Amino Acids (2013) 44:1347–1355
DOI 10.1007/s00726-013-1470-8

ORIGINAL ARTICLE

Senegalin: a novel antimicrobial/myotropic hexadecapeptide from the skin secretion of the African running frog, *Kassina senegalensis*

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Received: 24 November 2012 / Accepted: 11 February 2013 / Published online: 22 February 2013

Abstract Amphibian skin is a rich and unique source of novel bioactive peptides most of which are endowed with either antimicrobial or pharmacological properties. Here, we report the identification and structural characterization of a novel peptide, named senegalin, which possesses both activities. Senegalin is a hexadecapeptide amide (FLPFLI PALTSLISSL-NH_2) of unique primary structure found in the skin secretion of the African running frog, *Kassina senegalensis*. The structure of the biosynthetic precursor of senegalin, deduced from cloned skin cDNA, consists of 76 amino acid residues and displays the typical domain organization of an amphibian skin peptide precursor. Both natural senegalin and its synthetic replicate displayed antimicrobial and myotropic activities. Senegalin was active against *Staphylococcus aureus* (MIC 50 μM) and *Candida albicans* (MIC 150 μM) but was non-haemolytic at concentrations up to and including 150 μM. In contrast, senegalin induced a dose-dependent contraction of rat urinary bladder smooth muscle (EC_{50} 2.9 nM) and a dose-dependent relaxation of rat tail artery smooth muscle (EC_{50} 37.7 nM). Senegalin thus represents a prototype biologically active amphibian skin peptide and illustrates the fact that amphibian skin secretion peptidomes continue to be unique sources of such molecules.

Keywords Amphibian · Peptide · Antimicrobial · Smooth muscle · Molecular cloning

Introduction

The potential of amphibian skin secretions for the discovery of novel pharmacologically active peptides is now well recognized by scientists from many disciplines in the biosciences. The pioneering work of the Italian scientist Vittorio Erspamer and his colleagues in the 1960s was largely responsible for bringing this potential to the attention of researchers with the discoveries of peptides such as caerulein and bombesin and the selective opiate receptor ligands, the dermorphins and deltorphins (Erspamer 1994). These reports and the many discoveries have shown unequivocally that amphibian skin is an extraordinary source of biologically active peptides, most of which fall into two broad classes: (1) pharmacologically active and (2) potent broad-spectrum antimicrobials (Bevins and Zasloff 1990; Erspamer 1994). Many of the pharmacologically active skin peptides are either identical to or contain the same biologically active core sequences of endogenous vertebrate regulatory peptides (Bevins and Zasloff 1990; Erspamer 1994). Often the amphibian skin homologs are more potent than their endogenous regulatory peptide counterparts due to selective post-translational modifications or specific amino acid substitutions (Bevins and Zasloff 1990; Erspamer 1994). Occasionally, peptides that display profound and potent effects on vertebrate tissues are found to be of unique primary structures having no

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obvious counterparts in contemporary databases of natural peptide and protein structures, even within those that are generated from translation of gene sequences from systematic sequencing projects. This phenomenon has led to the speculation that these peptides are either three-dimensional mimics of existing molecules or indeed may have arisen through natural selection for purpose as defensive agents (Clarke 1997; Duda et al. 2002; Pukala et al. 2006). Nevertheless, such peptides could provide much useful information to pharmaceutical scientists for drug design, and this type of approach has been hailed as 'chemical prospecting' which involves utilizing clues from an animal's behavior or its interaction with its environment or with other animals to indicate the presence of such compounds (Clarke 1997; Baker et al. 2007; Tempone et al. 2007).

Here, we describe a novel 16-mer C-terminally amidated peptide, named senegalin, isolated from the defensive skin secretion of the African running frog, *Kassina senegalensis*. Senegalin has no obvious counterparts in contemporary databases of peptide or protein structures and thus represents a new chemical entity. Molecular cloning of the cDNA encoding its biosynthetic precursor, indicated that it is encoded as a single copy within a precursor protein of 76 amino acid residues consisting of a signal peptide domain, an acidic amino acid residue-rich spacer domain and a mature peptide-encoding domain flanked N-terminally by a classical –Lys-Arg- (KR) propeptide convertase processing site and C-terminally by a Gly (G) residue amide donor. A synthetic replicate of senegalin displayed broad-spectrum activity against the Gram-positive bacterium, *Staphylococcus aureus,* and the yeast, *Candida albicans*. In addition, senegalin induced potent (low nanomolar) and dose-dependent contractions of rat urinary bladder and relaxation of tail artery smooth muscle preparations.

Materials and methods

Specimen biodata and harvesting of skin secretion

Specimens of *K. senegalensis* ($n = 3$) were obtained from several sources and had all been captive-bred. The frogs were adults (4 cm snout-to-vent length) and were maintained for a period of 4 months prior to secretion harvesting. They were housed in our purpose-designed amphibian facility at between 18 and 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 dorsal skin by gentle transdermal electrical stimulation using the technique of Tyler et al. (1992). Briefly, the moistened skin was stimulated by platinum electrodes (6 V DC; 4-ms pulse-width; 50 Hz) for two periods of 20-s duration following which the secretion was washed from the skin using deionized water, snap-frozen in liquid nitrogen and lyophilized. Lyophilizates were stored at −20 C prior to analysis. All procedures were carried out following approval under local university and UK national animal experimentation rules.

Fractionation of skin secretion by reverse-phase HPLC

Five milligram of lyophilized skin secretion was dissolved in 0.5 ml of trifluoroacetic acid (TFA)/water (0.1:99.9; v/v), clarified by centrifugation and the decanted supernatant was then subjected to reverse-phase HPLC fractionation using a Cecil Adept Binary HPLC system (Cambridge, UK) fitted with a Jupiter C-5 analytical column (250 × 4.6 mm). Bound peptides were eluted with a linear gradient formed from 0.05/99.5 (v/v) TFA/water to 0.05/19.95/80.0 (v/v/v) TFA/water/acetonitrile in 240 min at a flow rate of 1 ml/min. Fractions (1 ml) were collected at minute intervals and the effluent absorbance was continuously monitored at λ214 nm. Two 100-µl samples from each chromatographic fraction were removed, lyophilized and stored at −20 °C prior to analysis for smooth muscle and antimicrobial activities, respectively.

Structural analyses and chemical synthesis of the isolated peptide

Reverse-phase HPLC fraction #117 was found to possess both smooth muscle and antimicrobial activities and a sample of this fraction was subjected to tandem MS/MS fragmentation sequencing using an LCQ-Fleet electrospray ion-trap mass spectrometer (Thermo Fisher Scientific, CA, USA). The sample was introduced into the mass spectrometer in positive-ion mode at a flow rate of 100 µl/min and a spray voltage of 4.5 kV. The doubly charged ion at m/z 866.56 was trapped and subjected to CID fragmentation at normalized collision energy of 60 arbitrary units. After establishment of the unequivocal primary structure of the peptide using Proteome Discoverer vs.1 software, a replicate was synthesized by solid-phase Fmoc chemistry using a PS3 automated peptide synthesizer (Protein Technologies, Tucson, AZ, USA). After completion of all amino acid coupling cycles, peptides were cleaved from the synthesis resin, deprotected and purified of contaminants by reverse phase HPLC. The degree of purity and authentication of structure of synthetic peptide was determined using MALDI-TOF MS and reverse phase HPLC.

Molecular cloning of the cDNA encoding the biosynthetic precursor of the novel peptide

A 5-mg sample of lyophilized skin secretion was dissolved in 1 ml of cell lysis/mRNA protection buffer supplied by Dynal Biotec, UK. Polyadenylated mRNA was isolated by the use of magnetic oligo-dT beads as described by the manufacturer (Dynal Biotec, UK). The isolated mRNA was subjected to 5′ and 3′-rapid amplification of cDNA ends (RACE) procedures to obtain full-length peptide biosynthetic precursor nucleic acid sequence data using a SMART-RACE kit (Clontech, UK) essentially as described by the manufacturer. Briefly, the 3′-RACE reaction employed a nested universal (NUP) primer (supplied with the kit) and a degenerate sense primer (S: 5′-CCTTTYH-TIHTICCIGCIHT-3′) that was complementary to the internal amino acid sequence, -P–F-L/I-L/I–P-A-L/I- of the novel peptide. The 3′-RACE reaction was purified and cloned using a pGEM-T vector system (Promega Corporation) and sequenced using an ABI 3100 automated sequencer. The sequence data obtained from the 3′-RACE product was used to design a specific antisense primer (AS: 5′-GACTTCCACAGAGGTGGGAGTT-3′) to a conserved site within the 3′-non-translated region of the novel peptide precursor-encoding cDNA. 5′-RACE was carried out using this specific primer in conjunction with the NUP RACE primer and resultant products were purified, cloned and sequenced.

Rat smooth muscle bioassays

Male Wistar rats (250–300 g) were euthanized by carbon dioxide asphyxiation followed by cervical dislocation under appropriate UK animal licences and following local ethical committee approval. The rats were placed dorsal surface down and the abdomen was opened by means of an incision along the mid ventral line and subcutaneous fat was carefully dissected. The exposed urinary bladder was removed from each rat, emptied of urine and placed in ice-cold Kreb's solution (118 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.15 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.1 mM $MgCl_2$ and 5.6 mM glucose), equilibrated with 95 % O_2, 5 % CO_2. Muscle strips, 2×10 mm^2, were dissected from the bladder under a dissection microscope. These were tied at each end with a fine silk ligature (0.2 mm) with one end subsequently attached to a fixed pin and the other, to a transducer in a 2 ml organ bath containing Kreb's solution at 37 °C flowing at 2 ml/min with constant bubbling of 95 % O_2, 5 % CO_2. After a 20 min equilibration period, muscle strips were tested for viability using 60 mM KCl.

For arterial smooth muscle preparations, the tail artery vascular bed was identified and moistened with Krebs' solution. The membrane and the connective tissue beneath the main artery were carefully removed. The proximal region of the tail artery was excised and immediately placed in ice-cold Kreb's solution. Two-millimeter-wide rings of artery were cut and mounted on a transducer prior to placing in 2-ml capacity organ baths containing Kreb's solution flowing through at 2 ml/min and maintained at 37 °C with constant bubbling of carbogen gas mixture. Muscle rings were equilibrated for 1 h before experimental procedures were initiated.

The synthetic replicate of the novel peptide was initially prepared as a stock solution in Kreb's buffer at a concentration of 10^{-3} M. Sequential molar concentrations of the peptide were prepared from these in the range of 10^{-5} to 10^{-11} M prior to each experiment. Dose–response curves were constructed for the peptide on individual urinary bladder and arterial smooth muscle preparations ($n = 6$ in each case). Tension changes in the smooth muscle preparations were detected by force transducers connected to a Power Lab System (AD Instruments Pty Ltd.). Data were analyzed to obtain the mean and standard errors of responses by Student's *t*-Test and dose–response curves were constructed using a best-fit algorithm through the data analysis package provided.

Antimicrobial assays

The test organisms, *E. coli* (NCTC 10418)—Gram-negative, *S. aureus* (NCTC 10788)—Gram-positive, and *C. albicans* (NCPF 1467)—a yeast, were grown in Muellar–Hinton Broth (MHB) for 18 h in an orbital incubator at 37 °C. Assays with antimicrobial peptides were carried out essentially as described previously (Zhang et al. 2010). The lowest concentration showing no growth was recorded as the minimal inhibitory concentration (MIC).

Hemolysis assay

A 2 % (v/v) suspension of horse erythrocytes in sodium phosphate-buffered saline (PBS), pH 7.2, was prepared from defibrinated horse blood (TCS Biosciences Ltd, UK). Erythrocyte suspensions (200 µl) were incubated with a range of concentrations of the novel peptide, similar to those employed for antimicrobial activity assays, at 37 °C for 60 and 120 min. Cell lysis was assessed by measurement of optical density at $\lambda = 550$ nm using an ELISA plate reader (Biolise BioTek EL808). Negative controls employed consisted of a 2 % (v/v) erythrocyte suspension and PBS in equal volumes and positive controls consisted of a 2 % (v/v) erythrocyte suspension and an equal volume of PBS containing 2 % (v/v) of the non-ionic detergent, Triton X-100 (Sigma–Aldrich).

Results

Isolation and structural characterization of senegalin

Screening of the reverse-phase HPLC fractions from the skin secretion of the African running frog, *K. senegalensis* (Fig. 1), identified a fraction that possessed both myotropic and antimicrobial activities. Fraction #117, containing both of these activities, was analyzed and the primary structure of its main component peptide was determined by LC/MS/MS fragmentation sequencing (Fig. 2). The lack of unequivocal assignation of L (Leu) and I (Ile) was due to their isobaric nature but this was made possible following acquisition of translated open-reading frame amino acid sequence from the cloned biosynthetic precursor-encoding cDNA (Fig. 3).

Interrogation of contemporary protein/peptide databases by FASTA and BLAST Internet sequence alignment tools indicated that the peptide exhibited little structural similarity with any known peptide. By nature of its novel structure and bioactivity, this peptide was named, senegalin, as a consequence of its unique structure and frog species of origin.

Molecular cloning of senegalin biosynthetic precursor-encoding cDNA and bioinformatic analyses

A cDNA encoding the biosynthetic precursor of senegalin was successfully and repeatedly cloned from the skin secretion library using the RACE protocol described (Fig. 3). The open-reading frame consisted of 76 amino acid residues organized into the readily identifiable domain architecture of an amphibian skin peptide precursor (Fig. 4). The putative signal peptide consisted of 22/23 residues followed immediately by the first of six typical KR-(-Lys-Arg-) propeptide convertase processing sites. A tripeptide sequence (-SDG-) separated this first processing site from the second that was upstream of the first of three identical acidic amino acid residue-rich spacer peptides (-ADEEGED-). This organization was repeated several times up to the pair of processing sites that occur in tandem, flanking the N-terminus of the mature peptide, senegalin. The single copy of senegalin was flanked C-terminally by a canonical glycyl residue that acts as an amide donor to generate the terminal leucinamide. BLAST analysis of senegalin using the online portal of the US National Center for Biotechnological Information (NCBI) revealed that this peptide displayed no structural identity with any archived peptide from any origin. In contrast, the same analysis with the signal peptide sequence, showed that it displayed a high degree of identity with the signal peptides of at least ten other cloned skin peptide precursors from *Kassina* frogs (Fig. 5a). In addition, BLAST analysis of the acidic amino acid residue-rich spacer peptide domain showed that it displayed total identity with that of the kassinakinin S precursor and just a single amino acid difference (D/E) with the kassorin S precursor both from *K. senegalensis* (Fig. 5b). Secondary structure prediction analysis of senegalin using the Swiss-Model Workspace (Arnold et al. 2006) found that senegalin was of insufficient chain length (16 residues) to perform this. However, performing this analysis using the entire biosynthetic precursor sequence (Fig. 6) showed that the senegalin-encoding domain was indeed predicted to be α-helical. The membrane-interacting signal peptide domain was likewise predicted to contain significant α-helix. The nucleotide sequence of the senegalin precursor has been deposited in the EMBL Nucleotide Sequence Database under the accession code HE863807.

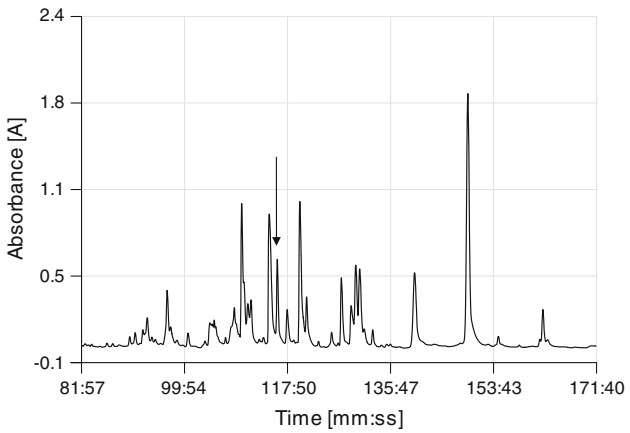

Fig. 1 Region of reverse phase HPLC chromatogram of *Kassina senegalensis* skin secretion. The elution position/retention time of the antimicrobial/myotropic peptide corresponding to senegalin is indicated by an *arrow*

Antimicrobial/hemolytic activity of senegalin

Senegalin was found to be ineffective against the Gram-negative bacterium, *E. coli*, but displayed growth inhibitory activity against the Gram-positive bacterium, *S. aureus* with a minimal inhibitory concentration (MIC) of 50 μM, and against the pathogenic yeast, *C. albicans*, with an MIC of 150 μM. The peptide possessed little significant hemolytic activity up to and including concentrations of 150 μM.

Smooth muscle pharmacology

Senegalin was found to produce a dose-dependent contraction of rat urinary bladder smooth (EC_{50} 2.9 nM) (Fig. 7a) and a dose-dependent relaxation of rat arterial smooth muscle (EC_{50} 37.7 nM) (Fig. 7b).

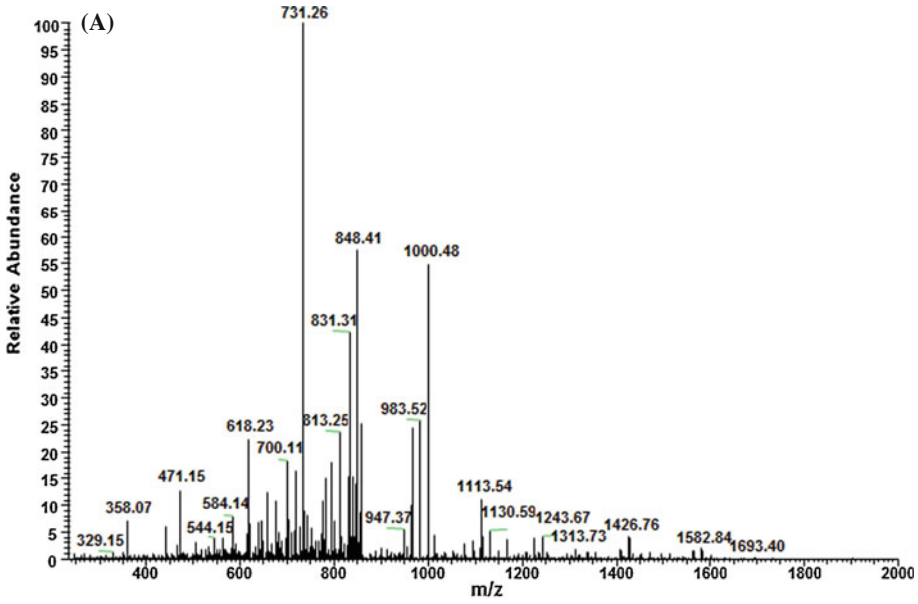

Fig. 2 Structural characterization of natural senegalin. (**a**) MS/MS fragmentation spectrum of the doubly charged ion (*m/z* 866.56) of senegalin. (**b**) Predicted singly- and doubly-charged *b*-ions and *y*-ions arising from MS/MS fragmentation of senegalin. Fragment ions observed are indicated in *bold typeface* and are *underlined*. The primary structure of senegalin is indicated. Note the equivocal identification of isobaric Leu (L) and Ile (I) amino acid residues at positions 2, 5, 6, 9, 12, 13 and 16

(B)

#1	b(1+)	b(2+)	Seq.	y(1+)	y(2+)	#2
1	148.07570	74.54149	F			16
2	**261.15977**	131.08352	L/I	**1583.97717**	**792.49222**	15
3	**358.21254**	179.60991	P	**1470.89310**	**735.95019**	14
4	**505.28096**	**253.14412**	F	**1373.84033**	**687.42380**	13
5	**618.36503**	**309.68615**	L/I	**1226.77191**	**613.88959**	12
6	**731.44910**	366.22819	L/I	**1113.68784**	**557.34756**	11
7	**828.50187**	**414.75457**	P	**1000.60377**	**500.80552**	10
8	**899.53899**	**450.27313**	A	**903.55100**	**452.27914**	9
9	**1012.62306**	506.81517	L/I	**832.51388**	**416.76058**	8
10	**1113.67074**	**557.33901**	T	**719.42981**	360.21854	7
11	**1200.70277**	**600.85502**	S	**618.38213**	**309.69470**	6
12	**1313.78684**	**657.39706**	L/I	**531.35010**	**266.17869**	5
13	**1426.87091**	**713.93909**	L/I	**418.26603**	209.63665	4
14	**1513.90294**	**757.45511**	S	**305.18196**	153.09462	3
15	**1600.93497**	**800.97112**	S	218.14993	109.57860	2
16			L/I-Amidated	131.11790	66.06259	1

Discussion

The defensive skin secretions of anuran amphibians have long been known to contain many diverse bioactive compounds, especially a striking abundance and diversity of biologically active peptides (Bevins and Zasloff 1990; Erspamer 1994; Clarke 1997). However, the skin secretions of the vast majority of species remain unstudied and as a consequence, a true picture of this vast diversity remains to be revealed. Although novel peptides continue to be discovered in the secretions of members of well-studied taxa, such as phyllomedusine and ranid frogs (Conlon et al. 2004; Azevedo Calderon et al. 2011), the greatest potential for discovery is proving to be in those that have been established as having complex secretions yet have been poorly studied to date, such as the African kassinid frogs (Matutte et al. 2000; Wang et al. 2009a, 2009b, 2009c; Chen et al. 2011; Wang et al. 2012).

The emergence in recent years of strains of microorganisms that are resistant to commonly used antibiotics has stimulated a search for new naturally occurring bactericidal and fungicidal lead compounds. The amphipathic polypeptides of amphibian skin granular glands represent an important feature of the defense strategy of these animals and currently constitute one of the largest cohorts of this class of natural antibiotic chemicals (Simmaco et al. 1998; Conlon et al. 2004; Azevedo Calderon et al. 2011). These antimicrobial peptides are usually different in structure between species and are invariably present in multiple structurally related isoforms within a species with each

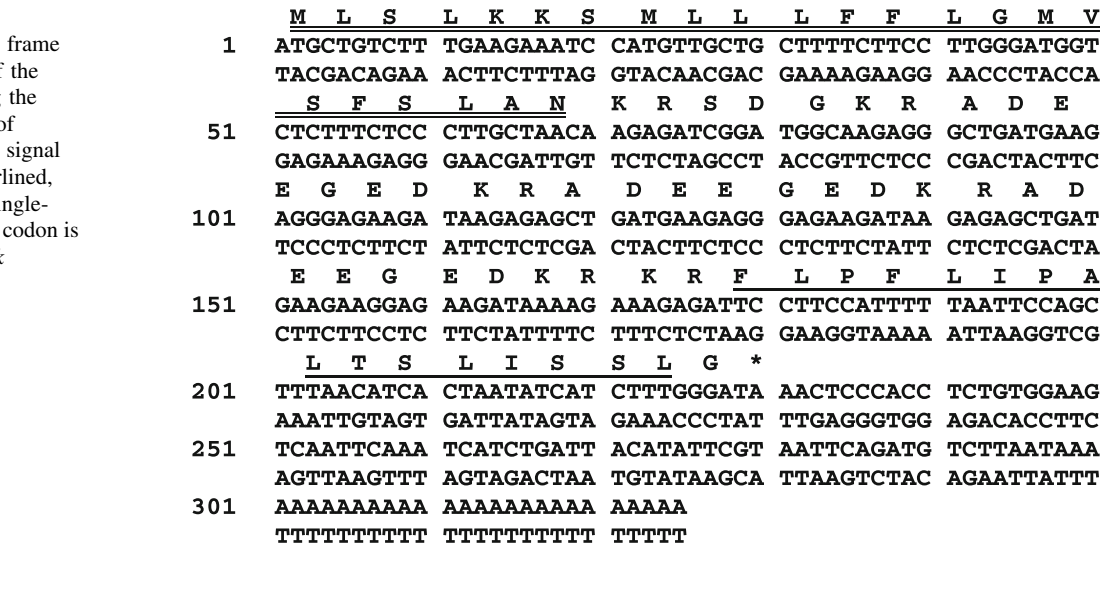

Fig. 3 Nucleotide and translated open-reading frame amino acid sequence of the cloned cDNA encoding the biosynthetic precursor of senegalin. The putative signal peptide is double-underlined, the mature peptide is single-underlined and the stop codon is indicated by an *asterisk*

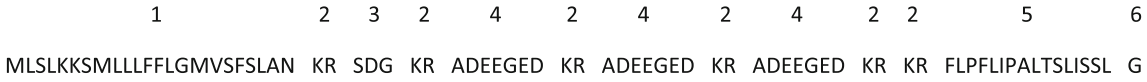

Fig. 4 Domain architecture of the senegalin precursor. *1* Putative signal peptide. *2* Propeptide convertase dibasic amino acid processing sites ($n = 6$). *3* Extension peptide. *4* Acidic amino acid residue-rich spacer peptides ($n = 3$). *5* Mature senegalin. *6* C-terminal glycyl residue amide donor

(A)

	Peptide precursor	Signal peptide	Accession No.
1.	**Senegalin**	MLSLKKSMLLLFFLGMVSFSLA	
2.	**Kasseptin 1Ma**	----------	CAX33885.1
3.	**Kassorin M**	--T----------	CBK52153.1
4.	**Kasseptin 1Md**	----------I-	CAX33888.1
5.	**Kasseptin 1Mc**	----------I-	CAX33887.1
6.	**Kasseptin 1Mb**	----------I-	CAX33886.1
7.	**Kassorin S**	--T----------	CBL43005.1
8.	**IF-8**	--T-RT----------	CAX53224.1
9.	**Kassorin SL**	--T----------S---	CBL51549.1
10.	**Kasstasin**	-M----------	GOLWV9.1
11.	**Galensin**	-T----------L--V---	Q90W78.1

(B)

	Peptide precursor	Acidic spacer peptide	Accession No.
1.	**Senegalin**	KRADEEGEDKRADEEGEDKRADEEGEDKRKR	
2.	**Kassinakinin S**	----------	CAI43965.1
3.	**Kassorin S**	----------E----	CBL43005.1

Fig. 5 BLAST analysis/alignment data arising from interrogation of the NCBI protein database with the amino acid sequences of two domains from the biosynthetic precursor of senegalin. **a** The signal peptide of the senegalin precursor was found to be highly conserved between skin peptide precursors from *K. senegalensis* and *K. maculata*, and likewise in (**b**), the primary structure of the acidic spacer peptide was identical to or exhibited one amino acid substitution with the corresponding domains of the precursors of *kassinakinin S* and *kassorin S*. *Accession numbers* for all database sequence entries are shown

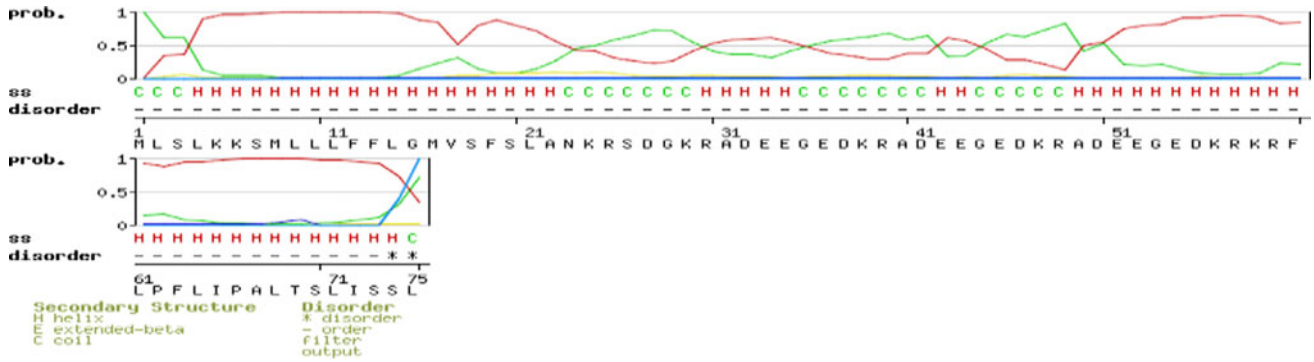

Fig. 6 Secondary structure prediction analysis of the senegalin precursor protein. Residues 1–23 represent the putative signal peptide domain that is essentially predominantly α-helical. Residues 60–75 represent the mature senegalin peptide domain that is likewise essentially α-helical. The mature senegalin peptide itself was too short in chain length to be recognized by the predictive software employed in Swiss-Model

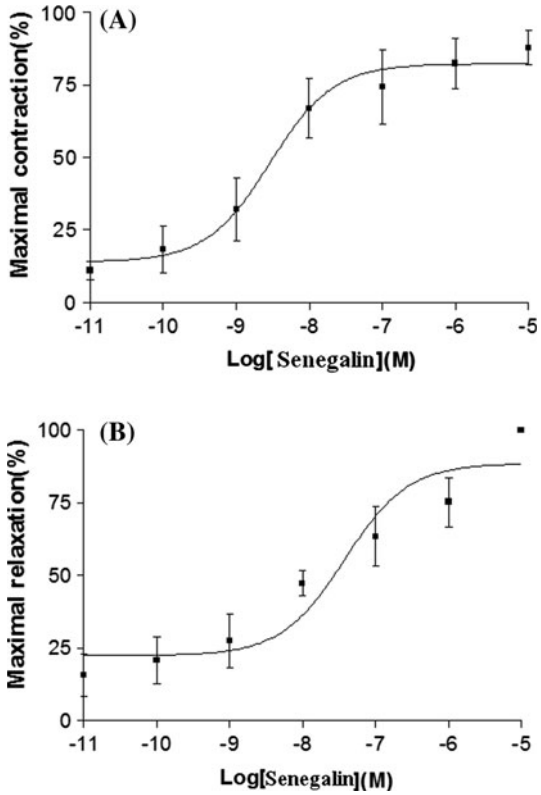

Fig. 7 Pharmacological characterization of synthetic senegalin using rat smooth muscle preparations. **a** Dose–response curve of contraction of urinary bladder smooth muscle and **b** dose–response curve of relaxation of tail artery smooth muscle. Each data-point represents the mean and standard error of six replicates. EC_{50} for contraction of urinary bladder smooth muscle was 2.9 nM and for relaxation of tail artery smooth muscle was 37.7 nM

isoform often displaying differential activity against a range of pathogenic microorganisms (Simmaco et al. 1998; Conlon et al. 2004; Azevedo Calderon et al. 2011). The defensive skin secretions of African kassinid frogs contain complex peptidomes that have yielded several groups of novel peptides with antimicrobial activities that include kassinatuerins 1 and 2 (Matutte et al. 2000), kassinatuerin 2-related peptides (Wang et al. 2009c) and kassorins (Chen et al. 2011).

Here, we report the isolation and structural characterization of a novel peptide, named senegalin, from the skin secretion of *K. senegalensis*, which displays both selective antimicrobial and myotropic activities. A cDNA encoding the senegalin biosynthetic precursor was also cloned from a skin-secretion derived cDNA library and the translated amino acid sequence of the mature senegalin peptide confirmed the sequence obtained through MS/MS fragmentation and resolved the ambiguity and positioning of the isobaric Leu (L) and Ile (I) residues. The C-terminal amidation status of the mature peptide was also confirmed by the strategic positioning of an amide donating Gly (G) residue. NCBI BLAST analysis of the structure of senegalin failed to produce any hits among known antimicrobial peptides from any origin, indicative of its novelty. However, a similar analysis of the putative signal peptide domain established its very high degree of primary structural conservation with the corresponding domains of previously reported kassinid frog skin peptides including kasseptins, kassorins, kasstasin, galensin and peptide IF-8 (Fig. 5a). Likewise, the acidic amino acid residue-rich spacer domain was identical to that of the kassinakinin S precursor (Chen et al. 2005) and with one residue exception, and to that of the kassorin S precursor (Chen et al. 2011). These data elegantly illustrate how our "shotgun" cloning approach directed toward transcripts encoding amphibian skin peptide precursors can work efficiently when one is forearmed with the knowledge of highly conserved regions of nucleotide and amino acid sequences for universal primer design that can work on cDNA libraries from a single species, from species within the same genus or family, and even in

some instances, for anurans in general (Vanhoye et al. 2003; Zhou et al. 2007).

Many amphibian skin peptides that display antimicrobial activity have predicted secondary structures that are predominantly α-helical in nature—a feature that facilitates interaction with their bacterial target membranes (Simmaco et al. 1998; Haney et al. 2009; Amiche and Galanth 2011). However, the 16-mer senegalin was found to be too short to perform the secondary structure prediction analysis using the Swiss Model workspace (Arnold et al. 2006), so the entire sequence of the precursor was employed. As expected, the signal peptide was predicted to be essentially α-helical due to its fundamental function in membrane translocation of synthesized proteins destined for secretion and in addition, the domain encoding senegalin was also predicted to be essentially α-helical (Fig. 6).

In terms of biological functions, senegalin was found to be of moderate antimicrobial potency against two of the test micro-organisms employed, the Gram-positive bacterium, *S. aureus* and the yeast, *C. albicans*, but was not active against the Gram-negative bacterium, *E. coli.* However, senegalin displayed little hemolytic activity at concentrations up to and including 150 μM. Despite the moderate antimicrobial activity, it should be remembered that senegalin occurs in the defensive skin secretion alongside other types of antimicrobial peptides and it may be that interaction and possibly synergism takes place between these different types of peptide in nature, as has been demonstrated for the magainins of *Xenopus laevis* (Maloy and Kari 1995).

However, in contrast to the moderate antimicrobial potency displayed by senegalin, it was found to be a highly potent in contracting the smooth muscle of rat urinary bladder (EC_{50} of 2.9 nM) and in relaxing the smooth muscle of rat tail artery (EC_{50} of 37.7 nM) (Fig. 7). These potencies are as high if not higher than many established physiological agents and would be consistent with rather specific target interactions rather than by non-specific effects. Such targets could most likely be G protein-linked receptors (GPCRs) and many receptors of this class, both those predicted from the human genome and those demonstrated to be expressed, remain as "orphans" with no high-affinity specific ligands identified to date (Levoye et al. 2006; Allen and Roth 2011). It may be that some of the novel peptides identified in amphibian skin secretions represent cognate ligands for some of these "orphan" receptors and studies focused on this possibility are underway at present.

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