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Identification of three novel *Phyllomedusa sauvagei* dermaseptins (sVI–sVIII) by cloning from a skin secretion-derived cDNA library

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Abstract

The defensive skin secretions of many amphibians contain a wide spectrum of biologically active compounds, particularly antimicrobial peptides that act as a first line of defence against bacterial infection. Here we describe for the first time the identification of three novel dermaseptin-related peptides (dermaseptins sVI–sVIII) whose primary structures were deduced from cDNAs cloned from a library constructed from lyophilised skin secretion of the South American hylid frog, *Phyllomedusa sauvagei*. The molecular masses of each were subsequently confirmed by interrogation of archived LC/MS files of fractionated skin secretion followed by automated Edman degradation sequencing. The heterogeneity of primary structures encountered in amphibian skin antimicrobial peptides may in part be explained by individual variation—a factor essential for selective functional molecular evolution and perhaps, ultimately in speciation.

Keywords: Phyllomedusinae; Skin; Antimicrobial peptides; Mass spectroscopy; Molecular evolution; cDNA cloning

1. Introduction

Extracts of amphibian skin have been used for centuries in folk medicine and witchcraft due to their possession of a wide spectrum of pharmacological effects [1]. The source of these biologically active compounds are the dermal granular glands and biochemically, the constituent molecules are representative of many classes including biogenic amines, peptides, proteins, alkaloids and heterocyclics [1–3]. The structural diversity of peptides in these amphibian defensive skin secretions probably reflects different roles, either in the regulation of physiological functions of the skin or in defence against predators or microorganisms [4,5].

Over the past three decades, the ready availability of broad-spectrum antibiotics has caused complacency about the threat of bacterial infection. However, bacteria have become resistant to most of these antimicrobial agents as a result of chromosomal mutations or the exchange of genetic material via plasmids and transposons [6]. This ubiquitous problem of resistance demands renewed efforts in the quest for novel antibacterial agents effective against pathogenic bacteria resistant to current antibiotics. Amphibian skin peptides have been the subject of intense research interest for many years from both academic and pharmaceutical groups due to their potential applications in biophysical research, biochemical taxonomy and in lead compound development for new pharmaceuticals [7–10].

Bombinin, isolated from the skin of the European yellow-bellied toad, *Bombina variegata*, some 30 years ago, was the first reported amphibian skin peptide with demonstrable antibacterial and haemolytic activity [11]. However, it was not until a decade later, when Michael Zasloff isolated the magainins from *Xenopus laevis* skin, that research was focussed on this field, resulting in identification of a wide range of antimicrobial peptides with different structural motifs [1,4,12]. The subfamily of South and Central American leaf frogs, the Phyllomedusinae, contains about 50 species in several genera [13]. The largest genus, *Phyllomedusa*, contains species with skin secretions that have been described as a ‘‘huge factory and store-house of a variety of (biologically)

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active peptides'' [14]. Numerous peptides have been isolated from this source and clearly these can be classified, on a primary structural basis, into several main families [15]. In particular, a number of dermaseptin peptides have been isolated from the skin of *Phyllomedusa* species and characterized as displaying different pharmacological, antimicrobial or inhibitory activities [16–19]. To date, five dermaseptins (sI–sV), 24–34 amino acid residues in length, have been identified in and structurally characterised from the skin secretion of *Phyllomedusa sauvagei* [19] but the structure and organisation of their homologous cDNAs remain unreported.

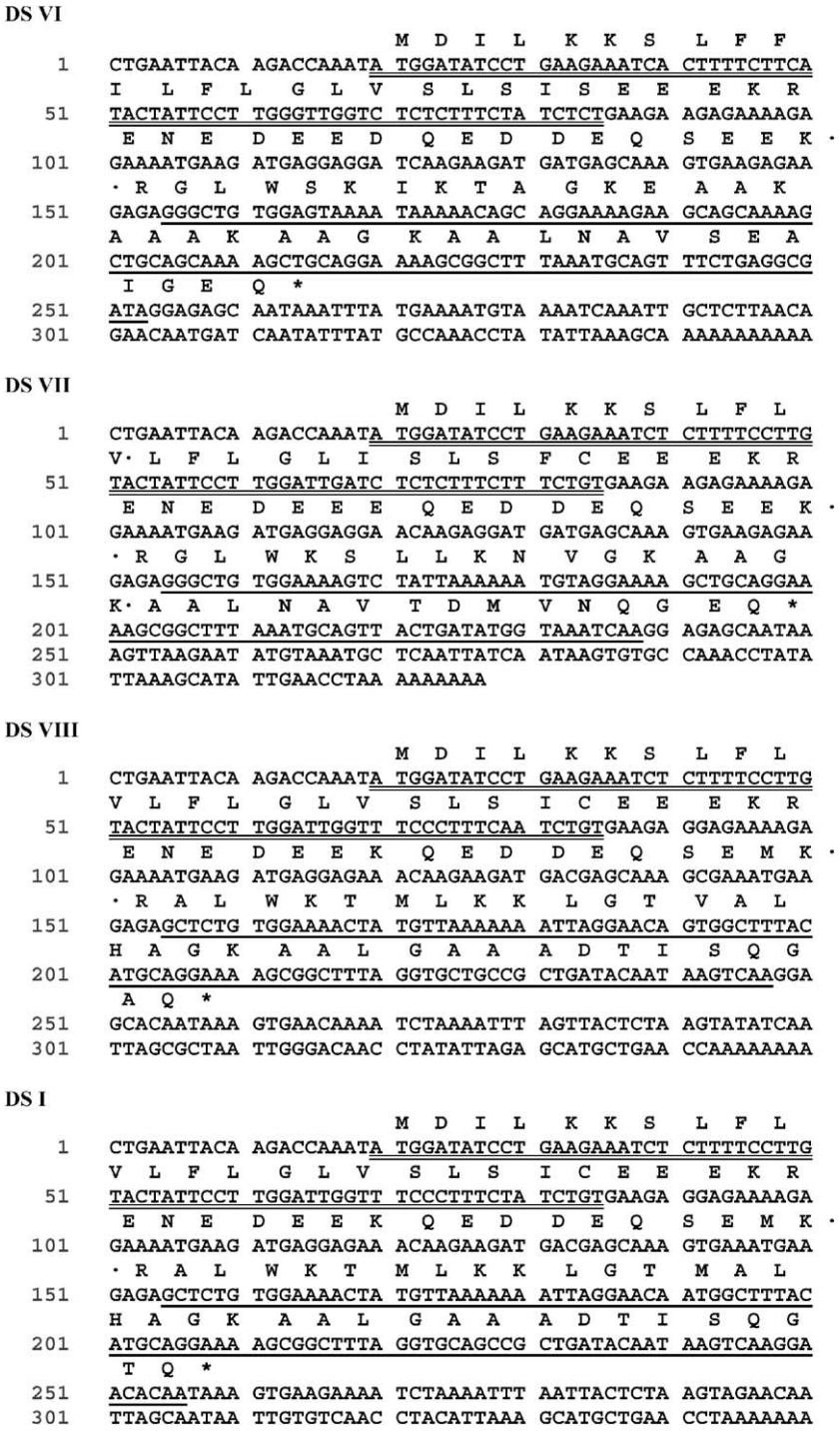

Fig. 1. Nucleotide sequences of precursor cDNAs encoding dermaseptins (sVI–sVIII and sI) cloned from a *P. sauvagei* skin secretion library. The putative signal peptides (double-underlined), mature peptides (single-underlined) and stop codons (asterisks) are indicated.

Here we report a more detailed study of the dermaseptins present in the defensive skin secretion of this leaf frog. We describe for the first time three different full-length cDNAs, each encoding one novel deduced dermaseptin peptide. These have been named in accordance with established nomenclature as dermaseptins sVI–sVIII(s for sauvagei). These were cloned using a recently described technique developed in our laboratory, by which amphibian granular gland peptide precursors can be cloned from cDNA libraries constructed from lyophilised skin secretion [20].

Subsequent to deduction of novel dermaseptin primary structures from cloned cDNAs, the peptides were identified by molecular mass in fractions of skin secretion collected following LC/MS and structures were confirmed by automated Edman degradation. Rather than suggest that these peptides were overlooked in previous studies of this species, we contend that there exists primary structural variation between individual specimens or discrete populations. This may represent a situation analogous to single nucleotide polymorphisms (SNPs) much studied in higher vertebrates that provides the basis for molecular natural selection ultimately leading to speciation events.

2. Materials and methods

2.1. Specimen biodata and secretion harvesting

P. sauvagei ($n = 4$) were obtained from a commercial source in the United States having been caught in the wild and imported from Paraguay. The frogs were adults (two males and two females—5 and 8 cm snout-to-vent length, respectively) and were kept for a period of 4 months prior to secretion harvesting. They were maintained in our purpose-designed amphibian facility at 18–25 °C under a 12/12-h light/dark cycle and fed multivitamin-loaded crickets three times per week. Defensive skin secretions were obtained from the paired paratoid and tibial glands by gently squeezing using a thumb and the obvious viscous white secretion was washed from the skin using deionised water, snap-frozen

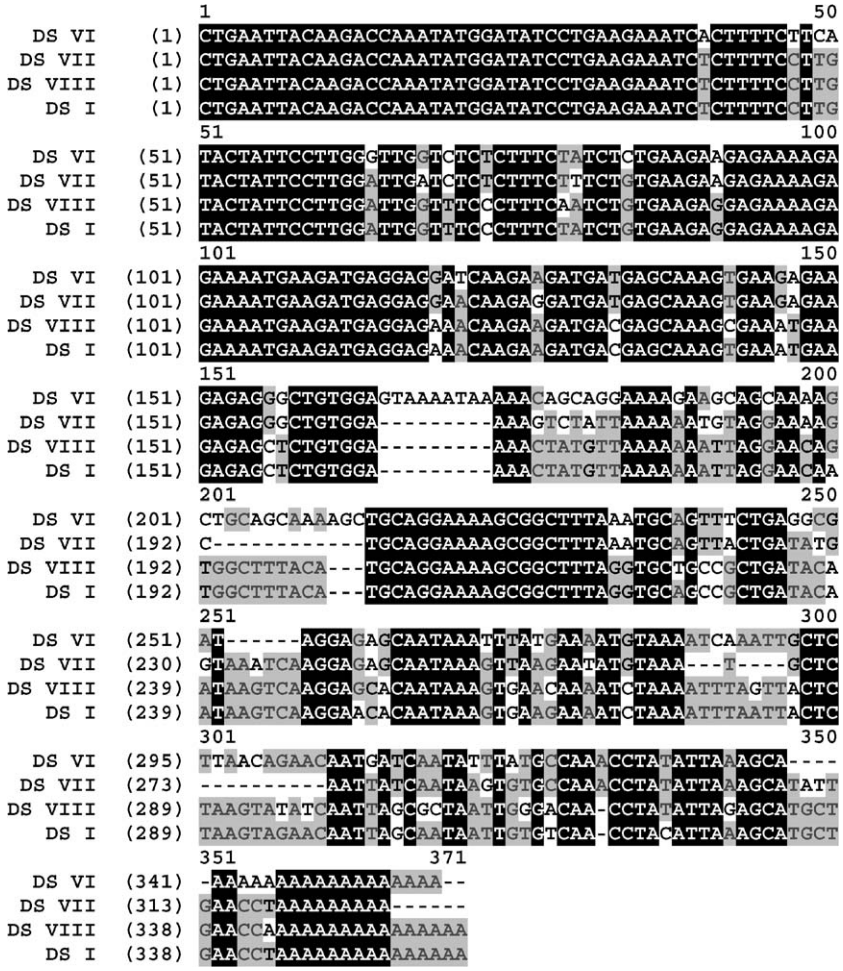

Fig. 2. Alignment of nucleotide sequences of dermaseptin precursor cDNAs cloned from the *P. sauvagei* skin secretion library. Identical bases in all three shaded in black. Consensus bases in two sequences shaded grey. Gaps inserted to maximise alignment.

in liquid nitrogen and lyophilised. Lyophilisate was stored at − 20 °C prior to analysis.

2.2. Cloning of dermaseptin cDNAs

Five milligrams of lyophilised skin secretion were dissolved in 1 ml of cell lysis/mRNA stabilisation solution (Dynal, UK). Polyadenylated mRNA was isolated using magnetic oligo-dT beads as described by the manufacturer (Dynal Biotech, UK). The isolated mRNA was subjected to 3′-RACE procedures to obtain full-length preprodermaseptin nucleic acid sequence data using a SMART-RACE kit (Clontech UK) essentially as described by the manufacturer. Briefly, the 3′-RACE reactions employed a UPM primer (supplied with the kit) and a sense primer (S1; 5′-ACTTTCYGAWTTRYAAGMCCAAABATG-3′) that was designed to a segment of the 5′-untranslated region of phylloxin cDNA from *Phyllomedusa bicolor* (EMBL Accession no. AJ251876) and the opioid peptide cDNA from *Pachymedusa dacnicolor* EMBL Accession no. AJ005443). The PCR cycling procedure was carried out as follows: Initial denaturation step: 90 s at 94 °C; 35 cycles: denaturation 30 s at 94 °C, primer annealing for 30 s at 58 °C; extension for 180 s at 72 °C. PCR products were gel-purified and cloned using a pGEM-T vector system (Promega) and sequenced using an ABI 3100 automated sequencer.

2.3. Identification and structural analysis of novel deduced dermaseptins

Five milligrams of lyophilised skin secretion were dissolved in 0.5 ml of 0.05/99.5 (v/v) trifluoroacetic acid (TFA)/water and clarified of microparticulates by centrifugation. The supernatant was then subjected to LC/MS using a gradient formed from 0.05/99.5 (v/v) TFA/water to 0.05/29.95/70.0 (v/v/v) TFA/water/acetonitrile in 180 min at a flow rate of 1 ml/min. A Thermoquest gradient reversed phase HPLC system, fitted with an analytical column (Jupiter C-5, 5 μ particle, 300 Å pore, 250 × 10 mm, Phenomenex, UK) and interfaced with a Thermoquest LCQ™ electrospray ion-trap mass spectrometer, was employed. The effluent from the chromatographic column was flow-split with approximately 10% entering the mass spectrometer source and 90% directed towards a fraction collector. Dead volume between column and fraction collector was minimal (20 μl). The molecular masses of polypeptides in each chromatographic fraction were further analysed using matrix-assisted laser desorption/ionisation, time-of-flight mass spectrometry (MALDI-TOF MS)

Table 1
Predicted *b*- and *y*-ion MS/MS fragment ion series (singly- and doubly-charged) of dermaseptin sI (DS I). Observed ions are indicated in bold typeface

Sequence	#	Single charged ions			Sequence	#	Double charged ions		
		b (*m*/*z*)	*y* (*m*/*z*)				*b* (*m*/*z*)	*y* (*m*/*z*)	
A	1	72.1	3456.1	34	A	1	36.5	**1728.5**	34
L	2	185.2	3385.0	33	L	2	93.1	1693.0	33
W	3	371.5	3271.8	32	W	3	186.2	**1636.4**	32
K	4	499.6	3085.6	31	K	4	250.3	**1543.3**	31
T	5	600.7	2957.5	30	T	5	300.9	**1479.2**	30
M	6	**731.9**	2856.3	29	M	6	366.5	1428.7	29
L	7	**845.1**	2725.2	28	L	7	423.0	**1363.1**	28
K	8	**973.3**	2612.0	27	K	8	487.1	1306.5	27
K	9	**1101.4**	2483.8	26	K	9	551.2	1242.4	26
L	10	**1214.6**	2355.6	25	L	10	607.8	1178.3	25
G	11	**1271.6**	2242.5	24	G	11	636.3	**1121.7**	24
T	12	**1372.7**	2185.4	23	T	12	686.9	1093.2	23
M	13	**1503.9**	2084.3	22	M	13	752.5	1042.7	22
A	14	**1575.0**	**1953.1**	21	A	14	788.0	977.1	21
L	15	**1688.2**	**1882.1**	20	L	15	**844.6**	941.5	20
H	16	**1825.3**	**1768.9**	19	H	16	913.2	885.0	19
A	17	**1896.4**	**1631.8**	18	A	17	948.7	816.4	18
G	18	**1953.4**	**1560.7**	17	G	18	977.2	780.8	17
K	19	2081.6	**1503.6**	16	K	19	1014.3	752.3	16
A	20	2152.7	**1375.5**	15	A	20	1076.9	688.2	15
A	21	2223.8	**1304.4**	14	A	21	**1112.4**	652.7	14
L	22	2336.9	**1233.3**	13	L	22	**1169.0**	617.2	13
G	23	2394.0	**1120.1**	12	G	23	**1197.5**	560.6	12
A	24	2465.1	1063.1	11	A	24	**1233.0**	532.0	11
A	25	2536.2	**992.0**	10	A	25	**1268.6**	496.5	10
A	26	2607.2	**920.9**	9	A	26	**1304.1**	461.0	9
D	27	2722.3	849.8	8	D	27	**1361.7**	425.4	8
T	28	2823.4	**734.8**	7	T	28	**1421.2**	367.9	7
I	29	2936.6	633.7	6	I	29	**1468.8**	317.3	6
S	30	3023.7	520.5	5	S	30	**1512.3**	260.8	5
Q	31	3151.8	433.4	4	Q	31	**1576.4**	217.2	4
G	32	3208.8	305.3	3	G	32	**1604.9**	153.1	3
T	33	3309.9	248.2	2	T	33	**1655.5**	124.6	2
Q	34	3438.1	147.1	1	Q	34	**1719.5**	74.1	1

Fig. 3. Alignment of translated open-reading frame amino acid sequences of *P. sauvagei* dermaseptin precursors deduced from cloned cDNAs. Identical amino acid residues shaded in black. Gaps inserted to maximise alignment.

on a linear time-of-flight Voyager DE mass spectrometer (Perseptive Biosystems, MA, USA) in positive detection mode using alpha-cyano-4-hydroxycinnamic acid as the matrix. Internal mass calibration of the instrument with known standards established the accuracy of mass determination as ±0.1%. The polypeptides with masses coincident with those deduced from cloned cDNAs were each subjected to primary structural analysis by automated Edman degradation using an Applied Biosystems 491 Procise sequencer in pulsed-liquid mode. The limit for detection of phenylthiohydantoin (PTH) amino acids was 0.1 pmol.

3. Results

3.1. In vitro cDNA library construction from skin secretion

From the skin secretion-derived cDNA library, four preprodermaseptins cDNAs were consistently cloned and each encoded a single copy of a dermaseptin. However, only one of these encoded a previously documented dermaseptin (dermaseptin sI) from this species. The others encoded dermaseptins of novel primary structures and these were named, in accordance with established nomenclature, dermaseptins sVI–sVIII) (Fig. 1), respectively. Alignment of both nucleotide sequences (Fig. 2) and open-reading frame amino acid sequences (Fig. 3), using the AlignX programme of the Vector NTI Bioinformatics suite (Informax), revealed a high degree of primary structural similarity between the novel dermaseptin peptides. A BLAST search in the EMBL Nucleotide Sequence Database revealed that they exhibit high degrees of structural similarity to other reported preprodermaseptin cDNAs from phyllomedusine frogs (data no shown). The nucleotide sequences have been deposited in EMBL Nucleotide Sequence Database under the accession codes AJ564791 to AJ564794.

3.2. Isolation and structural characterization of novel deduced dermaseptins

Only one previously reported dermaseptin (dermaseptin sI) (3454.9 Da), was located by interrogation of the archived LC/MS files of *P. sauvagei* skin secretion. Its primary structure was confirmed by MS/MS fragmentation sequencing (Table 1). Interestingly, this was the only previously documented dermaseptin whose cDNA was cloned using the procedure described here. The additional three novel dermaseptins were not located using masses deduced from the -KR- processing site upstream of the dermaseptin encoding domain to the termination of the open-reading frame. However, by sequential deletion of three C-terminal amino acid residues (-GEQ,-GEQ and -GAQ, in from the open-reading frames of each novel peptide-encoding cloned cDNA, respectively), all three processed dermaseptin peptides were identified. These three polypeptides, with molecular masses of 3195.6 Da (dermaseptin sVI), 2897.2 Da (dermaseptin sVII) and 3135.3 Da (dermaseptin sVIII), were all completely resolved by HPLC, with retention times of 115, 121 and 127 min, respectively (Fig. 4). Their unequivocal primary structures were also established by automated Edman degradation (Table 2). The NCBI-BLAST search revealed that dermaseptin sVI and dermaseptin sVII showed 87% and 85% sequence identity, respectively, with adenoregulin (dermaseptin bII) and dermaseptin bVI from *P.*

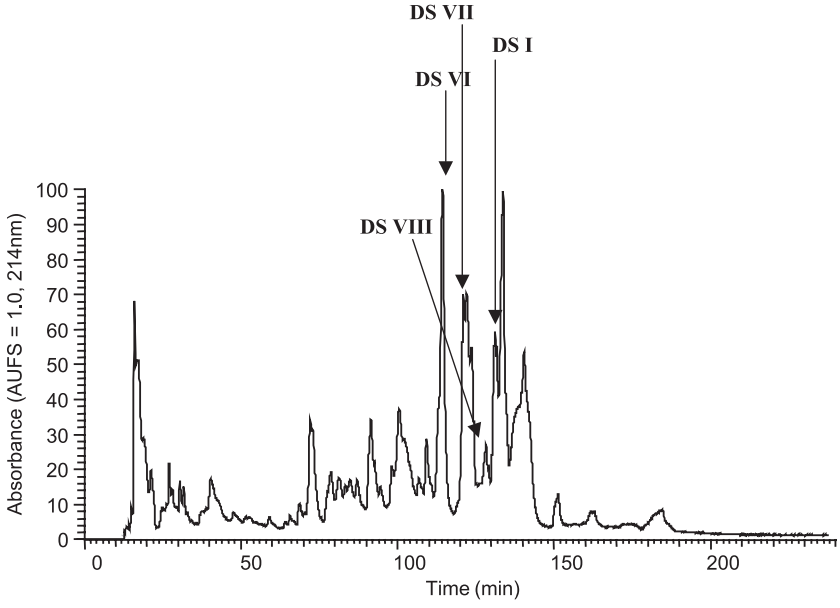

Fig. 4. Reverse phase HPLC chromatogram of *P. sauvagei* skin secretion. The retention times of dermaseptin sI (DSI) and the novel dermaseptins sVI–sVIII (DS VI–DS VIII) are indicated.

Table 2
Complete automated Edman degradation data for each predicted novel dermaseptin (sVI–sVIII)

Cycle no.	DS VI	DS VII	DS VIII
1	G (1168)	G (1184)	A (529)
2	L (706)	L (1720)	L (480)
3	W (254)	W (685)	W (126)
4	S (127)	K (1096)	K (276)
5	K (346)	S (348)	T (254)
6	I (665)	L (920)	M (300)
7	K (323)	L (1160)	L (278)
8	T (448)	K (888)	K (228)
9	A (628)	N (729)	K (266)
10	G (498)	V (693)	L (237)
11	K (452)	G (705)	G (244)
12	E (206)	K (764)	T (194)
13	A (516)	A (747)	V (186)
14	A (611)	A (1064)	A (240)
15	K (320)	G (594)	L (187)
16	A (496)	K (574)	H (175)
17	A (544)	A (781)	A (187)
18	A (563)	A (1104)	G (112)
19	K (253)	L (504)	K (102)
20	A (387)	N (488)	A (163)
21	A (479)	A (701)	A (210)
22	G (200)	V (427)	L (106)
23	K (214)	T (287)	G (93)
24	A (302)	D (332)	A (147)
25	A (354)	M (420)	A (178)
26	L (204)	V (236)	A (183)
27	N (215)	N (168)	D (52)
28	A (244)	Q (126)	T (36)
29	V (174)		I (64)
30	S (58)		S (6)
31	E (24)		Q (9)
32	A (58)		
33	I (26)		

Amino acid residue identity and quantitative yield (pmol) are indicated for each cycle.

bicolor [21,22]. An interesting observation was that dermaseptin sVIII has only one site of amino acid substitution when compared with dermaseptin sI [23]. Unlike dermaseptin s1, mature dermaseptin sVI–sVIII peptides lacked the C-terminal tripeptide (-GE/AQ) of the precursor open-reading frame deduced from their respective cDNAs. MS/MS fragmentation of each peptide using a QTOF-Ultima instrument (Micromass, UK), established that each was C-terminally amidated with either an isoleucine amide (sVI) or a glutamine amide (sVII–sVIII) (data not shown). The glycyl residue downstream of each mature peptide thus acts as an amide donor.

4. Discussion

The defensive skin secretions of frogs are known to contain a plethora of biologically active peptides, some of which share common primary structural features with endogenous vertebrate regulatory peptides and others that appear to have no obvious structural counterparts in higher vertebrates [7]. The dermaseptins constitute a group of peptides that fall into the latter category displaying broad spectrum antimicrobial activity against both bacteria and fungi [15–19]. The role of this peptide family, typically consisting of 27–34 amino acid residues, is thought to be in defending the naked moist skin of the frogs against environmental or pathogenic microbial invasion. One must bear in mind that the integument of the amphibian plays many important physiological roles including respiration and osmoregulation—fundamental processes that could impinge upon the well-being of the animal if impaired by microbial colonisation. In fact, dermaseptins were the first vertebrate peptides to exhibit lethal effects against the filamentous fungi responsible for severe opportunistic infections accompanying immunodeficiency syndrome and the use of immunosuppressive agents [15]. These peptides thus play an important role in amphibian survival as the habitats of most if not all species also contain rich microbial floras. In chemical terms, these peptides are cationic, amphipathic peptides whose mode of antimicrobial action is thought to involve cell membrane interaction, integration and subsequent lysis [23]. In contrast to other peptides in the defensive skin secretion that interact with specific membrane receptors on entry into the tissues of the ingesting predator (bradykinins, tachykinins, bombesins, etc.), in a stereo-constrained fashion, the cationic amphipathic peptides can accumulate and accommodate more primary structural mutations without loss of efficacy. In the past, studies on amphibian skin peptides employed the pooled skin extracts or electrically stimulated secretions from many specimens of a given species resulting in the isolation and structural characterisation of many structural variants of antimicrobial peptides. We believe that these data represent the spectrum of variants within the species but may not reflect the structures as occurring in any one individual or related group. The data presented here in part substantiate this hypothesis. Here we report for the first time the isolation and structural characterisation of three novel dermaseptins (sVI–sVIII) from the defensive skin secretion of the previously and extensively studied South American leaf frog, *P. sauvagei.* We first cloned their respective encoding cDNAs using a novel method recently developed in our laboratory that permits both isolation and sequencing of peptides and cloning of homologous cDNAs from a single samples of lyophilised secretion [20,24]. Until now, whilst isolation and structural characterization of proteins/peptides could be achieved using lyophilised venom, construction of cDNA libraries for the purpose of molecular cloning of precursors necessitated sacrifice of the living specimen followed by removal of skin. These procedures thus made it extremely difficult to perform both aspects of study on the same specimens and made it impossible to perform sequential experiments in time. While it is acknowledged that the presence of mRNA in a particular tissue does not necessarily mean that a given protein/peptide is expressed there, the parallel identification of both transcript and translate in the

same biological specimen attributes extreme scientific robustness to the present data. The data also unequivocally attest to the fact that the sacrifice of frogs for skin cDNA library construction, hitherto regarded as a sine qua non for the cloning of granular gland secretion components, is unnecessary and that generation of appropriate molecular genetic data is not compromised.

As mentioned previously, the synthesis and secretion of broad-spectrum antimicrobial peptides by the skin granular glands of frogs and toads is an important feature of the defence strategy of these vertebrates [23]. Some of these are also synthesised in gastric and intestinal mucosa [25,26]. Many studies have found that antimicrobial peptides are frequently produced in multiple structurally related isoforms with each isoform displaying differential activity against a range of pathogenic microorganisms [23]. This may provide the explanation as to why within a given species pool, such diversity is evident. Each species of amphibian usually occupies a large geographical range and the species studied here, *P. sauvagei*, is an inhabitant of the Chaco (dry prairie) of Argentina, Brazil, Bolivia and Paraguay [13]. It would thus be expected that over such a wide range, the species would be subjected to a broad spectrum of different environmental and pathogenic microbes and hence it would be very likely that an endogenous genetic plasticity in innate defence mechanisms or molecules would reflect this. This may thus in part provide an insight into the development of races and sub-species that ultimately provides the basis for speciation events. Several studies are ongoing at present in our laboratories to address these questions in an objective manner. The relevance of these findings, however, is of more than academic interest. The emergence in recent years of strains of microorganisms that are resistant to commonly used antibiotics has stimulated the search for new naturally occurring bactericidal and fungicidal agents that may have clinical utility [6]. If the data presented in the present study can be extrapolated to other species, and there is some evidence that it can, then the numbers of permutations of cationic amphipathic antimicrobial peptides from amphibian sources is vastly greater than the current state of knowledge would suggest. Thus the likelihood that somewhere in the biosphere the molecular survival machinery of an amphibian has solved our problem of treating multidrug-resistant pathogenic infections in humans with an efficaceous peptide is greatly increased. Apart from this, the study of such molecules, generated by natural combinatorial chemistry events over millions of years and naturally selected for effectiveness, can provide much insight into structure/activity requirements for therapeutic antimicrobial drug design.

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