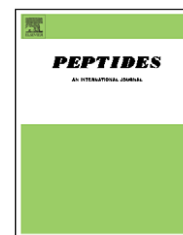


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Histamine-releasing and antimicrobial peptides from the skin secretions of the Dusky Gopher frog, *Rana sevosa*

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ABSTRACT

Seven novel peptides were isolated from the skin secretions of the North American dusky gopher frog, *Rana sevosa*, on the basis of antimicrobial activity and histamine release from rat peritoneal mast cells. The peptides were purified to homogeneity using HPLC and characterized by electrospray ion-trap mass spectrometry, MALDI-TOF mass spectrometry and Edman sequencing. Bioinformatic analysis of primary structures revealed that the novel peptides could be assigned to four established families of ranid frog antimicrobial peptides, namely esculentin-1, esculentin-2, brevinin-1 and ranatuerin-2. The peptides were named in accordance with accepted terminology as ranatuerin 2SEa, etc., reflecting the peptide family name, the species of origin (SE for *sevosa*) and the isotype (a). Of major interest was the fact that brevinin 1SE displayed significant structural similarity to ponerin W5, an antibacterial venom peptide from the ant, *Pachyconyla goeldii*. This is a further example of amphibian skin defensive peptides showing striking structural similarities to peptides from insects. These data may shed some light on the functional biological relevance of defensive peptides that possess both antimicrobial and histamine-releasing activities.

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1. Introduction

The innate immune system is an ancient evolutionary mechanism for recognizing prokaryotic pathogens. It serves as the first-line of host defence in limiting microbial infection in the early hours after exposure and, unlike the adaptive immune system, previous exposure to pathogens creating immunologic memory is not required [14,30]. A widespread characteristic of the innate immune system of invertebrates and vertebrates is the production of antimicrobial peptides, with the induction pathways for these peptides being highly-conserved [2,13]. Antimicrobial peptides are relatively non-specific and rapidly kill invading microbes without the

inherent delay involved in de novo antigen-dependent responses [1,6,28]. While many conventional antibiotics kill bacteria over a period of days, antimicrobial peptides kill within minutes, making them an attractive model for the development of new bacteriostatic therapeutics [10,11]. Studies indicate that these peptides act directly on the membrane of the target microorganism, and that effects are not receptor-mediated [10,19,21].

Frogs and toads synthesize antimicrobial peptides in their granular glands, with the peptides being released upon adrenergic stimulation, stress or injury [29]. Skin secretions generally contain multiple antimicrobial peptides with distinct spectra of activity. It has been postulated that this

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diversity is required to protect the animal from invasion by a wide array of different microorganisms [9]. Antimicrobial peptides have been shown to be involved in many aspects of innate host defences associated with acute inflammation [11]. Mast cells play a central role in acute inflammatory response and allergic reactions through the release of a variety of biologically-active compounds, either stored in granules, such as histamine and proteases, or de novo synthesized on demand, such as prostaglandins, leukotrienes and some cytokines [18,7]. The release of histamine from rat peritoneal mast cells may be utilized as a screen for putative bioactive peptides. Indeed, several peptides have been identified in this manner, including peptide Leucine Arginine (pLR) from *Rana pipiens* [26], peptide Tyrosine Arginine from *Rana sevososa* [8] and granuliberin R from *Rana rugosa* [31].

Degranulation of mast cells by antimicrobial peptides results in the release of histamine and various pro-inflammatory mediators. It has therefore been proposed that cationic antimicrobial peptides are effectors of the innate immune system through their role in the degranulation of mast cells. This leads to histamine release with subsequent vasodilation and polymorphonuclear leukocyte (PMN) recruitment to the sites of acute inflammation [20]. Ultimately, this results in reducing the spread of bacteria at the wound site [11].

In the present study, skin secretions from the dusky gopher frog, *R. sevososa*, were screened for potential histamine-releasing and antimicrobial peptides. These frogs are listed as endangered in the US Endangered Species Act and Critically Endangered in the Red List of the International Union for Conservation of Nature and Natural Resources (IUCN), with only two known populations [25]. Several new peptides with bacteriostatic and histamine-releasing activity were isolated and fully characterized using HPLC, mass spectrometry and automated Edman degradation.

2. Materials and methods

2.1. Collection of skin secretions

Adult male ($n=5$) and female ($n=6$) *R. sevososa* frogs were massaged at multiple sites on the dorsal skin. The frogs readily secreted. After the procedure the frogs were returned unharmed to their protected habitat in the State of Mississippi. The skin secretions were collected into a chilled beaker containing glacial acetic acid (5 ml) by washing the frog's skin with distilled water. Skin secretions from each sex were separately pooled, snap-frozen in liquid nitrogen and lyophilized.

2.2. Reverse phase HPLC coupled with electrospray mass spectrometry

The HPLC system used was supplied by ThermoQuest and comprised a P4000 pump, AS 3000 autosampler, 200 μ l injector loop, on line UV 1000 UV detector and an SCM 1000 vacuum membrane degasser. The column used for chromatographic separations was a 250 mm \times 4.6 mm Phenomenex, Luna C₁₈ column (Macclesfield, Cheshire, UK). A security guard cartridge (Phenomenex, Macclesfield, Cheshire, UK) was positioned just before the analytical column. For mass

spectrometric studies, the HPLC system was interfaced with a ThermoFinnigan electrospray ion trap (LCQTM) mass spectrometer (Hemel, UK). Ten milligrams of lyophilized skin secretions (5 mg from each sex mixed) was reconstituted in 1 ml of 0.05% (v/v) trifluoroacetic acid (TFA)/water, clarified by centrifugation and 200 μ l of clear supernatant was subjected to reverse phase HPLC. The column was equilibrated in 0.05% TFA/water (solution A) and elution of the peptides was achieved by increasing the concentration of solution B (0.05% TFA in 80% acetonitrile/water) from 0 to 100% B in 80 min at a flow rate of 0.5 ml/min. Absorbance was monitored at 214 nm. The molecular masses of the peaks were determined from electrospray ionisation mass spectra using multiply-charged ion profiles. If additional purification of the peaks was required, separation was achieved using a 250 mm \times 4.6 mm Vydac diphenyl column on 2158 Unicord SD LKB HPLC pump system (Brommn, Sweden). HPLC aliquots (100 μ l) requiring further purification were separately lyophilized, reconstituted in 1 ml of (0.1% TFA/water) and chromatographed on the Vydac diphenyl column. The column was equilibrated in 0.1% TFA/water (solution A) and elution of the peptides was achieved by increasing the concentration of solution B (0.1% TFA in 70% acetonitrile/water) from 0 to 100% B in 70 min at a flow rate of 1 ml/min. Peaks were hand collected and the molecular masses of peptides in the fractions were determined using MALDI-TOF mass spectrometry.

2.3. Matrix-assisted, laser desorption, ionisation, time-of-flight mass spectrometry (MALDI-TOF MS)

Molecular mass determination of the peptides in the purified HPLC peaks was performed using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) (Voyager DE, PerSeptive Biosystems, Framingham, MA). An aliquot (1 μ l) of the sample was mixed with 1 μ l of matrix solution, placed on a well of a 100-well stainless steel sample plate and allowed to dry at room temperature. The matrix solution was a 10 mg/ml solution of α -cyano-4-hydroxycinnamic acid in acetonitrile/ethanol (1/1). The mass spectra were recorded as a mass/charge ratio against abundance.

2.4. Primary structural analysis of the purified peptides by automated Edman degradation

Automated Edman degradation was employed for the sequencing of the bioactive peptides. Microsequencing was performed on an Applied Biosystems 491 Procise Sequencer (Foster City, California, USA) with pulsed liquid phase delivery. The limit for detection of phenylthiohydantion (PTH) amino acids was 0.5 pmol. The primary structures of the peptides were compared with those deposited in SWISSPROTTM database. The protein database screening for similar sequences was performed using the FASTA program available in the bioinformatic program GCG₁₀ package at the human genome management program available at <http://www.hgmp.mrc.ac.uk>.

2.5. Antimicrobial assay

Antimicrobial activity of the peptides was monitored by incubating lyophilized aliquots of HPLC purified fractions on

bacteria-seeded agar lawns using the lysozyme-enhanced inhibition zone assay as described by Chalk et al. [4,5]. Standard bacterial strains used in antimicrobial assays were *Escherichia coli* K12 (University of Ulster Culture Collection), as a model Gram-negative bacterium, and *Micrococcus luteus* NCT C2665 as a model Gram-positive bacterium. *E. coli* seeded plates were prepared by the addition of 7 ml of molten 0.7% w/v LB agar (pH 7.2), 7 μ l of 0.01% w/v streptomycin sulphate solution and 1 μ l of *E. coli* sub-culture into a 9-cm triple vent Petri dish containing 350 μ l of 0.1% w/v lysozyme solution. Similarly, *M. luteus* plates were made by the addition of 7 μ l of *M. luteus* (0.4 O.D. 600 nm) to 7 ml of the molten nutrient agar in a 9-cm triple vent petri dish. After cooling, the agar was pierced with a sterile hypodermic needle to allow for the application of the purified HPLC skin secretions fractions. Of the purified HPLC fractions, 100 μ l was dried under reduced pressure and reconstituted in 20 μ l of sterile 0.1 M phosphate buffered saline (pH 7.3). Doubling dilutions of cecropin A were prepared ranging from 4 to 500 μ g/ml for inclusion as a positive control. From the lyophilized HPLC fractions, 2 μ l was added to the wells and incubated statically overnight at 37 °C. The diameters of the inhibition zones were subsequently measured.

2.6. Histamine release assay

Male Hooded Lister rats (150–250 g body weight) were lightly anaesthetized with CO₂ and then killed by cervical dislocation and exsanguination. Mixed peritoneal cells were obtained and the cells were washed twice in Tyrode's buffer (NaCl (137 mM), glucose (5.6 mM), HEPES (10 mM), KCl (2.7 mM), MgCl₂·6H₂O (1 mM), CaCl₂·2H₂O (1 mM) and NaH₂PO₄·2H₂O (0.4 mM), pH 7.4) and recovered by centrifugation (100 g, 4 °C, 2 min). Isolated peritoneal cells (100 μ l) were aliquoted into conical

polystyrene test tubes and prewarmed to 37 °C for 5 min. Lyophilized aliquots of the HPLC purified fractions were reconstituted in Tyrode's buffer (100 μ l) and added to cells 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 the supernatants removed for histamine assay. The remaining pellets were resuspended in buffer (3 ml) and then placed in a boiling water bath (10 min) to release the residual histamine. The histamine content was determined in both the supernatants and the cell pellets using the fluorimetric method based on Shore et al. [27]. Histamine release was expressed as the percentage of total content and values were corrected for spontaneous release in the absence of peptides not exceeding 5%.

3. Results

Peptides in the skin secretions of *R. sevosia* frogs were initially separated by HPLC-MS (Fig. 1). Fractions were subsequently screened for their ability to release histamine from rat peritoneal mast cells and their growth inhibitory activity against the Gram-negative bacterium, *E. coli*, and the Gram-positive bacterium *M. luteus*. As shown in Fig. 2, antimicrobial activity was limited to peptides eluting between 40 and 49 min. Histamine-releasing activity was in the main, associated with the same fractions (Fig. 2). Homogeneity was assessed by electrospray and MALDI-TOF mass spectrometry. Fractions eluting at 41, 43 and 45 min that contained more than one peptide as determined by mass spectrometry, were subjected to further purification on a diphenyl VYDAC column (Fig. 3). Ultimately, seven novel peptides were structurally characterized and bioinformatic analysis indicated that they belonged

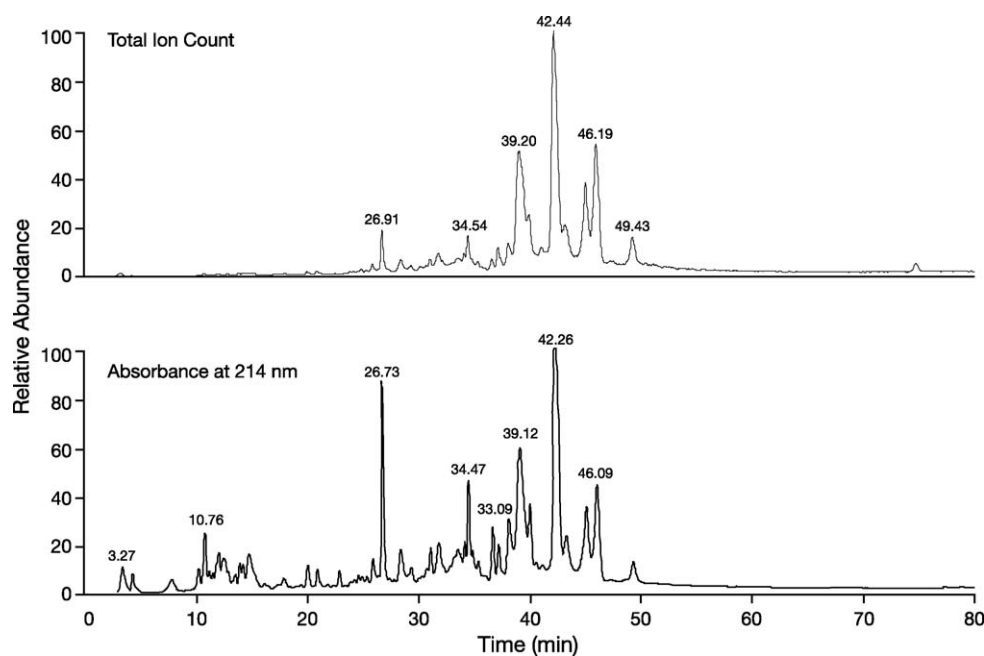


Fig. 1 – Reverse phase HPLC purification of *Rana sevosia* skin secretions. Pooled skin secretions from five frogs was applied to a Phenomenex Luna C18 column and eluted at a flow rate of 0.5 ml/min. Top panel shows the total ion count, the bottom panel displays absorbance at 214 nm.

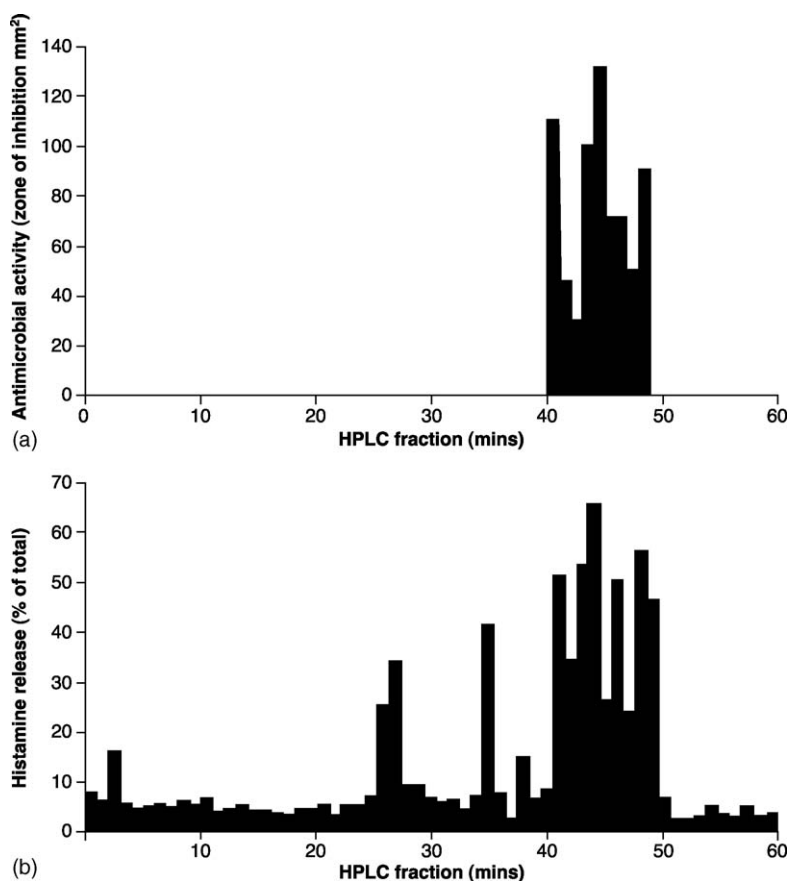


Fig. 2 – (a) Antimicrobial screen of the HPLC purified fractions from the skin secretions of male *Rana sevos*a frogs, antimicrobial activity on *E. coli* seeded plates is shown. (b) Screen for histamine-releasing activity in the same fractions.

to the esculentin, brevinin and ranatuerin families of peptides (Table 1). The spectrum of antimicrobial activity in relation to the peptides' action on *E. coli* and *M. luteus* is shown in Table 2. The terminology adopted in the naming of the peptides is one in which we have named the individual peptides by using the first two initial letters of the species to indicate their origin (SE for *sevos*a as S has already been used) and have designated isoforms by the letters a, b, and c, where appropriate [29]. Two other peptides that showed histamine-releasing activity eluted in fractions 26/27 and 35. Fractions 26/27 contained the 2160 Da peptide, named peptide Tyrosine Arginine, an

immunomodulatory peptide belonging to the peptide Leucine Arginine family of peptides [8]. The second fraction, number 35, contained three peptides of 2220, 2255 and 2489 Da, respectively. These peptides did not show any antimicrobial activity and were not present in sufficient quantities to permit structural characterization.

There was no gender difference in the antimicrobial activity of skin secretions and both sexes displayed similar LC-MS profiles. However, a peptide eluting in fraction 12 from a separately chromatographed sample of female frog skin secretions (Fig. 4) evoked a high degree of mast cell histamine-

Table 1 – Primary structures of the antimicrobial and histamine-releasing peptides from *Rana sevos*a skin secretions

Peptide name	Sequence	Mass (Da)	Peptide family
Esculentin-1SEa	GLFSKFNKKKIKSGLIKIITAGKEAGLEALRTGIDVIGCKIKGEC	4903	Esculentin-1
Esculentin-1SEb	GLFSKFNKKKIKSGLFKIITAGKEAGLEALRTGIDVIGCKIKGEC	4936	Esculentin-1
Esculentin-2SE	GFFSLIKGVAKIATKGLAKNLGKMGDLVIGCKISKEC	3837	Esculentin-2
Brevinin-1SE	FLPLVRGAAKLIPSVVCAISKRC	2481	Brevinin-1
Ranatuerin-2SEa	GFISTVKNLATNVAGTVIDTIKCKVTGGC	2910	Ranatuerin-2
Ranatuerin-2SEb	AIMDTIKDTAKTVAVGLLNKLCCKITGC	2948	Ranatuerin-2
Ranatuerin-2SEc	GIMDTIKDTAKTVAVGLLNKLCCKITGC	2935	Ranatuerin-2

The yields of the peptides upon purification were (μmol) esculentin-1SEa, 1.68; esculentin-1SEb, 0.80; esculentin-2SE, 0.52; brevinin-1SE, 0.55; ranatuerin-2SEa, 1.86; ranatuerin-2SEb, 0.31 and ranatuerin-2SEc, 0.27. The molecular masses were determined by electrospray and MALDI-TOF mass spectrometry.

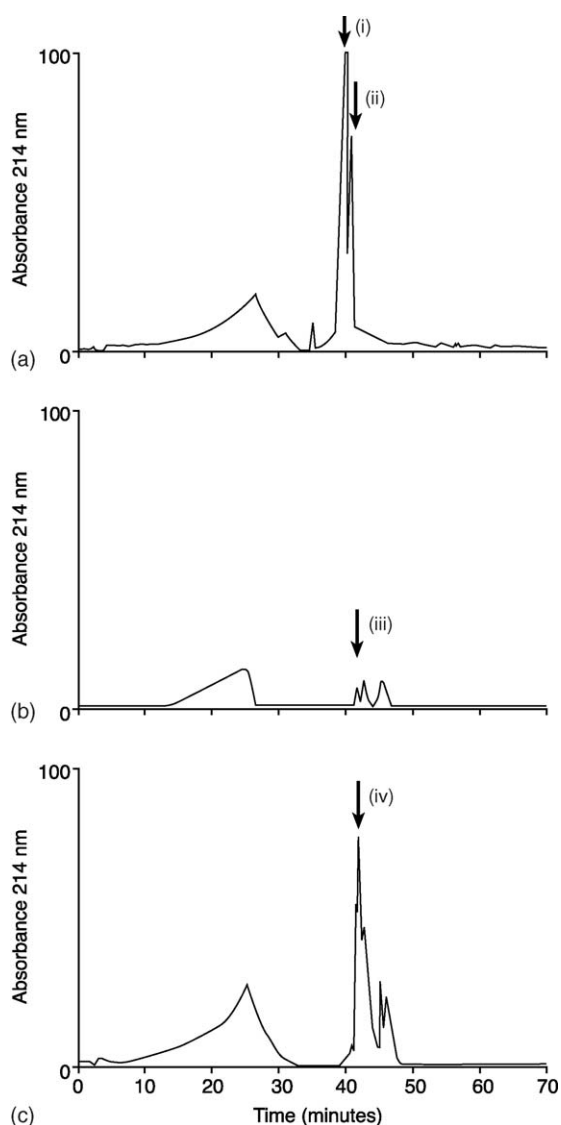


Fig. 3 – Reverse phase HPLC (Vydac diphenyl column) of: (a) fraction 41, arrow denotes fraction containing (i) esculentin-1SEa and (ii) esculentin-1SEb; (b) fraction 43, arrow denotes fraction containing (iii) ranatuerin-2SEa; and (c) fraction 45, arrow denotes fraction containing (iv) esculentin-2SE. Absorbance was monitored at 214 nm.

release (56%). This was not observed in separately chromatographed skin secretions from male frogs (Fig. 2). Edman degradation of this peptide revealed that it constituted the C-terminal heptapeptide loop of esculentin-2SE, $-C^{30}KISKEC^{36}$. Despite the potent histamine-releasing ability of this peptide, it was devoid of antimicrobial activity. Systematic peptidomic analysis of *R. sevos*a skin secretions revealed the presence of the amphipathic central domain of esculentin-2SE ($-K^{11}IATKGLAKNLGKMGDLVG^{29}$) in HPLC fraction 33 of the female frog skin secretions (Fig. 4). This peptide was devoid of both antimicrobial and histamine-releasing activities and was not present in fractions of male frog skin secretions.

Table 2 – Antimicrobial activities of peptides isolated from an extract of the skin secretions of *Rana sevos*a

Peptide name	Zone of inhibition on <i>E. coli</i> seeded plates (mm ²)	Zone of inhibition on <i>M. luteus</i> seeded plates (mm ²)
Esculentin-1SEa	110	121
Esculentin-1SEb	30	36
Esculentin-2SE	131	152
Brevinin-1SE	90	90
Ranatuerin-2SEa	100	152
Ranatuerin-2SEb	71	142
Ranatuerin-2SEc	50	100
Cecropin A (100 µg/ml)	56	144

Antimicrobial activity is expressed as the square of the inhibition zone diameter (mm²). Cecropin A was used as a positive control.

4. Discussion

Antimicrobial peptides are found in all animal species with more than 880 peptides having been discovered to date [3]. Many species of frogs possess a large array of antimicrobial peptides in their defensive skin secretions and individual specimens produce multiple, structurally-related isoforms with each often exhibiting differential activity against a range of pathogenic microorganisms [21]. Originally the proposed site of action for antibacterial peptides has been thought to be the cytoplasmic membrane of the microorganism that contains the electron transport chain and the enzymes responsible for oxidative phosphorylation [24,19]. Cationic peptides interact with the negatively charged bacterial phospholipids and permeate the membrane through the formation of transmembrane pores. However, it has now been shown that antimicrobial peptides can also use the passive transport system to enter bacteria and from there they are able to disrupt multiple cellular processes within the bacteria [24]. Antimicrobial peptides have been shown to act synergistically. When administered in combination, the *Xenopus laevis* skin antimicrobial peptides, magainin 2 and PGLa, show a marked functional synergism in bacteria, tumor cells and structurally-defined artificial membranes. These peptides, in the presence of membrane-mimicking large unilamellar vesicles, form complexes with each other and exhibit a sigmoidal dependence of activity on concentration, indicating that they are acting in a cooperative manner [19]. It is thus not unlikely that the seven peptides identified in this study may act in such a cooperative manner.

Although no differences were observed in antimicrobial activity between the male and female skin secretions, there were observed sex-related differences in the ability of respective skin secretions to release histamine from rat peritoneal mast cells. This observation inadvertently led to a structure/function insight into the actions of one of the novel peptides, esculentin-2SE. The full-length peptide was present in both male and female skin secretions and possesses both antimicrobial and histamine releasing activity. However, the identification of a significant additional histamine-releasing but non-antimicrobial peptide in female frog secretions, as the

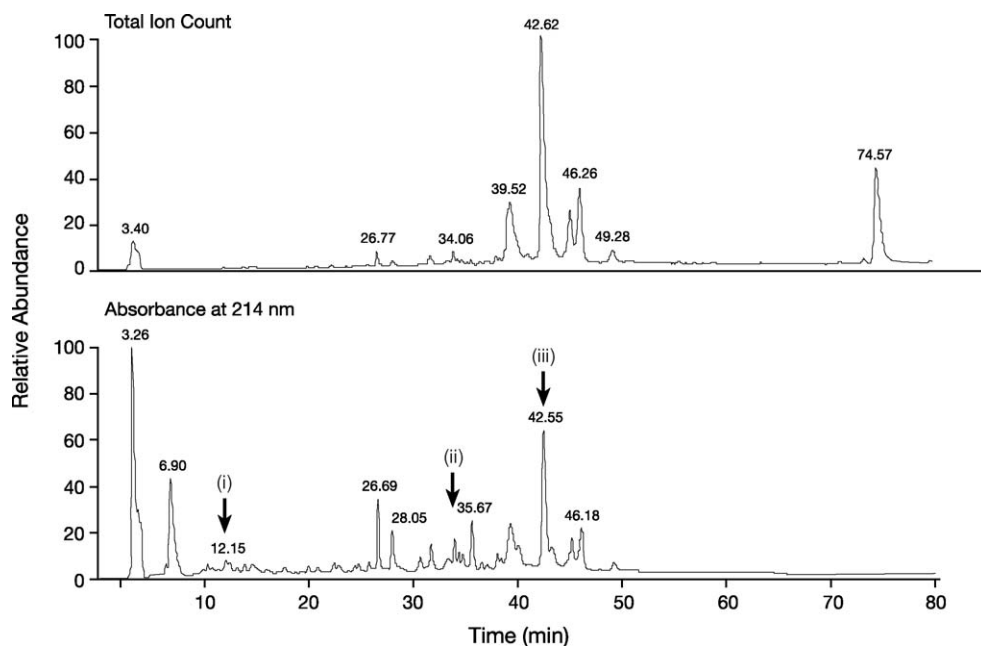


Fig. 4 – Reverse phase HPLC purification of female *Rana sevosia* skin secretions. Pooled skin secretions from six frogs was applied to a Phenomenex Luna C18 column and eluted at a flow rate of 0.5 ml/min. Top panel shows the total ion count, the bottom panel displays absorbance at 214 nm. (i) C-terminal heptapeptide loop of esculentin-2SEa, (ii) central amphipathic domain (residues 11-30) of esculentin-2SE and (iii) full length peptide, esculentin-2SE.

C-terminal heptapeptide loop of this peptide and additionally, the non-bioactive central amphipathic domain, gives an insight into the structure-activity requirements for each bioactivity. Mast cell histamine release is effected only by the intact esculentin and the C-terminal heptapeptide loop. Antimicrobial activity is only possessed by the intact peptide and not by the central amphipathic domain or C-terminal heptapeptide loop. These data would imply that the cationic loop is the region of the esculentin molecule that interacts in an electrostatic manner with the highly-negatively charged surface of the mast cell eliciting degranulation, and that this electrostatic interaction may be the first event to occur at the negatively-charged glycocalyx of the bacteria. Antimicrobial activity is dependent on the ability of the peptides to cross the membrane, with stabilized secondary structure of an amphipathic helix or sheet and a cationic nature essential for activity [11,12]. Perhaps surprisingly, the amphipathic central domain of esculentin-2SE does not appear to possess antimicrobial or mast cell degranulation activity even though it possesses both intrinsic cationic character (net positive charge +3) and a propensity for amphipathicity. The esculentins, and perhaps other *Rana* frog skin antimicrobial peptides that possess these structural features, are thus exquisitely bioengineered and optimized to perform their functions as antimicrobial agents. However, their potent mast cell degranulation ability that affects histamine release may be an important factor in their unsuitability as systemic therapeutics. Interestingly, while many publications focus on the establishment of microorganism sensitivity to this class of antimicrobial, few perform parallel mast cell histamine-release assays, a fact that is quite difficult to understand when the prototype *Rana* frog cationic amphipathic skin

peptide, pipinin-1, was actually isolated as a consequence of this bioactivity [15].

Mast cells are an integral component of the innate immune system, with bacteria being able to activate mast cells in the absence of specific antibodies to the pathogens [17]. The antimicrobial peptides found in the species *R. sevosia*, are able to degranulate mast cells with the concomitant release of histamine. As with other amphibian skin secretions peptides found to elicit the release of histamine from rat peritoneal mast cells, the peptides from *R. sevosia* are likely to cause histamine release in a receptor-independent mechanism linked to the activation of G-proteins [16,22]. This may indicate a potential role of antimicrobial peptides in the inflammatory process [20]. Degranulation of mast cells results in the release of histamine and other proinflammatory mediators including tumor necrosis factor alpha (TNF α) [17], that results in attraction of inflammatory cells, mainly polymorphonuclear leukocytes (PMN), to the sites of acute inflammation and vasodilation, which ultimately helps to reduce the bacterial spread [10].

In summary, the present study describes the isolation, from extracts of the skin of the North American dusky gopher frog *R. sevosia*, of seven peptides with histamine releasing activity on rat peritoneal mast cells and growth-inhibiting activity toward the bacteria *M. luteus*, and *E. coli*. These peptides are all novel and belong to the following four families; esculentins 1 and 2, brevinin-1 and ranatuerin-2. This is consistent for a ranid frog of North American origin where SE refers to the species (*sevosia*) and isoforms are designated by lower case letters [29]. Brevinin 1SE displayed significant structural similarity (52% identity) to poneracin W5, an antibacterial venom peptide from the predatory ant, *Pachyconyla goeldii* [23]. The entire central domain

of brevinin 1SE-G⁷AAKLIPSVV¹⁶ is present in ponericin W5. Ranatuerin 2SEa is unusual among ranatuerins in having a C-terminal heptapeptide rather than hexapeptide loop that hitherto has been considered unique to the family and is in fact displayed by ranatuerins 2SEb and 2SEc.

In conclusion, the skin secretions of the dusky gopher frog, *R. sevos*, contains at least seven antimicrobial peptides of novel structure, which can also elicit histamine release from rat peritoneal mast cells. These new peptides can thus, in addition to having bacteriostatic properties, modulate the immune system through their ability to degranulate mast cells. This spectrum of activity dictates new avenues of research in selecting amphibian skin antimicrobial peptides as therapeutic lead compounds and to further elucidate their exact mechanism of action.

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