Volume 324, number 2, 159–161 FEBS 12571 June 1993

Novel antimicrobial peptides from skin secretion of the European frog *Rana esculenta*

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Received 13 April 1993

Three antimicrobial peptides were isolated from skin secretion of the European frog, *Rana esculenta*. Two of them show similarity to brevinin-1 and brevinin-2, respectively, two antimicrobial peptides recently isolated from a Japanese frog [Morikawa, N., Hagiwara, K. and Nakajima, T. (1992) Biochem. Biophys. Res. Commun 189, 184–190]. The third one, named esculentin, is 46 residues long and represents a different type of peptide. All these peptides have as a common motif an intramolecular disulfide bridge located at the COOH-terminal end. The peptides from *R. esculenta* show distinctive antibacterial activity against representative Gram-negative and Gram-positive bacterial species. In particular, esculentin is the most active against *Staphylococcus aureus*, and has a much lower hemolytic activity.

Antibacterial peptide; Hemolysis; Amphibian skin; *Rana esculenta*

1. INTRODUCTION

As has been known for quite some time, skin extracts of frogs contain peptides with antimicrobial activity. The first observations were made in *Bombina* [1,2], although a thorough description of the structure and activity of the bombinins and their precursors has only recently been achieved [3,4]. The magainins, another class of potent antimicrobial peptides, were isolated from skin secretion of *Xenopus laevis* [5]. Dermaseptin is an antimicrobial peptide which was isolated from skin of *Phyllomedusa sauvagei* [6]. All these molecules have since been the subject of intense multidisciplinary research in order to clarify their mechanism of action, biosynthesis, activity towards different microorganisms and potential therapeutical applications [7,8].

In the course of a research project aimed at the isolation and pharmacological characterization of bradykinin-like peptides in methanolic extracts from the skin of the European green frog, *Rana esculenta*, we have isolated a family of hydrophobic peptides which possessed cytolytic activity [9]. Preliminary structural analysis of these peptides aroused suspicion about their numerosity as being at least in part originated from artifacts due to the relatively crude extraction procedure. We therefore decided to investigate the possibility of obtaining a peptide fraction using a more physiological procedure and to undertake a detailed study of both its structural and lytic properties.

Here we present our results on the structure and the antimicrobial and hemolytic activities of three different peptides isolated from skin secretion of *Rana esculenta*. While this paper was in preparation, the sequence of two related peptides was presented which were isolated from skin extracts of the Japanese frog *Rana brevipoda porsa* [10].

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2. MATERIALS AND METHODS

2.1. *Collection and purification of skin secretion*

The peptide-containing secretion, induced by mild electrical shock, was collected from the surface of the skin of 1 specimen of *R. esculenta* by washing the dorsal region of the frog with 120 ml of 0.9% NaCl. The solution was then lyophilized and redissolved in 12 ml bidistilled water. 1-ml aliquots were filtered and fractionated by HPCL on a Beckman model 332 system using a reverse-phase column (Aquapore RP-300, 7 mm × 250 mm, Brownlee Labs, Applied Biosystems) eluted with a 35 min-gradient of 10 70% acetonitrile/isopropanol (4:1) in 0.2% (by vol) trifluoroacetic acid, at a flow rate of 2.0 ml/min. Elution was monitored on a Beckman 165 spectrophotometer at 220 nm. The effluent from three such HPLC separations, developed under identical experimental conditions, was collected in 2-ml fractions in the same set of tubes and lyophilized. A 1% aliquot of the material from each tube was then used for assay of biological activity.

2.2. *Antibacterial assays*

The antibacterial activity was tested using an inhibition zone assay on agarose plates seeded with *Escherichia coli* D21, *Bacillus megaterium* Bm11 or *Staphylococcus aureus* Cowan I [11]. The peptide fractions (3 μl) were placed in small wells of thin agarose plates containing rich medium and about 1×10^5 bacterial cells. The plates were incubated overnight at 30°C. The zones of inhibition were measured, and the lethal concentration (LC, the lowest concentration that inhib-

its growth) was calculated from the diameter of the zones obtained in serial dilutions of the test substance by using the formula given in [11].

2.3. *Hemolytic activity*

The hemolytic activity was recorded by an adaptation of the antibacterial inhibition zone assay on agarose plates with human red blood cells according to [12]. Sterile agarose plates contained 6 ml of medium with 1% agarose, 0.9% NaCl and 10% human red cells obtained according to [13]. The plates were incubated at 30°C for 24 h. Clear zones were measured and lytic concentration values calculated as described above.

2.4. *Structural analysis*

Amino acid analyses were performed with a Pharmacia Alpha Plus 4151 analyzer after vapor phase hydrolysis of the peptides (1–2 nmol) in 6 N HCl for 24 h. Peptide sequences were determined by automated Edman degradation with Applied Biosystems model 475A or 476A sequencers. Cysteine residues were identified after alkylation with 4-vinylpyridine [14] of peptides coupled to Sequelon-AA membranes (Millipore) via carbodiimide activation according to the manufacturer's instructions. After coupling of the peptides (about 1 nmol), the membranes were wetted with 40 μl of 0.5 M Tris-HCl, pH 7.5 (containing 2 mM EDTA and 1.4 μmol dithiothreitol) and incubated for 2 h at room temperature. Then, 4-vinylpyridine (9 μmol, 1 μl) was added and after 10 min incubation, the membrane was washed in bidistilled water, dried and then placed in the blott-cartridge (Applied Biosystems) for sequencing.

3. RESULTS AND DISCUSSION

A typical chromatogram obtained by RP-HPLC of the peptide fraction collected from the skin secretion of *Rana esculenta* is presented in Fig. 1. An aliquot corresponding to 1/12 of the total material secreted after electrical stimulation by a single frog was loaded onto the column. Two-ml fractions were collected and tested for different cytolytic activities. Fractions displaying the highest hemolytic activity or antimicrobial activities against *Escherichia coli* or *Staphylococcus aureus* (Fig. 1) were further purified by RP-HPLC using different experimental conditions. Three peptides were investigated further by amino acid analysis, which showed the presence of cysteine/cystine in all of them, and by

Table I

Antibiotic and lytic activity of *Rana esculenta* peptides

Peptide	Lethal concentration (μM)			
	E. coli D21	*B. megaterium* Bm11	*S. aureus* Cowan 1	Red blood cells
Brevinin-1E	1.8	0.4	0.6	0.5
Brevinin-2E	0.5	0.2	2.0	>100
Esculentin	0.2	0.1	0.4	>100
A1	>50	18.3	>200	~20
Bombinin[a]	3.0	0.8	14.0	>50
Cecropin A[b]	0.3	0.5	>200	>400
Melittin	0.8	0.6	0.2	0.9

[a] Bombinin is used as a reference molecule, since the same LC values were obtained in this study and in the Department of Microbiology, University of Stockholm.

[b] Data from [17].

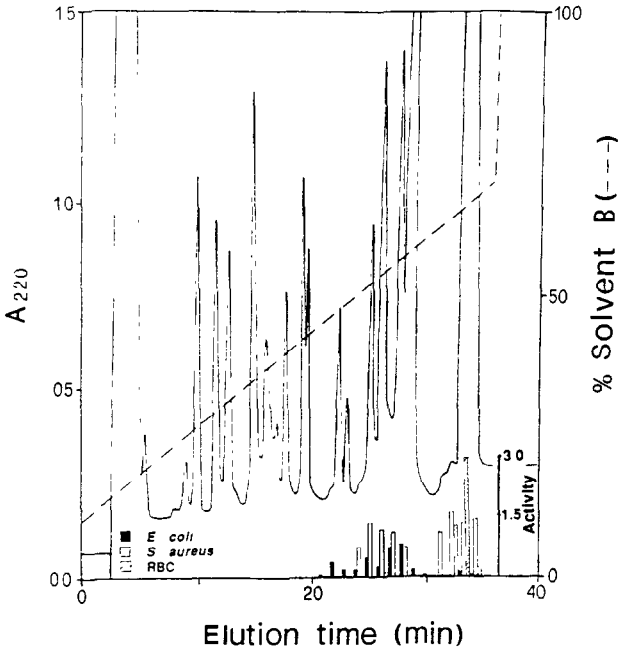

Fig. 1. Reverse-phase HPLC of skin secretion of *R. esculenta*. Antimicrobial activity against *E. coli* is expressed in cecropin A units ($\times 10^{-3}$). Activity against *S. aureus* and hemolytic activity are expressed in diameter^2 (cm) of growth inhibition or lytic zone.

Edman degradation, where the sequencing yield was progressively decreasing in the COOH-terminal part of each peptide. Therefore, Edman degradation was carried out after reduction and treatment with 4-vinylpyridine [14] of peptides coupled to Sequelon-AA membranes via carbodiimide activation. Using this method, the complete sequence of the peptides was obtained and it could be shown that each peptide contains two cysteine residues, which were detected as the PTH-derivative of *S*-pyridylethylcysteine. These experiments also demonstrated that the peptides were not amidated at the COOH-terminus. If Edman degradation was performed without prior reduction, the cysteine derivative could not be detected. This indicates that the two cysteines present in each peptide form a disulfide bridge.

The structure of the three peptides from skin secretion of *R. esculenta* is shown in Fig. 2. The shortest peptide contains 24 amino acids and has a net charge of +4. The two cysteines, of which one is the COOH-terminal residue, are separated by five amino acids. Its sequence is similar to the sequence of brevinin-1, an antimicrobial peptide recently isolated from the skin of another *Rana* species [10]. The two peptides have 16 amino acids in common, including the position of the two cysteines. Accordingly, we named this peptide brevinin-1E (E for esculenta).

The second peptide is comprised of 33 amino acids, including two cysteines which are positioned like in brevinin-1E. This peptide is homologous to brevinin-2,

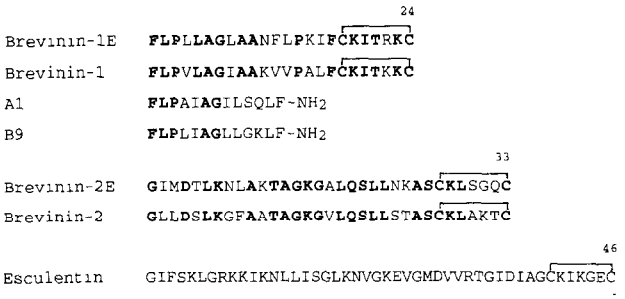

Fig. 2. Amino acid sequence of antimicrobial peptides from skin secretion of *R. esculenta*. The sequences of brevinin-1E and brevinin-2E are compared with those of brevinin-1 and brevinin-2, respectively, from *Rana brevipoda porsa* [10]. Identical residues are boldfaced. The sequences of peptides A1 and B9 from methanol skin extracts of *R. esculenta* [9] are also reported for comparison.

with which it has 21 residues in common (see Fig. 2). We have thus termed it brevinin-2E.

Finally, a third antimicrobial peptide composed of 46 amino acids was detected in skin secretion of *R. esculenta*. This apparently represents a new type of peptide whose sequence is not related to any known one. However, like the brevinins, it also contains two cysteine residues located at position 1 and 6 from the COOH-end (see Fig. 2). This peptide has been named esculentin.

Also included in Fig. 2 are the amino acid sequences of the peptides A1 and B9 from skin extracts of *R. esculenta*, which have been described earlier [9]. The amino-terminal sequences of these tridecapeptides are similar to those of brevinin-1 and brevinin-1E.

The antimicrobial and hemolytic activities of these three peptides are shown in Table I compared to those of bombinin from skin of *Bombina variegata* [3], cecropin A from moth hemolimph [15], and melittin from bee venom [16]. It is evident that the three peptides from skin secretion of *R. esculenta* all show high antimicrobial activity against the two Gram-positive and one Gram-negative bacteria tested, with esculentin being the most potent one. Moreover, brevinin-1E shows a very high hemolytic activity, being more potent than melittin. The peptide A1 described earlier [9] has only low antimicrobial but some hemolytic activity (see Table I).

Like other antimicrobial peptides from amphibian skin, the ones from *R. esculenta* also have a positive net charge. Interspersed between lysine and arginine residues are sequences containing primarily amino acid with hydrophobic side chains. These structural features may be necessary for the interaction with phospholipid bilayers. Many of these antimicrobial peptides from diverse sources can form amphipatic helices. This is also true for those segments of brevinin-2E and esculentin which precede the first cysteine residue. On the other hand, brevinin-1E contains, besides the common COOH-terminal disulfide loop, two proline residues separated by ten amino acids. Therefore, in this peptide only a short α helix of about three turns could potentially be formed.

It is now becoming apparent that antimicrobial peptides with very different amino acid sequences are present in frogs of different families. This has now been shown for *Bombina variegata* and *Bombina orientalis* (family Discoglossidae) [3,4], *Xenopus laevis* (family Pipidae) [5], *Phyllomedusa sauvagei* (family Hylidae) [6], and two *Rana* species (family Ranidae) [10 and present work]. It can thus be expected that many additional peptides with antimicrobial activity will be found in other amphibian species.

Acknowledgements. This work was supported in part by grants from the Italian Ministerio dell'Università e della Ricerca Scientifica e Tecnologica and Consiglio Nazionale delle Ricerche no. 92.02146.CT14. Part of this work will be submitted in partial fulfillment of the requirements of a Ph.D. degree at the Università La Sapienza, Roma (by G M.) We thank Prof. Vittorio Erspamer and Prof. Gunther Kreil for continued encouragement and helpful discussions. The support and advice of Prof. Hans G. Boman to M S. during the EMBO sponsored visit to the Department of Microbiology, University of Stockholm, was invaluable.

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