FROG SKIN ACTIVE PEPTIDES (FSAP) AS A HIGHY EFFECTIVE ANTIMICROBIAL AGENTS

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ABSTRACT

During last ten to fifteen years a considerable scientific attention has been paid to biologically active substances as known as *frog skin active peptides* (FSAP). These substances are naturally produced by many *Amphibian* organisms and could be found within skin secretion of most frog families, especially *Ranidae*. Biomolecules of that class possess extremely valuable properties: show high antimicrobial and antifungal activity – especially effective against multiresistant bacterial infections; act as protease inhibitors and components of an innate immune system; demonstrate relatively weak hemolytic activity. Such a combination makes frog skin active peptides a potential candidates for drug development with possible application in therapy, traumatology, surgery, etc. In present paper an origin, classification, function as well as methods for determination of FSAP are summarized. It is underlined that Bulgarian region is definitely suitable for extensive FSAP investigations due to the naturally abundance of genus *Rana* species.

Key words: frog skin active peptides (FSAP), antimicrobial peptides, multiresistant bacterial infections, drug development

What are FSAP?

Frog skin active peptides (FSAP) are naturally produced polypeptides of 20-30 AA length and 2000-3000 Da molecular weight in average; there are extreme exceptions though. It was shown many times that FSAP are important skin secretion components of organisms belonging to class *Amphibia*, subclass *Lissamphibia* (frogs, toads, salamanders, newts, etc.).

Due to the increasing interest nowadays, the number of isolated and studied FSAP substances constantly grows (now exceeds 200); there are even some correspondent nomenclature problems raised (6).

The systematical research on FSAP has begun in the early 1990s. It appears that frog skin active peptides could be categorized in groups (peptide lines) and in peptide families within these lines. On the basis of limited structural similarity, at least 11 well-established peptide lines have been identified so far (6). In Table 1 a brief information concerning known FSAP lines is given; more detailed data on major representatives could be found in Table 2.

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Why world scientists are interested in FSAP?

Table 1. FSAP lines identified so far (data retrieved from (39,40)).

№	FSAP line name	Known members	First reported	in Ref.
1	Brevinins	62	1992	(21)
2	Esculentins	12	1993	(29)
3	Ranalexins	3	1994	(5)
4	Temporins	40	1995	(31)
5	Rugosins	3	1995	(32)
6	Ranatuerins	24	1998	(12)
7	Palustrins	11	2000	(1)
8	Nigrocins	5	2001	(24)
9	Japonicins	3	2002	(13)
10	Ranacyclins	2	2003	(16)
11	Pipinins	1	2005	(20)

Immediately after the isolation of first frog skin active peptides, an attention has been drawn upon their significant biological activity. In general, **antimicrobial function** appears to be the most important FSAP' feature. Combining the antimicrobial function with some other FSAP' advantages, it becomes clear why these peptides are often spoken of as **"natural antibiotics"** as well as propositions for drug development on their basis are periodically being discussed. The FSAP and their modifications may become extremely valuable due to **high efficiency against multiresistant bacterial infections** (18).

Table 2. Some typical FSAP details.

Peptide name	AA Length	Molecular weight	AA Sequence
Brevinin-1	24	2531	FLPVLAGIAA[10] KVVPALFCKI[20] TKKC *(disulfide bridge: 18-24)*
Brevinin-2	33	3254	GLLDSLKGFA[10] ATAGKGVLQS[20] LLSTASCKLA[30] KTC *(disulfide bridge: 27-33)*
Esculentin-1	46	4888	GIFSKLGRKK[10] IKNLLISGLK[20] NVGKEVGMDV[30] VRTGIDIAGC[40] KIKGEC *(disulfide bridge: 40-46)*
Japonicin-1	14	1650	FFPIGVFCKI[10] FKTC *(disulfide bridge: 8-14)*
Japonicin-2	21	2358	FGLPMLSILP[10] KALCILLKRK[20] C *(disulfide bridge: 14-21)*
Nigrocin-2	21	2031	GLLSKVLGVG[10] KKVLCGVSGL[20] C *(disulfide bridge: 15-21)*
Ranacyclin-E	17	1906	SAPRGCWTKS[10] YPPKPCK *(disulfide bridge: 6-16)*
Ranalexin	66	7615	MFTLKKSLLL[10] LFFLGTINLS[20] LCEEERNAEE[30] ERRDNPDERD[40] VEVEKRFLGG[50] LIKIVPAMIC[60] AVTKKC *(disulfide bridge: 60-66)*
Ranatuerin-1	25	2651	SMLSVLKNLG[10] KVGLGFVACK[20] INKQC *(disulfide bridge: 19-25)*
Temporin-A	13	1398	FLPLIGRVLS[10] GIL
Pelophylaxin-2	74	8070	MFTMKKSLLF[10] FFFLGTIALS[20] LCEEERGADE[30] EENGAEITDE[40] EVKRGILLNT[50] LKGAAKNVAG[60] VLLDKLKCKI[70] TGGC

Brevinins are known to be active against a wide range of *Gram*-positive and *Gram*-negative bacteria and against strains of pathogenic fungi. Brevinin-1 is active against *Staphylococcus aureus* and *Escherichia coli*; inactivates infectious *Herpes simplex* viruses HSV-1 and HSV-2 (35) as well as stimulates insulin release from BRIN-BD11 cells (19). It has been reported (23) that Brevinin-1BYa shows growth inhibitory activity against a range of reference strains of *Gram*-positive and *Gram*-negative bacteria, against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA), and against reference strains and clinical isolates of the opportunistic yeast pathogens *Candida albicans*, *Candida tropicalis*, *Candida krusei* and *Candida parapsilosis*. The peptide is highly hemolytic for human erythrocytes, but derivatives in which the cysteine residues are replaced by serine to produce an acyclic analog reduces hemolytic activity while retaining high antimicrobial potency. Brevinin-ALb has been shown to induce mast cell degranulation and histamine release, and is cytotoxic for solid tumor cell line HepG2 (14).

Esculentins are very active against *Staphylococcus aureus*, and have a much lower hemolytic activity than Brevinin line members. It has been stated that Esculentin-1 also inhibits the growth of *Pseudomonas aeruginosa*, *Candida albicans*, and *Saccharomyces cerevisiae* (30). Chemically synthesized peptide segments corresponding to Esculentin-1 (amino acids 1-15 and 9-27) show antimicrobial activity against *Escherichia coli*, *Mos blue*, *E. coli 2*, *Bacillus brevis*, *B. megaterium*, *Pseudomonas HTL*, and *Vibrio mimicus* (26). Transgenic tobacco plants expressing Esculentin-1 with the substitution Met-28Leu has been created; these plants have been reported to show enhanced resistance against bacterial or fungal phytopathogens (25).

Ranalexins have been shown to be most active against *Gram*-positive bacteria (8), but also against *Candida spp*. and *Cryptosporidium parvum* (9), methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* (10). There are evidences that Ranalexin can prevent bacterial growth, endotoxemia, and mortality in rats with septic shock and also reduces plasma levels of TNF-alpha (7).

Table 3. Natural occurrence of order Anura (Frogs) species in Bulgarian habitat.

	Family (5)	Genus (5	Species (12)
Order Anura (Frogs)	**Bombinatoridae**	**Bombina**	*Bombina bombina* (European Fire-bellied Toad)
			Bombina variegate (Yellow-bellied toad)
	Bufonidae	**Bufo**	*Bufo bufo* (Common Toad)
			Bufo viridis (European Green Toad)
	Hylidae	**Hyla**	*Hyla arborea* (European tree frog)
	Pelobatidae	**Pelobates**	*Pelobates fuscus* (Common Spadefoot)
			Pelobates syriacus (Eastern Spadefoot Toad)
	Ranidae	**Rana**	*Rana dalmatina* (Agile Frog)
			Rana kl. Esculenta (Edible Frog)
			Rana graeca (Greek Frog)
			Rana ridibunda (Marsh Frog)
			Rana temporaria (Common Frog)

Temporins – both natural and synthetic – have antibacterial activity directed mainly against *Gram*-positive bacteria. Temporin-A and some synthetic analogs have been shown to possess variable antibiotic activities against a broad spectrum of micro-organisms, including clinically important methicillin-sensitive and -resistant *Staphylococcus aureus* as well as vancomycin-resistant *Enterococcus faecium* strains (33); allow the leakage of large-size molecules from the bacterial cells (15); are active against the chytrid fungus (*Batrachochytrium dendrobatidis*) associated with global amphibian declines (28); induce the migration of human monocytes, neutrophils and macrophages, and stimulates Ca^{2+} flux in monocytes (3); display anti-*Leishmania* activity at micromolar concentrations, with no cytolytic activity against human erythrocytes (17). Some hybrid molecules retain significant antifungal activity, are less hemolytic than Temporin-A, and inhibit blood coagulation (34). It is also noted that transgenic potatoes expressing N-terminally modified Temporin-A (MsrA3) are resistant to late blight caused by *Phytophthora infestans* and pink rot caused by *Phytophthora erythroseptica* (22). Temporin-L readily penetrates into lipid monolayers; intercalation is enhanced in the presence of the common bacterial negatively charged phospholipid phosphatidylglycerol, whereas eukaryotic cholesterol counteracts penetration into lipid films to some extent (36); it has been shown that the simultaneous administration of Temporin-L and beta-lactams produce the highest antimicrobial activities and the strongest reduction in plasma endotoxin and TNF-alpha levels, resulting in the highest survival rates in two rat models of septic shock causes by *Gram*-negative bacteria (11). Temporin-Ma has been shown to induce mast cell degranulation and histamine release, and has cytotoxic activity toward solid tumor cell line HepG2 (14).

Ranatuerins are peptides with antimicrobial activity towards *Staphylococcus aureus*. Ranatuerin-1 shows the broadest spectrum of antimicrobial action with inhibitory activity against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. Ranatuerin-2P appears to inactivate frog virus 3, a potentially pathogenic iridovirus infecting anurans, and channel catfish herpesvirus by direct action rather than inhibition of replication in infected cells (4). Ranatuerin-2TRa has been shown to be active against the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* (27).

Nigrocins are cationic antimicrobial peptides which possess a broad spectrum of antimicrobial activity against various microorganisms with different specificity (24).

Japonicins are bacteristatic for *Escherichia coli* and the *Gram*-positive bacterium *Staphylococcus aureus*.

It has been shown that Ranacyclins and pLR have antimicrobial and antifungal activity but differ in their activity spectra (16).

Pipinin-1, yet the only known member of Pipinins family, is antimicrobial and has insulin releasing activity (20).

Why Bulgarian scientists may be interested in FSAP?

As a south-eastern part of European region, Bulgaria demonstrates a natural abundance of *Amphibian* organisms. There are 17 *Amphibia* species that inhabit the territory of Bulgaria (38). They include 12 frog species from 5 families (see Table 3 for details) and 5 salamander species from the family *Salamandridae*. The most recently discovered species is the Edible Frog which was first registered in 1966. Some of the most common species include the European Green Toad, Yellow-bellied toad and Marsh Frog.

Some of the *Anura* species listed above are not specific to Bulgarian habitat only and have been investigated by foreign authors. For some of the species it has been firmly shown to produce FSAP (*Rana dalmatina, Rana kl.*

Esculenta, Rana temporaria, Bufo bufo); other are known to produce biologically active neuropeptides (*Bombina bombina*); the rest are not examined yet (*Bombina variegate, Bufo viridis, Hyla arborea, Pelobates fuscus, Pelobates syriacus, Rana graeca* and *Rana ridibunda*). This opens up a space for Bulgarian scientists to prepare research projects mainly on two directions:

a. To confirm occurrence of known biologically active substances (and especially FSAP) in skin secretion of *Anura* species with Bulgarian origin;
b. To investigate the possibility for unexamined species to produce yet unknown biologically active substances (and especially FSAP).

It may sound reasonable to pay attention firstly at *Rana kl. Esculenta* as a most reliable source of experimental material; moreover it should be mentioned here, that there are some recent traditions in Bulgarian Edible Frog breeding. Some preliminary clinical observations testify to the positive influence of *R. Esculenta* secretion on surface injuries elimination.

As a final goal of investigations should be set the answer of the question: "Is it possible to develop novel antibacterial drugs on the basis of naturally produced or synthetically made FSAP?".

Which are preferable methods in FSAP investigation?

The process of biochemical analysis regarding frog skin active peptides follows the routine way of polyaminocarboxylic acids examination. From physicochemical point of view it could be separated to steps as follows:

Biosampling. Crude secretion is usually obtained by mild electrical stimulation of the dorsal frog skin surface (20). Average biosample volume collected in this manner is about 5-10 mg. (±)-Norepinephrine hydrochloride stimulation is also possible (1).

Preliminary purification. High-performance liquid chromatography (HPLC) techniques are preferred here. Widely used solvent system is tri?uoroacetic acid (TFA)/water/acetonitrile; absorbance monitoring at 214 nm (1). C-18 reversed-phase HPLC yields 50-100 fractions, which can be examined for bioactivity and processed further.

Deep purification. Chosen fractions often undergo rechromatographing (once or more) to enhance separation level. Final purification by C-4 HPLC leads to single homogenous peaks.

Sequence determination. Positive and negative ion electrospray mass spectrometry (ES-MS) could be used as a primary method to investigate the amino acid sequence (2). Edman automated degradation is helpful for additional clarification. Also, skin peptide precursor cDNA cloning techniques have been reported to be useful for FSAP family identification (37).

Biological activity study. After FSAP purification and isolation (or, in some cases, after their chemical synthesis) a series of tests should be done in order to examine peptide's antibiotic activities against specific micro-organisms and determine antimicrobial and/or antifungal activity. In accordance to opportunity of utilizing the FSAP' therapeutic potential, attention should be paid to their citotoxicity and hemolytic properties.

CONCLUSION

In present paper a naturally produced biologically active substances, as known as Frog Skin Active Peptides (FSAP) are discussed. The known general information regarding their origins as well as main biomedical properties are summarized. A brief classification has been presented. Preferable methods for FSAP analysis have been outlined. It has been demonstrated that FSAP are natural biomolecules of significant interest to the modern science, including chemistry, biochemistry, medicine and pharmacy.

Attention has been drawn to the opportunity of running systematical research on local Bulgarian *Anura* species as well as to actuality of studying the possibilities for drug development on the FSAP basis.

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