

## Kassinakinin S: A novel histamine-releasing heptadecapeptide from frog (*Kassina senegalensis*) skin secretion

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Received 1 September 2005

Available online 21 September 2005

### Abstract

Amphibian defensive skin secretions remain a largely untapped resource for the peptide biochemist with an interest in the identification, structural characterization, and precursor cDNA cloning of novel bioactive peptides. Here we report the isolation, structural characterization, functional profiling, and nucleotide sequence of precursor cDNA of a novel histamine-releasing heptadecapeptide, FIPVTLALHKEKLN-amide, from the defensive skin secretion of the African running frog, *Kassina senegalensis*. This peptide was found to be a potent histamine secretagogue ( $EC_{50} = 6 \mu\text{M}$ ; maximal release =  $25 \mu\text{M}$ ) in a rat peritoneal mast cell model system and was accordingly named kassinakinin S. The open-reading frame of the cDNA encoding prepro-kassinakinin S was found to consist of 71 amino acid residues containing a single copy of kassinakinin S and its glycyl residue amide donor at the C-terminus. Kassinakinin S can thus be added to the growing list of amphibian skin bioactive peptide prototypes.

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**Keywords:** Amphibian; Mast cells; Degranulation; Molecular cloning; Mass spectrometry

An acute inflammatory response often rapidly follows envenomation [1]. The classical local symptoms, such as pain, edema, and vasodilation, have been related to mast cell histamine release at the site of injection and potentially life-threatening systemic sequelae, manifest as anaphylactic shock, occur in a relatively small number of individuals, especially those that have been previously sensitized [2]. In the majority of cases, individuals have been stung by insects, most commonly wasps, hornets, and bees [3]. It is therefore not surprising that most biochemical research has been directed at the determination of the chemical nature of the evocative agents within venoms derived from these species [4,5]. Several classes of peptides have been isolated from these sources including mastoparan (histamine-releasing peptide I) and histamine-releasing peptide II from the venom of the Oriental hornet (*Vespa orientalis*) [6], melittin and mast cell-degranulating peptides (MCDP) from honeybee (*Apis mellifera*) venom [7,8], bombolitin and

MCDP from bumblebee (*Bombus pennsylvanicus*) venom [9,10], and several interesting bradykinin-like peptides from a wide range of wasp and hornet venoms including megascoliakinin from the garden dagger wasp (*Megascolia flavifrons*) [11], waspkinin from *Parapolybia indica* [12], and vespakinin M from *Vespa mandarinia* [13].

Amphibians, unlike other venomous animals such as jellyfish, cone shells, insects, scorpions, spiders, and snakes, do not employ their “venom” primarily for prey capture but rather utilize their toxic skin secretions in defense against predators which may explain why they have not evolved penetrative structures to deliver their noxious secretions directly into the tissues of the assailant. The buccal cavity is thus the first site of contact between the defensive secretions and the tissues of the predator, and application of skin secretions of the African clawed toad, *Xenopus laevis*, to the oral cavity of colubrid snakes rapidly induces yawning and gaping behaviors and other oral dyskinesias [14]. While it is well recognized that amphibian skin secretions are complex mixtures of biologically active molecules, especially peptides, the total inventory of such

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in even a single species remains to be realized. As histamine-releasing peptides are well represented in the venoms of invertebrates, it is not unlikely that such peptides exist in amphibian defensive skin secretions. In fact, while a few peptides with such activity have previously been identified in these [15–18], there has been little focused research effort using this bioactivity as an end-point.

Here, we report the primary structure of a prototype histamine-releasing peptide isolated and structurally characterized from the defensive skin secretion of the African running frog, *Kassina senegalensis*. The peptide displays little significant structural similarity to any peptide/protein in contemporary databases at the National Center for Biotechnological Information (NCBI), with the exception of histamine-releasing peptide II from the venom of the Oriental hornet (*Vespa orientalis*) [6,19]. The peptide was named kassinakinin S and the cDNA encoding its precursor was cloned from a library constructed from the same sample of lyophilized venom used in its isolation [20,21].

## Materials and methods

**Specimen biodata and secretion harvesting.** *Kassina senegalensis* ( $n = 3$ ) were obtained from a commercial source and had been captive bred in the United States. The frogs were adults on receipt (2 males, 3 cm snout-to-vent length, 1 female, 5 cm snout-to-vent length) and were settled into their new surroundings for 4 months prior to secretion harvesting. They were maintained in our purpose-designed amphibian facility at 20–25 °C under a 12 h/12 h light/dark cycle and fed multivitamin-loaded crickets three times per week. Skin secretion was obtained from dorsal skin by mild transdermal electrical stimulation (5 V; 3 ms pulses) for 30 s. The skin secretion was washed from the skin using deionized water, snap-frozen in liquid nitrogen, and lyophilized. Lyophilizate was stored at –20 °C prior to analysis.

**Semi-preparative reverse phase HPLC.** Lyophilized skin secretion (35 mg) from *K. senegalensis* was reconstituted in 1 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 0.05/19.95/80.0 (v/v/v) TFA/water/acetonitrile in 80 min at a flow rate of 2 ml/min. A Thermoquest gradient reversed phase HPLC system, fitted with a semi-preparative column (Jupiter C-5, 5 $\mu$  particle, 300 Å pore, 300 × 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  $\mu$ l). Fractions of approximately 1.8 ml were collected at minute intervals. Samples (100  $\mu$ l) from each chromatographic fraction were removed, lyophilized, and subjected to assay for histamine release.

**Histamine release assay.** Male Hooded Lister rats (150–205 g body weight) were lightly anesthetized with CO<sub>2</sub> and then killed by cervical dislocation and exsanguination. Mixed peritoneal cells were obtained as described by Cross et al. [22]. The cells were washed twice in Tyrode's buffer (NaCl (137 mM), glucose (5.6 mM), Hepes (10 mM), KCl (2.7 mM), MgCl<sub>2</sub>·6H<sub>2</sub>O (1 mM), CaCl<sub>2</sub>·2H<sub>2</sub>O (1 mM) and NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O (0.4 mM), pH 7.4) and recovered by centrifugation (100g, 4 °C, 2 min). Isolated peritoneal cells (100  $\mu$ l) were dispensed into conical polystyrene test tubes and pre-warmed to 37 °C for 5 min. Lyophilized aliquots of chromatographic fractions were reconstituted in Tyrode's buffer (100  $\mu$ l) and added to peritoneal cell suspensions. Following incubation (10 min, 37 °C), the reaction was quenched by addition of ice-cold

Tyrode's buffer (2.8 ml). The cell suspensions were centrifuged as above and both the supernatants and the cell pellets were analyzed for histamine content using the fluorimetric method based on Shore et al. [23]. Histamine release was expressed as the percentage of the total cellular content and values were corrected for spontaneous release. Spontaneous histamine-release in the absence of peptides did not exceed 5%.

**Identification and structural analysis of kassinakinin S.** Semi-preparative reverse phase HPLC fractions containing histamine-releasing activity (#s 35, 40, 47, and 50) were lyophilized and separately subjected to LC/MS using a gradient formed from 0.05/99.5 (v/v) trifluoroacetic acid (TFA)/water to 0.05/19.95/80.0 (v/v/v) TFA/water/acetonitrile in 80 min at a flow rate of 1 ml/min. The remainder of the chromatographic conditions and hardware employed were the same as described for semi-preparative fractionation of crude secretion, except for the use of an analytical column of different column chemistry (Jupiter C-18, 5 $\mu$  particle, 300 Å pore, 250 × 4.6 mm, Phenomenex, UK). Fraction size was approximately 900  $\mu$ l. Samples of 100  $\mu$ l from each fraction were removed, lyophilized, and following reconstitution in appropriate buffer, were assayed for histamine-releasing activity. The fraction in each case, containing maximal activity and containing a single predominant peptide, was subjected to primary structural analyses by automated Edman degradation using an Applied Biosystems 491 Procise sequencer in pulsed-liquid mode or by MS/MS fragmentation sequencing using the LCQ. Secondary structural prediction analysis of kassinakinin S was performed using on-line programs available through Network Protein Sequence Analysis on the HUPO website (Human Proteome Organisation (HUPO), <http://211.32.65.137/link/tools.htm>).

**Synthesis of kassinakinin S and dose–response evaluation.** Kassinakinin S was synthesized automatically using the solid-phase method (Wang resin, 0.6 mmol/g) and standard Fmoc chemistry using an Applied Biosystems 433 peptide synthesizer. After cleavage of the peptide from the resin and deblocking, the resultant material was purified and the final product was structurally verified by LC/MS. Dose–response curves of histamine release using the rat peritoneal mast cell preparation previously described were constructed using the synthetic replicate of kassinakinin S at a range of concentrations up to 25  $\mu$ M.

**Cloning of prepro-kassinakinin S cDNA.** A five milligram 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 end (RACE) procedures to obtain full-length prepro-kassinakinin S nucleic acid sequence data using a SMART-RACE kit (Clontech UK) essentially as described by the manufacturer. Briefly, the 3'-RACE reactions employed a nested universal (NUP) primer (supplied with the kit) and a sense primer (S: 5'-TTYATHCCIGTIACIYTIYTIGC-3') that was complementary to the amino acid sequence, -FIPVTLA-, of kassinakinin S. The 3'-RACE reaction was purified and cloned using a pGEM-T vector system (Promega) and sequenced using an ABI 3100 automated sequencer. The sequence data obtained from the 3'-RACE product were used to design a specific antisense primer (AS: 5'-GAATTGACTTCCAAAGAGGTGG GA-3') to a conserved site within the 3'-non-translated region. 5'-RACE was carried out using this specific primer in conjunction with the NUP RACE primer and resultant products were purified, cloned, and sequenced.

## Results

### Isolation and characterization of kassinakinin S

Screening of semi-preparative reverse phase HPLC fractions of the skin secretion from *K. senegalensis*, for the presence of histamine-releasing activity, detected such in fractions 35, 40, 46/47, and 50 (Fig. 1). Following further analytical reverse phase HPLC analysis of samples

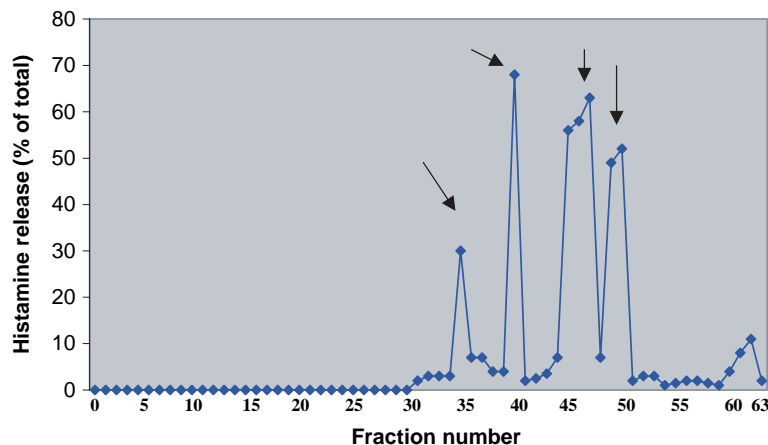


Fig. 1. Histamine releasing (mast cell degranulating) activity in semi-preparative reverse phase HPLC fractions of the skin secretion from the frog, *K. senegalensis*.

from each of these fractions, a histamine-releasing peptide of novel primary structure was found in the sample of fraction 40 and this peptide eluted as a single peak (Fig. 2). Electrospray ionization MS analysis of this peptide resolved a series of related multiply charged ions with a deduced molecular mass of 1976.9 Da (Fig. 3). This peptide elicited 70% release of the total cellular histamine in the rat peritoneal mast cell preparation employed in the bioas-

say. The primary structure of this novel peptide was established as, FIPVTLLALHKIKEKLN-amide, by using a combination of automated Edman degradation and MS/MS fragmentation (data not shown). The additional peptides with histamine-releasing activity were found to correspond to (1) kassinatuerin 2 (4–18 fragment), (3) kassinatuerin 1, and (4) kassinatuerin 2 (4–20) fragment (Table 1) [24].

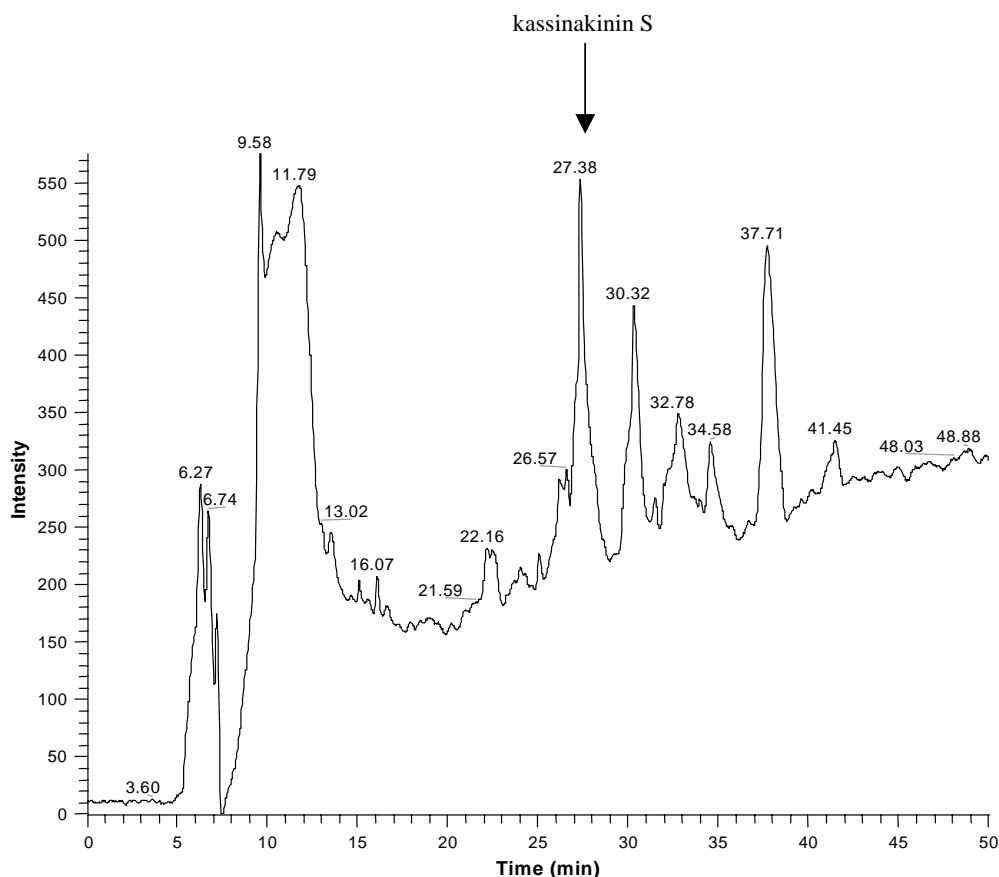


Fig. 2. Reverse phase HPLC chromatogram of semi-preparative reverse phase HPLC fraction #40 that exhibited mast cell histamine-releasing activity. The arrow indicates the peak containing the novel peptide, kassinakinin S.

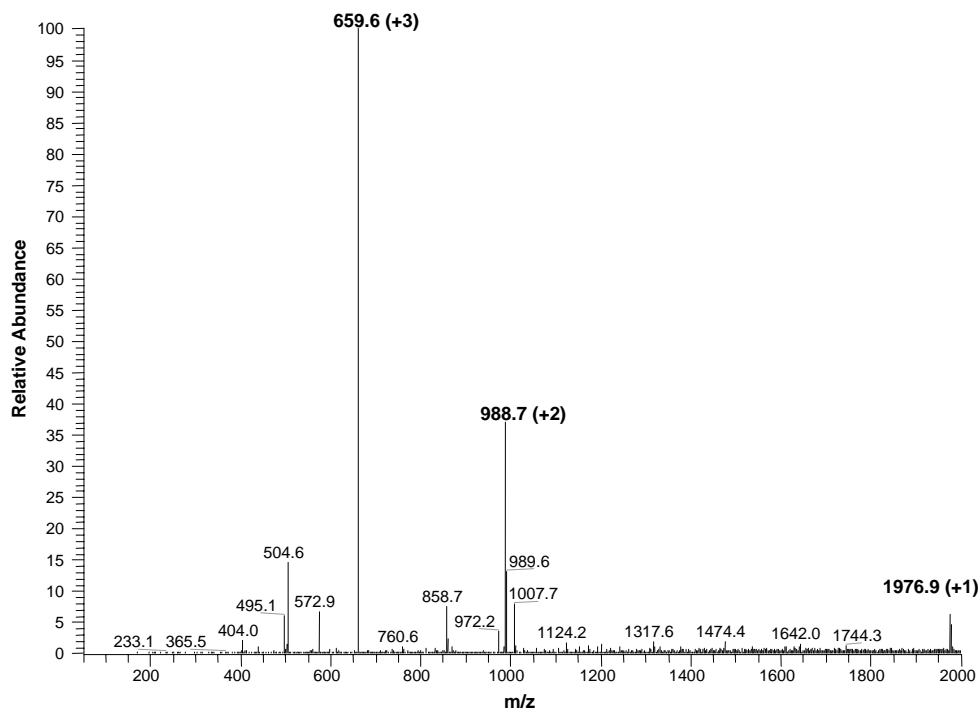


Fig. 3. Electrospray ionization mass spectrum of purified kassinakinin S. Singly charged  $(M+H)^+ = m/z$  1976.9, doubly charged  $(M+2H)^{2+} = m/z$  988.7, and triply charged  $(M+3H)^{3+} = m/z$  659.6, ions are indicated in bold typeface.

Table 1

Molecular masses, primary structures, and identities of histamine releasing peptides present in semi-preparative reverse phase HPLC fractions 35, 40, 47, and 50, respectively, of *K. senegalensis* skin secretion

Peptide	Original fraction	Mass (Da)	Primary structure	Identity
1	#35	1607.9	YLAPLIPHAVKAIKD	Kassinatuerin 2 (4–18) fragment
2	#40	1975.9	FIPVTLLALHKIKEKLN <sup>a</sup>	Kassinakinin S
3	#47	2282.8	GFMKYIGPLIPHAVKAIKSDLI <sup>a</sup>	Kassinatuerin 1
4	#50	1833.2	YLAPLIPHAVKAIKSDLI <sup>a</sup>	Kassinatuerin 2 (4–20) fragment

For kassinatuierins see [25].

<sup>a</sup> C-terminally amidated amino acid residues.

Interrogation of contemporary protein/peptide databases within the National Center for Biotechnology Information (NCBI-USA) using FASTA and BLAST algorithms, with the established primary structure of the novel peptide, indicated little significant rational structural similarity with any known peptide or protein using the top 100 entries. However, extending these searches outside specified limits revealed a limited structural similarity with histamine-releasing peptides from wasp/hornet venoms, particularly with HR-II from the venom of the Oriental hornet (*V. orientalis*) (Fig. 4). These wasp/hornet venom peptides are named vespakinins with a single capital letter following this to indicate the species of origin, for example, vespakinin M from the hornet, *V. mandarinia* [13]. In accordance, we named the novel amphibian skin secretion-derived histamine-releasing peptide described here, kassinakinin S. Synthetic kassinakinin S was found to be identical to the natural peptide in terms of molecular mass, HPLC

retention time, and MS/MS fragmentation spectrum (data not shown). The dose–response curve for mast cell histamine release constructed with this synthetic replicate of kassinakinin S indicated an  $EC_{50}$  of 6  $\mu$ M with maximal release of ca. 80% of histamine content being achieved by 25  $\mu$ M (Fig. 5). Several secondary structure prediction tools were employed and consensus was achieved by HNN (hierarchical neural network) and SOPMA (secondary protein structure by prediction from multiple alignments). These indicated that kassinakinin S is essentially  $\alpha$ -helical with an  $\alpha$  helical content of 70.59% for residues 4 through 15 (Fig. 6).

#### *Cloning of prepro-kassinakinin S cDNA*

The kassinakinin S precursor (prepro-kassinakinin S) encoding cDNA was successfully cloned from the skin secretion library using the RACE protocol described. The

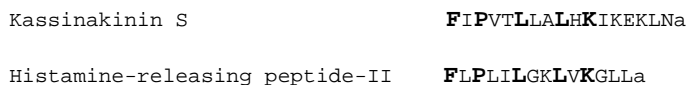


Fig. 4. Comparison of the primary structures of kassinakinin S from African running frog (*K. senegalensis*) defensive skin secretion and histamine-releasing peptide II (HR-II) from the venom of the Oriental hornet (*V. orientalis*). Identical residues are indicated in bold typeface.

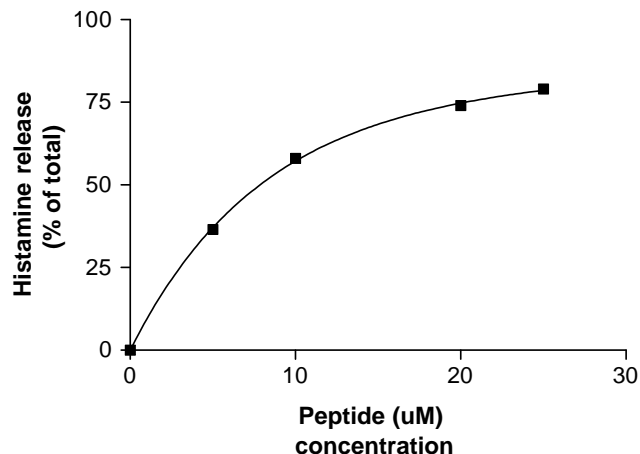


Fig. 5. Dose–response curve of histamine releasing (mast cell degranulating) activity obtained using a synthetic replicate of kassinakinin S. Each point represents the mean and standard error of five experiments.

open-reading frame consisted of 71 amino acid residues (Fig. 7). The domain topology of the precursor is illustrated in Fig. 8. The putative signal peptide is followed by a domain rich in acidic amino acid residues and containing classical -Lys-Arg- (-KR-) propeptide convertase processing sites. In fact, three copies of an identical acidic heptapeptide (ADEEGED) are encoded here between these classical cleavage sites. The N-terminal Phe (F) residue of the mature kassinakinin S is flanked by a double processing site (-KRKR-) and the C-terminal asparaginyl residue is

flanked C-terminally by a glycyl residue that serves as an amide donor. The nucleotide sequences of kassinakinin S has been deposited in EMBL Nucleotide Sequence Database under Accession codes [AJ874341](http://www.ebi.ac.uk/EMBL/Sequence/Database/entry/AJ874341).

## Discussion

The defensive skin secretions of anuran amphibians have long been known to contain many diverse bioactive compounds, especially an overabundance of biologically active peptides [25,26]. With respect to peptides that induce mast cell degranulation/histamine release, amphibian skin secretions have been poorly researched with only four examples alluded to in the literature, namely peptide XO-4 from *Hylambates maculatus* [15], granuliberin-R from *Rana rugosa* [16], and both pipinin [17] and peptide leucine arginine (pLR) [18] from *Rana pipiens*. Both XO-4 and pipinin are classical frog skin cationic, amphipathic peptides, and most if not all such molecules in this class effect mast cell degranulation/histamine release to some degree. However, pLR is quite different in structure from this group of peptides, possessing a disulfide-bridged internal loop, but represents a most potent histamine secretagogue by nature of its highly cationic nature ( $pI = 10.2$ ) and net positive charge of +4 at physiological pH [18]. Likewise, kassinakinin S represents a prototype amphibian skin peptide with potent mast cell degranulation/histamine-releasing activity achieved in the low micromolar concentration range, and, in terms of structural similarity, exhibits limited

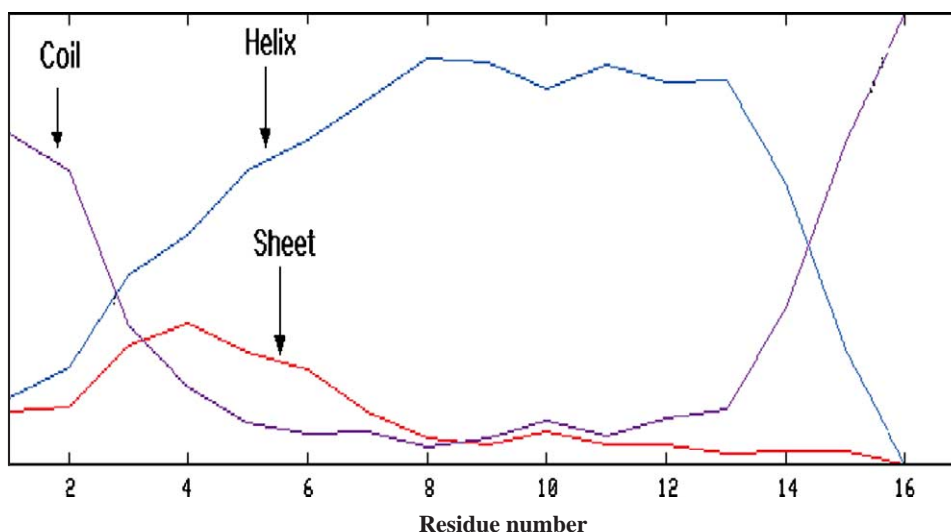


Fig. 6. Secondary structure prediction for kassinakinin S, indicating the predominance of  $\alpha$ -helix obtained using the Hierarchical Neural Network (HNN) programme available at <http://211.32.65.137/link/tools.htm>.

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                                M M K K S M L L L
1  ATTGGACTGA ACTCAATCCC CAACATGATG AAGAAATCCA TGTTGCTGCT
   TAACCTGACT TGAGTTAGGG GTTGTACTAC TTCTTTAGGT ACAACGACGA
   F F L G M V S L S L A Y N K R A
51  TTTCTTCCTT GGGATGGTCT CTCTTCTCT TGCTTATAAC AAGAGAGCTG
   AAAGAAGGAA CCCTACCAGA GAGAAAGAGA ACGAATATTG TTCTCTCGAC
   D E E G E D K R A D E E G E D K R
101  ATGAAGAGGG AGAAGATAAG AGAGCCGATG AAGAGGGAGA AGATAAGAGA
   TACTTCTCCC TCTTCTATT TCTCGGCTAC TTCTCCCTCT TCTATTCTCT
   A D E E G E D K R K R F I P V T L
151  GCTGATGAAG AGGGAGAAGA TAAGAGAAA AGATTCAATC CAGTTACACT
   CGACTACTTC TCCCTCTTCT ATTCTCTTC TCTAAGTAAG GTCAATGTGA
   L A L H K I K E K L N G *
201  TTTGGCGCTT CATAAAATAA AAGAAAAACT TAATGGATAA ACTCCACCT
   AAACCGCGAA GTATTTTATT TTCTTTTGA ATTACCTATT TGAGGGTGA

251  CTTTGGAAGT CAATTCATAT CATCTGATTA CATATTCATA ATTCAGATGT
   GAAACCTTCA GTTAAGTATA GTAGACTAAT GTATAAGTAT TAAGTCTACA

301  CTTAATAAAA AAAAAAAAAA AAAAAAAAAA
   GAATTATTTT TTTTTTTTTT TTTTTTTTTT

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Fig. 7. Nucleotide sequence of cloned skin secretion cDNA encoding prepro-kassinakinin S. The putative signal peptide is double underlined, the single copy of senegalalin is single underlined, and the stop codon (TAA) is indicated by an asterisk.

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1                               22
MMKKSMLLLFFLGMVSLAYN

23                               53
KRADEEGEDKRADEEGEDKRADEEGEDKRKR

54                               71
FIPVTLLALHKEKLNG

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Fig. 8. Domain topography of prepro-kassinakinin S. Residues 1–22 constitute the putative signal peptide. Residues 23–53 constitute the acidic spacer peptide region typified by classical -KR- (-Lys-Arg-) propeptide convertase processing sites (italicized and underlined). The single copy of mature kassinakinin S (residues 54–70) is in bold typeface and the C-terminal glycyl (G) residue that donates the amide is in italics.

but significant homology with histamine-releasing peptide II (HR-II) from the venom of the Oriental hornet (*V. orientalis*) (Fig. 5). However, like pLR, kassinakinin S is not amphipathic but rather is almost entirely  $\alpha$ -helical and highly cationic displaying a  $pI$  of 10.55—factors that certainly contribute to its activity Cross et al. [22]. Interestingly, three other major histamine-releasing peptides were identified in the skin secretion of *K. senegalensis* and structural characterization of each of these revealed that they were identical to intact kassinatuerin 1 and kassinatuerin 2 fragments 4–20 and 4–18, respectively. Kassinatuerins have been previously reported from the skin of this species and kassinatuerin 1 was found to be a potent and broad spectrum antimicrobial whereas the activity of kassinatuerin 2 could not be established [24]. These data however reveal that all three are capable of eliciting histamine release from a rat peritoneal mast cell preparation—an activity possessed by many “so-called” frog skin antimicrobial peptides, including pipinin from *Rana pipiens*

[17]. Although this biological effect may not be the definitive role of these peptides, it would nevertheless call into question their possible application as systemic anti-infectives.

Here we report for the first time, the integration of isolation/structural characterization and molecular cloning of a novel peptide (kassinakinin S) from a single sample of lyophilized skin secretion of the African running frog, *K. senegalensis*. Although this technique, developed in our laboratory to circumvent the need for specimen sacrifice, has been applied to many species of frogs from most major anuran families, the present species is the first to be examined from the family Hyperoliidae. The structural organization of prepro-kassinakinin S is illustrated in Fig. 5 and it very closely resembles the structures of precursors of other amphibian skin peptides determined from their corresponding cloned skin-derived cDNAs. The putative signal peptide domain (the “pre-” domain), rich in hydrophobic amino acid residues, is followed by a domain (the “pro-” domain) that is rich in acidic amino acid residues and often contains several classical -Lys-Arg- propeptide convertase processing sites. These domains are often highly conserved in primary structure within a given species but some areas of structural homology, at both nucleic acid and amino acid sequence levels, are highly conserved in all amphibian skin peptide precursors. The synthesis of polypeptides in the skin granular glands of frogs and toads, possessing a broad spectrum of bioactivities, is an important feature of the defense strategy of these vertebrates [25]. The cocktails of bioactive peptides are released from these glands upon application of a stressful or injurious stimulus to the amphibian by neurally induced contraction of myoepithelial cells that surround the glands [26]. Induction of histamine release from mast cells in the buccal mucosa of a predator would potentially produce a noxious painful sensation but would certainly induce local capillary dilation and plasma extravasation that might serve to

facilitate entry of other skin secretion components into the tissues.

The molecular and functional complexity of the components within defensive skin secretions from amphibians thus remains to be fully established and this research area provides the pharmacologist with an interesting model system for novel bioactive discovery.

### Acknowledgments

Mei Zhou is in receipt of an Overseas Ph.D. studentship from Queen's University Belfast and part of this work was performed by Cherith N. Reid as part of her Ph.D. research in 1999.

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