each component are unknown, has a very high probability of detecting some *m/z* signals equal to those from the previous analysis. For this reason, the analysis was conducted by alternating samples of short and long retention times to prevent contamination with components present in adjacent HPLC fractions. In addition, the *m/z* signals observed from previous injections were not taken into account. Another fundamental observation regarding the detection of false positive components is related to sodium and potassium adduct formation and oxidation of the methionines (Batista et al. 2007). In this way, peptide ion adducts formed by single, double, and triple addition of sodium and/or potassium ($[M+22]^+$ and $[M+39]^+$) were excluded from the molecular mass fingerprint list. Additional molecular masses with values increased by 15.99 Da were also removed from the list due to the high frequency of methionine oxidation. Double molecular mass values corresponding to the frequent dimerization of randomic molecules in gas phase were also excluded. However, all other possible but uncommon combinations of molecular mass values, e.g., loss of 44 Da (decarboxylation of gamma-glutamic acid), molecular masses with additional mass of 31.99 Da (double methionine oxidation/molecule), deamidation (−14 Da), among others were taken into account.

Dermaseptin-like peptides are very abundant in the skin secretion of the amphibians belonging to the Phyllomedusinae sub-family (Zairi et al. 2009; Thompson et al. 2007). However, only six different dermaseptins have been fully characterized from the species *P. dacnicolor* at primary structural level. Based on an initial fast CID-MS screen of HPLC fractions, we chose four unknown dermaseptin-like antimicrobial peptides to elucidate their primary structures. The main difficulty for de novo sequencing of unknown proteins and peptides by MS dissociation methods is the naturally occurring isobaric masses corresponding to the leucine and isoleucine amino acid residues. Although there are many reports using different stages of tandem fragmentation, [e.g., fragmentation of the 86 Da immonium ion from L or I to detect the preferential

Table 2 Assignment of the tryptic peptides obtained from the HPLC fractionation of the crude secretions of the frog *P. dacnicolor* by collision-induced dissociation (CID) and high energy collision dissociation (HCD)

HPLC (min)	Precursor ion (*m/z*)	$[M+H]^{1+}$	MS/MS-derived sequence	Peptide identification
22.75	335.17 z^{2+}	669.34	YPSFR	Unknown
24.40	545.79 z^{2+}	1,090.58	$RPP_{(OH)}$GFTPFR	Bradykinin (sp|P86157.1)
24.40	486.31 z^{2+}	971.62	RFPALLVR	Hyposin-4 (sp|P84957.1)
28.94	710.93 z^{2+}	1,420.86	-NLVLSK	Phylloseptin Bu-2 (sp|P86283.1)
29.84	404.72 z^{2+}	808.44	-PNPNK	Ranatachykinin-D (sp|P22691.1)
30.16	1,008.30 z^{2+}	2,015.60	-PTQSQGTNETLV-	Agglutinin beta-2 (sp|P83423.1)
30.16	488.25 z^{2+}	975.50	WQFEPLR	Unknown
32.46	537.79 z^{2+}	1,074.58	RPPGFTPFR	Bradykinin (sp|P84901.1)
33.00	565.34 z^{2+}	1,129.68	LLGGLLDTVTK	Caerin-2.1 (sp|P62570.1)
33.00	464.27 z^{2+}	927.54	SFKGLFTK	Unknown
34.64	478.81 z^{2+}	956.62	DLPPPLLPL	Tryptophyllin (sp|P84950.1)
37.76	304.65 z^{2+}	608.28	GMWSK	Dermaseptin (sp|P83642.1)
37.76	740.49 z^{2+}	1,479.98	EPGVLPKLLSVLSK	Brevinin-1T (sp|P82232.1)
40.90	578.27 z^{+}	578.27	GMWGK	Dermaseptin (sp|P83642.1)
40.90	731.38 z^{2+}	1,461.76	-SLDLTFDLLR	Sauvagin (sp|P01144.1)
40.90	412.73 z^{2+}	824.46	HNLELAK	Unknown
41.70	680.39 z^{2+}	1,359.78	AVLNAVTNMANQN	Dermaseptin (sp|P84922.1)
41.70	517.31 z^{+}	517.31	ALWK	Dermaseptin (sp|P84924.1)
41.70	578.27 z^{+}	578.27	GMWGK	Dermaseptin (sp|P83642.1)
42.50	680.39^{2+}	1,359.66	AVLNAVTNMANQN	Dermaseptin (sp|P84922.1)
42.50	479.76^{2+}:	957.51	NNAAAVSEAL-NH_2	Unknown
42.70	811.98 z^{2+}	1,622.96	FLSLLPHVLSALSN-	Phylloseptin (sp|P84570.1)
44.30	413.74 z^{2+}	826.48	FLSLLPH	Phylloseptin (sp|P84570.1)
44.30	388.23 z^{2+}	775.46	TLSDVLK	Citropin (sp|P81847.1)
44.30	703.40 z^{+}	703.40	VLSALSN	Phylloseptin (sp|P84568.1)
46.10	578.27^{1+}	578.27	GMWGK	Dermaseptin (sp|P83642.1)
46.10	517.3^{1+}	517.3^{1+}	ALWK	Dermaseptin (sp|P84924.1)
46.10	470.27 z^{2+}	939.54	FLFLPFR	Bradykinin (lsp|Q7LZ54.1)
46.10	589.86 z^{2+}	1,178.62	-PPSLPD	Unknown
46.70	388.23^{2+}:	775.46	TLSDVLK	Citropin (sp|P81847.1)
46.70	304.64^{2+}	608.28	GMWSK	Dermaseptin (sp|P83642.1)
47.64	380.25 z^{2+}	759.50	LLSVLSK	Temporin (sp|P82883.)
47.64	370.26 z^{2+}	739.52	LLGVLPK	Brevinin (sp|P82232.1)
47.64	422.72 z^{2+}	844.44	FLSMLPH	Phylloseptin (sp|P84938.1)
49.07	710.93 z^{2+}	1,420.86	VLSALPHVVSALSK	Phylloseptin (sp|P84904.1)
49.07	578.27^{1+}	578.27	GMWGK	Dermaseptin (sp|P83642.1)
49.07	680.39^{2+}	1,359.66	AVLNAVTNMANQN	Dermaseptin (sp|P84922.1)
49.07	517.31^{1+}	517.31	ALWK	Dermaseptin (sp|P84924.1)
50.80	409.23 z^{2+}	817.46	AVWNSLK	Dermaseptin (sp|P83914.1)
54.00	594.31 z^{2+}	1,187.62	-VMGTVVVNK	Unknown
55.12	883.49 z^{2+}	1,765.98	-LGGLLDTVTK	Caerin (sp|P62570.1)
55.12	449.80 z^{2+}	998.60	LAGQAALGAVK	Dermaseptin (sp|P84926.1)
59.80	405.72 z^{2+}	810.44	SFLTSQK	Unknown
59.80	554.83 z^{2+}	1,108.66	VLGNLLPHVF	Caerin (sp|P62548.1)

All amino acid residue written as L can be alternatively leucine or isoleucine

decomposition pathway of isoleucine immonium ion to produce the ions *m/z* 30, 44, and 69 in different amounts due to the lower energy barrier when compared to leucine (Armirotti et al. 2007)] the difficulty remains unsolved for real samples. Therefore, we used automatic Edman degradation to sequence part of the peptide up to the last L/I previously identified by mass spectrometry. Using this strategy, we successfully characterized two different dermaseptin-like peptides fully and two others partially. DMS-DA5 was sequenced up to position 28, since the last amino acid has molecular weight of 112 Da, which suggests the presence of an amidated L/I. Although many efforts were made to determine the terminal amino acid using tandem mass spectrometry (PQD/PQD) and Edman degradation, an unambiguous characterization was not possible. The last amino acid residue could have been determined by amino acid analysis, but there was insufficient material for this experiment. Similarly, DMS-DA7 was sequenced only by MS-fragmentation that gave an isobaric mass of 113.08 Da for L/I in three positions (2, 6, and 13).

DMS-DA5 showed potent activity against the four bacterial species. Experimental MIC values determined for DMS-DA5 are in the range of the most active peptides previously reported from amphibian skin of the Phyllomedusinae sub-family [Rydlo et al. 2006; Batista et al. 1999). Surprisingly, DMS-DA6 and DMS-DA8 are inactive against the bacteria tested, except for DMS-DA6, which inhibited *E. coli* with an MIC of 100 μM.

The de novo sequencing by mass spectrometry of the tryptic peptides generated from the HPLC fractions allows us to determine the general molecular contents of skin secretion of *P. dacnicolor*. The limited number of peptides sequenced de novo is due to numerous lysines in the sequences, which produced very short peptides composed of 3–5 amino acid residues; thus, it was impossible to obtain acceptable identification scores for the majority of the short sequence segments. Nevertheless, apart from the five well-defined structures of the peptides and the two partially sequenced protease inhibitors shown in Fig. 3, the 44 tryptic peptides sequenced de novo showed that the skin secretions of the amphibian *P. dacnicolor* are basically composed of dermaseptin-like peptides, bradykinins, tryptophyllins, cecropins, citropins, brevinins, hyposins, temporins, protease inhibitors, and unknown molecules (Table 2; Fig. 3). The general peptidomic analysis presented here clearly demonstrates that the skin secretion of this amphibian is an unexploited potential source of bioactive peptides with almost two hundred different molecules, including a number of distinct sub-families of antimicrobial peptides.

Acknowledgments This research was supported by grant from the Dirección General de Asuntos del Personal Académico (DGAPA) of the National Autonomous University of Mexico-UNAM (Grant number IN221508 to CVFB).

References

Amiche M, Ladram A, Nicolas P (2008) A consistent nomenclature of antimicrobial peptides isolated from frogs of the subfamily Phyllomedusinae. Peptides 29(11):2074–2082

Armirotti A, Scapolla C, Benatti U, Damonte G (2007) Electrospray ionization ion trap multiple-stage mass spectrometric fragmentation pathways of leucine and isoleucine: an ab initio computational study. Rapid Commun Mass Spectrom 21(19): 3180–3184

Auvynet C, Joanne P, Bourdais J, Nicolas P, Lacombe C, Rosenstein Y (2009) Dermaseptin DA4, although closely related to dermaseptin B2, presents chemotactic and Gram-negative selective bactericidal activities. FEBS J 276(22):6773–6786

Batista CV, da Silva LR, Sebben A, Scaloni A, Ferrara L, Paiva GR, Olamendi-Portugal T, Possani LD, Bloch C Jr (1999) Antimicrobial peptides from the Brazilian frog *Phyllomedusa distincta*. Peptides 20(6):679–686

Batista CV, D'Suze G, Gómez-Lagunas F, Zamudio FZ, Encarnación S, Sevcik C, Possani LD (2006) Proteomic analysis of *Tityus discrepans* scorpion venom and amino acid sequence of novel toxins. Proteomics 6(12):3718–3727

Batista CV, Román-González SA, Salas-Castillo SP, Zamudio FZ, Gómez-Lagunas F, Possani LD (2007) Proteomic analysis of the venom from the scorpion *Tityus stigmurus*: biochemical and physiological comparison with other *Tityus* species. Comp Biochem Physiol C Toxicol 146(1–2):147–157

Chen T, Orr DF, O'Rourke M, McLynn C, Bjourson AJ, McClean S, Hirst D, Rao P, Shaw C (2004) *Pachymedusa dacnicolor* tryptophyllin-1: structural characterization, pharmacological activity and cloning of precursor cDNA. Regul Pept 117(1):25–32

Conlon JM (2004) The therapeutic potential of antimicrobial peptides from frog skin. Rev Med Micro 15:17–25

Conlon JM, Iwamuro S, King JD (2009) Dermal cytolytic peptides and the system of innate immunity in anurans. Ann N Y Acad Sci 1163:75–82

Frost, DR (2009) Amphibian species of the world: an online reference. Version 5.3. Electronic database accessible at http://research.amnh.org/vz/herpetology/amphibia/index.php, American Museum of Natural History, New York, USA

Gottler LM, Ramamoorthy A (2009) Structure, membrane orientation, mechanism, and function of pexiganan—a highly potent antimicrobial peptide designed from magainin. Biochim Biophys Acta 1788(8):1680–1686

Hancock RE, Lehrer R (1998) Cationic peptides: a new source of antibiotics. Trends Biotechnol 16:82–88

Mahalka AK, Kinnunen P (2009) Binding of amphipathic α-helical antimicrobial peptides to lipid membranes: lessons from temporins B and L. Biochim Biophys Acta 1788:1600–1609

National Committee for Clinical Laboratory Standards (1997) Methods for dilution antimicrobial susceptibility tests for bacteria that grow, aerobically, approved standard M7-A4. NCCLS, Wayne, PA

Nicolas P, El Amri C (2009) The dermaseptin superfamily: a gene-based combinatorial library of antimicrobial peptides. Biochim Biophys Acta 1788:1537–1550

Nicolas P, Rosenstein Y (2009) Multifunctional host defense peptides. FEBS J 276(22):6464

Olamendi-Portugal T, Batista CV, Restano-Cassulini R, Pando V, Villa-Hernandez O, Zavaleta-Martínez-Vargas A, Salas-Arruz MC, Rodríguez de la Vega RC, Becerril B, Possani LD (2008) Proteomic analysis of the venom from the fish eating coral snake *Micrurus surinamensis*: novel toxins, their function and phylogeny. Proteomics 8(9):1919–1932

Rydlo T, Rotem S, Mor A (2006) Antibacterial properties of dermaseptin S4 derivatives under extreme incubation conditions. Antimicrob Agents Chemother 50(2):490–497

Shai Y (1999) Mechanism of the binding, insertion and destabilization of phospholipids bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides. Biochim Biophys Acta 1462:55–70

Thompson AH, Bjourson AJ, Orr DF, Shaw C, McClean S (2007) Amphibian skin secretomics: application of parallel quadrupole time-of-flight mass spectrometry and peptide precursor cDNA cloning to rapidly characterize the skin secretory peptidome of *Phyllomedusa hypochondrialis azurea*: discovery of a novel peptide family, the hyposins. J Proteome Res 6(9):3604–3613

Wang L, Zhou M, Chen T, Walker B, Shaw C (2009) PdT-2: a novel myotropic type-2 tryptophyllin from the skin secretion of the Mexican giant leaf frog, *Pachymedusa dacnicolor*. Peptides 30(8):1557–1561

Wang M, Wang Y, Wang A, Song Y, Ma D, Yang H, Ma Y, Lai R (2010) Five novel antimicrobial peptides from skin secretions of the frog, *Amolops loloensis*. Comp Biochem Physiol B Biochem Mol Biol 155(1):72–76

Wechselberger C (1998) Cloning of cDNAs encoding new peptides of the dermaseptin family. Biochim Biophys Acta 1388:279–283

Zairi A, Tangy F, Bouassida K, Hani K (2009) Dermaseptins and magainins: antimicrobial peptides from frogs' skin-new sources for a promising spermicides microbicides—a mini review. J Biomed Biotechnol 2009:452–567 PMID: 19893636 [PubMed-in process]