Mol. Cells *30*, 435-441, November 30, 2010
DOI/10.1007/s10059-010-0135-7

Molecules and Cells

Solution Structure of Antimicrobial Peptide Esculentin-1c from Skin Secretion of *Rana esculenta*

Su-Jin Kang, Woo-Sung Son, Kyung-Doo Han, Tsogbadrakh Mishig-Ochir[1], Dae-Woo Kim[2], Jae-Il Kim[2], and Bong-Jin Lee*

Granular glands in the skins of frogs synthesize and secrete a remarkably diverse range of peptides capable of antimicrobial activity. These anuran skin antimicrobial peptides are commonly hydrophobic, cationic and form an amphipathic α-helix in a membrane mimetic solution. Recently, they have been considered as useful target molecules for developing new antibiotics drugs. Esculentin-1c is a 46-amino acid residue peptide isolated from skin secretions of the European frog, *Rana esculenta*. It displays the most potent antimicrobial activity among bioactive molecules. Esculentin-1c has the longest amino acids among all antimicrobial peptides. The present study solved the solution structure of esculentin-1c in TFE/water by NMR, for the first time. We conclude that this peptide is comprised of three α-helices with each helix showing amphipathic characteristics, which seems to be a key part for permeating into bacterial membranes, thus presenting antimicrobial activity.

INTRODUCTION

Membrane-active peptides produced by most organisms exhibit antibiotic, fungicidal, hemolytic, virucidal and tumoricidal activities (Bechinger, 1997; Boman, 1995; Dennison et al., 2005; Gabay, 1994). Many membrane-active antimicrobial peptides have little toxicity against animal cells, whereas they exhibit a broad spectrum of antimicrobial activity against a range of microorganisms (Goraya et al., 1999; Hancock and Scott, 2000; Mor and Nicolas, 1994; Rinaldi, 2002; Won et al., 2004a; 2004b). Antimicrobial peptides are important components of an innate and rapid defense against harmful microorganisms in living organisms (Hancock and Scott, 2000; Koczulla and Bals, 2003; Nicolas and Mor, 1995; Shai, 2002; Zasloff, 2003). These antimicrobial peptides are synthesized and stored in dermal glands, and are secreted into the skin mucous upon alarm or injury (Simmaco et al., 1998). Most antimicrobial peptides are believed to kill pathogens by disrupting their membranes. These molecules commonly carry a net positive charge, and most of them have the propensity to form an amphipathic α-helix (Simmaco et al., 1994). Recently, interest in the antimicrobial peptides has increased due to their potential as therapeutic agents. Several antimicrobial peptides have been successful in pharmaceutical and commercial development (Hancock and Scott, 2000; Koczulla and Bals, 2003; Zasloff, 2003). The anuran skin has served as an exceedingly rich source of antimicrobial peptides, and a number of cationic a-helical antimicrobial peptides have been isolated from the granular glands of the skin of amphibians (Barra and Simmaco, 1995; Rinaldi, 2002; Rozek et al., 2000; Simmaco et al., 1998; Won et al., 2004b; 2009). Thus, anuran-skin antimicrobial peptides are considered as useful target molecules for developing new antibiotic and/or anticancer drugs (Koczulla and Bals, 2003; Rinaldi, 2002; Won et al., 2002; Zasloff, 2003).

Since 1987, when magainin was isolated from the skin secretions of the African clawed frog, *Xenopus laevis* (Zasloff, 1987), numerous additional antimicrobial peptides have been identified in different anuran species (Koczulla and Bals, 2003; Rinaldi, 2002; Won et al., 2002; Zasloff, 2003). They now number into the hundreds of members with the length of these peptides ranging from 10 to 50 amino acids (Jenssen et al., 2006; Nascimento et al., 2003; Rinaldi, 2002; Simmaco et al., 1998; Zasloff, 2002). The largest number of anuran-skin antimicrobial peptides has been isolated from frogs of the genus Rana, a group distributed worldwide with over 250 different species. These Ranidae peptides are composed of 10-47 amino acid residues and are characterized by what has been termed a Rana-box, a 7-membered cyclic loop region with a single disulfide bridge at the C-terminal end (Vanhoye et al., 2003). The numerous Ranidae peptides are classified into at least 14 families based on sequence similarity (Basir et al., 2000; Vanhoye et al., 2003). From *Rana esculenta* skin secretions, numerous different antimicrobial peptides have been isolated and grouped into four families on the basis of both their structural and functional features. Esculentin, which has 46 amino acids with a C-terminal disulfide bridge, is an antimicrobial peptide isolated from skin secretions of the edible European Frog, *Rana esculenta*. It is one of the earliest characterized families (Ali et al., 2002; Basir et al., 2000; Morikawa et al., 1992; Simmaco et al., 1993; 1994). Esculenin is a highly potent anti-microbial mole-

Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul 151-742, Korea, [1]Department of Biophysics, Faculty of Biology, National University of Mongolia, Ulaanbaatar, Mongolia, [2]Department of Life Sciences, Gwangju Institute of Science and Technology, Gwangju 500-712, Korea
*Correspondence: lbj@nmr.snu.ac.kr

Received May 10, 2010; revised August 13, 2010; accepted August 16, 2010; published online September 10, 2010

Keywords: antimicrobial peptide, nuclear magnetic resonance, solution structure

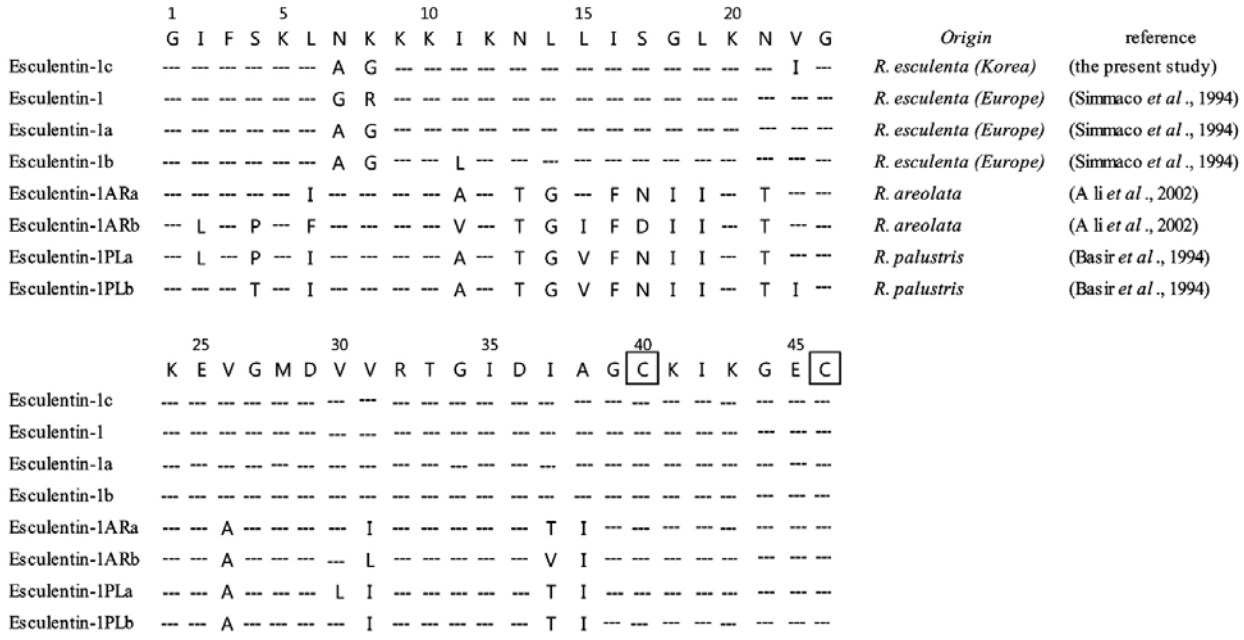

Fig. 1. Comparison of the amino-acid sequence of esculentin-1 peptides isolated from *Rana esculenta* and other Ranidae. The most conserved sequence is represented on the top line. For clarity, the sequence identity is represented by dashes. The Cys residues, indicated by boxes, form an intra-molecular disulfide bridge.

cule that is characterized by the most favorable spectrum of action with negligible effects on eukaryotic cell membranes. Moreover, this antimicrobial peptide is very active against *Staphylococcus aureus*, with a much lower hemolytic activity than brevinin-1 and brevinin-2, two other peptides also found in *Rana esculenta* (Simmaco et al., 1994; Won et al., 2004b). Esculentin-1c is a 46 residue antimicrobial peptide isolated from skin secretions of Korean *Rana esculenta*. It has two cysteine residues in positions 1 and 7 in the C-terminus (Won et al., 2004b). These cysteine residues are linked by a disulfide bridge. Figure 1 shows the amino acid sequences of esculentin-1 analogues, showing that the primary structure of esculentin-1 is relatively well conserved.

In a previous study, we searched for antimicrobial peptides from the skins of *Rana esculenta* inhabiting the Korean peninsula and investigated their antimicrobial and hemolytic activity (Won et al., 2004b). As a result, we found that esculentin-1c is strongly related to esculentin-1, as the former shows a broad range antimicrobial activity. In this study, we investigated the solution structures of the esculentin-1c, which is an antimicrobial peptide with longest amino acid sequence. The solution structure of esculentin-1c could be calculated for the first time among esculentin-like antimicrobial peptides.

MATERIALS AND METHODS

Preparation of peptide

Esculentin-1c peptide was synthesized automatically by solid-phase methods using standard Fmoc chemistry (Amblard et al., 2006; Wellings and Atherton, 1997). Fmoc (9-fluorenylmethoxycarbonyl)-protected amino acids and Rink Resins were obtained from Advanced Chemtech, Inc. and HPLC solvents were from Fisher Scientific. All other chemicals were either analytical or biotechnological grade, obtained from various manufacturers. To obtain the peptide amide, 4-(2′,4′-dimethoxypheyl-Fmoc-amino-methyl)phenoxy resin was used. Side-chain protection groups included Fmoc-O-butyl-L-Serine and N-Fmoc-N-Boc-L-Lysine. Double coupling procedures were performed with diisopropylcarbodiimide/1-hydroxybenzotriazole activation. Fmoc group removal from the peptide chain was performed with 25% piperidine in dimethylformamide. Cleavage of the resin and the protecting group was performed with 10% trifluoroacetic acid in dichloromethane. Purification and analysis of the products were performed by analytical reversed-phase HPLC on C-18 columns from Merck-Hitachi. Acetonitrile/water mixed with 0.1% trifluoroacetic acid was used as an eluent and a gradient of 20-80% acetonitrile was applied at a flow rate of 1 ml/min. The correct mass of the product peptides was identified by mass spectrometry.

CD spectroscopy

For CD spectroscopy, a precise quantity of powdered peptide was dissolved to a final concentration of 50 uM in various solvents: 10% TFE/water, 30% TFE/water, 50% TFE/water, 5 mM DPC and 10 mM SDS micelles. Before the measurement, the pH was adjusted to 4.0 with 0.1 N HCl or NaOH, and CD spectra were obtained on a JASCO J-720 spectropolarimeter, using a 0.2 cm path-length cell. CD scans were taken at 293 K from 250 to 190 nm, with a 1 nm bandwidth, 4 s response time, scan speed of 50 nm/min and 0.5 nm step resolution. Three scans were added and averaged, followed by subtraction of the CD signal of the solvent.

NMR spectroscopy

2,2,2-trifluoroethaol-d_3 99.5% was purchased from Aldrich (USA). All other chemicals were analytical grade obtained from various manufacturers. The sample for the NMR measurements contained 2 mM esculentin-1c peptide in a 70% TFE/water solution at pH 4.0. The 2D DQF-COSY, TOCSY (40 ms mixing time), and NOESY (150 and 200 ms mixing time) spectra were re-

corded on a Bruker 600 MHz spectrometer at 293 K. Solvent suppression was achieved by selective low-power irradiation of the water resonance. All NMR spectra were processed and analyzed with NMRPipe/NMRDraw software (Delaglio et al., 1995) and the NMRView program (Johnson and Blevins, 1994). ^{1}H chemical shifts were referenced to methyl signals of sodium 4,5-dimethyl-4-silapentane-1-sulphonate (DSS). Sequence-specific assignments of the proton resonances were achieved by spin system identification from the TOCSY and DQF-COSY spectra, followed by sequential assignment via the NOE connectivities.

Structure calculation

The sequence-specific assignments of the proton resonances were achieved by spin system identification from TOCSY and DQF-COSY spectra, followed by sequential assignments through the NOE connectivities. Distance restraints were obtained mainly by manual assignment of the NOE cross peaks in the NOESY spectra with 150 ms mixing times. The 150 and 200 ms NOESY spectra were compared to assess the possible contribution from spin diffusion. All NOE data were classified into three classes, strong, medium or weak, corresponding to upper bound interproton distance restrains of 3.0, 4.0 and 5.0 Å, respectively. Lower distance bounds were taken as the sum of the vander Waals radii of 1.8 Å. Three-dimensional structures were calculated using the simulated annealing and energy minimization protocol in the program CNS 1.1 (Brunger et al., 1998). Finally, 20 structures with the lowest energies were accepted to present an ensemble structure and to obtain the energy-minimized average structure.

RESULTS AND DISCUSSION

Backbone peaks assignment

The assignment of backbone peaks of esculentin-1c in TFE/water was mainly performed by the standard method proposed by Wüthrich (1986). The non-degenerated alpha protons of the glycine residues of esculentin-1c were easily identified in the DQF-COSY spectra. Two alanines were also found without ambiguity by the combination of TOCSY and DQF-COSY spectra. These spin systems were assigned to each amino acid types and were connected using the NOESY spectra. The sequential connections were completed mainly on the basis of the strong d_{NN} connections, starting from the unique amino acid types, such as glycines and alanines. These connections were compared with the $d_{\alpha N}$ (i, i + 3), $d_{\alpha N}$ (i, i + 4), and $d_{\alpha\beta}$ (i, i + 3) connections to ensure their initial assignments. We could achieve almost complete assignments of esculentin-1c NMR peaks. All of ^{1}HN except Gly 1, and 91% of αH and 98% of βH peaks were assigned. NMR peaks of Gly 1 residue were not observed in the spectrum and therefore could not be assigned. There were 8 Lys residues in the 46 amino acid sequences of esculentin-1c, and it takes 17% of total amino acid residues. As a result, almost NMR peaks of Lys residues were overlapped and couldn't be separated perfectly. Esculentin-1c has 11 Leucine and Isoleucine residues (24%). Moreover, their chemical shifts are similar to the point that these peaks appeared together and were difficult to be distinguished. These chemical shift values of the NH, αH, βH and others of esculentin-1c in the 70% TFE/water solution are given in the Table 1.

Table 1. Chemical shifts of ^{1}H of esculentin-1c in 70% TFE/water at pH 4.0 and 293 K

	NH	αH	βH	Others
Gly1	*ND	ND		
Ile2	8.410	3.972	1.723	γCH2, 1.332, 1.137, γCH3 0.711 δCH30.711
Phe3	7.920	4.374	3.200, 3.102	Hd/He 7.277 Hz 7.227
Ser4	8.059	4.163	3.975	
Lys5	7.860	4.165	1.978	γCH2 1.569 δCH2 1.447 εCH3 3.135 NH3 7.179
Leu6	7.980	4.129	1.572	γH 1.812 δCH3 0.902, 0.857
Ala7	8.266	4.002	1.323	
Gly8	7.962	3.876, 3.789		
Lys9	7.770	4.051	1.977	γCH2 1.597 δCH2 1.694
Lys10	7.979	4.032	1.977	
Ile11	8.313	3.756	1.920	γCH2 1.728, 1.179 δCH3 0.822
Lys12	8.082	3.948	1.932	
Asn13	7.948	4.431	2.965, 2.770	HNg 7.373, 6.635
Leu14	8.202	4.092	1.762	
Leu15	8.534	4.132	1.602	
Ile16	8.534	3.798	1.946	
Ser17	8.088	4.154	ND	
Gly18	8.220	3.952		
Leu19	8.255	4.168	1.569	
Lys20	8.361	4.074	1.980	
Asn21	7.936	4.633	2.952, 2.878	NHg 7.443, 6.659
Ile22	8.094	3.897	1.968	
Gly23	8.300	3.891, 3.790		
Lys24	7.828	4.164	1.975	
Glu25	8.023	4.118	2.215	γCH2 2.465
Val26	8.425	3.807	2.130	γCH3 1.045, 0.936
Gly27	8.090	3.896, 3,797		
Met28	8.034	4.304	2.216, 2.116	γCH2/εCH3 2.735, 2.575
Asp29	8.237	4.515	3.064, 2.838	
Val30	8.380	3.659	2.340	γCH3 1.070, 0.944
Val31	7.937	3.689	2.202	γCH3 1.067, 0.940
Arg32	8.415	3.963	1.968	γCH2 1.825, 1.675 δCH2 3.224, 3.1864 HNε 7.147
Thr33	8.077	4.012	4.339	γCH3 1.274
Gly34	8.147	3.943		
Ile35	8.126	3.818	1.956	
Asp36	8.237	ND	3.071	
Ile37	8.379	4.042	1.928	γCH2 1.604, 1,177 γCH3 0.897 δCH3 0.897
Ala38	8.193	4.040	1.485	
Gly39	8.219	3.953, 3.825		
Cys40	8.109	4.496	3.258, 3.122	
Lys41	8.742	4.158	2.148	
Ile42	8.444	3.890	1.963	γCH2 1.713, 1.230 γCH3 0.947 δCH3 0.856
Lys43	7.360	4.319	1.769	
Gly44	7.846	4.100, 4.033		
Glu45	8.251	4.460	1.850	γCH2 2.380, 2.290
Cys46	7.603	4.479	3.543, 2.896	

* ND, not detected

Determination of secondary structure

We used the Chemical Shift Index (CSI) method (Wishart and Sykes, 1994a; 1994b) to determine the secondary structure of esculentin-1c. This approach was developed to provide a NOE-independent method for structure determination. The CSI prediction result for esculentin-1c is shown in Fig. 2 along with the amino acid sequence. Generally, the values '1' and '-1' in the CSI indicate, respectively, β-strand and α-helical tendencies, while the other values (zero) indicate neither a β-strand nor a helical tendency. The consensus CSI for each residue was

Fig. 2. CSI plot of esculentin-1c. In the consensus CSI, the values '-1' and '+1' respectively indicate α-helical and β-sheet tendencies.

derived by a simple 'majority rule' (Wishart and Sykes, 1994). On the basis of our resonance assignments, three helical regions were identified using the CSI program as shown in Fig. 2 (Wishart et al., 1995). As a result, esculetine-1c consists of three α-helices corresponding to residues K5-S17 (αI), G23-R32 (αII), and A38-K43 (αIII).

Solution structure of esculentin-1c

As shown in Fig. 3, we examined the conformation of esculentin-1c in various membrane mimetic environments in this case TFE/water, DPC, and SDS micelles, by CD spectroscopy. Numerous membrane peptides and proteins are insoluble in water, which makes it difficult to study the solution structure using NMR spectroscopy. It has been reported that most of the antimicrobial peptides adopt an α-helical conformation in membrane-mimetic environments such as TFE/water, DPC micelles and SDS micelles, whereas they are nearly unstructured in an aqueous solution. We tried to solve the three-dimensional structure of esculentin-1c in SDS micelle or DPC micelle, which are used with bacterial membrane mimetic environments. However, the NMR peaks of the 2D NMR spectra measured in SDS micelles and DPC micelles showed severe overlapping as well as poor dispersion because esculentin-1c adopts the α-helical conformation and because its length of 46 amino acid residues is long. This prevented an analysis of the NMR spectra and a calculation of its structure. It has also been reported that the structures of several membrane peptides such as magainin and ranalexin in TFE/water are similar to those in SDS micelles and/or those in DPC micelles (Gesell et al., 1997; Vignal et al., 1998). In many cases, TFE has been used as helix-promoting solvent, as it induces helical structure only in peptides with a tendency toward helix formation. Moreover, it is assumed that the aqueous TFE-induced conformation is similar to the structure that would be observed in a biological membrane. In a similar fashion, the CD spectra of esculentin-1c in 5 mM DPC and 10 mM SDS were similar to the spectrum in TFE/water, also indicating a highly ordered helical conformation. Additionally, we found that the helical structure of esculentin-1c was stabilized by increasing concentrations of TFE, as shown by the CD intensification at 208 and 222 nm. This result indicates that the helicity of esculentin-1c increases in micellar environments. This conformation transition of esculentin-1c reflects its potential for interaction with a membrane. Not only was the α-helical structure of esculenitn-1c in TFE/water confirmed by CD spectroscopy, as with the SDS and DPC micelles, but the NMR spectra of esculentin-1c in TFE/water were well resolved. In addition, it was reported that peptides adopts a more α-helical conformation when they exist at a higher TFE concentration (Reed and Kinzel, 1993; Sivaraman et al., 1996; Sonnichsen et al., 1992). To enhance the NMR peak resolution, we selected 70% TFE/water as a solvent. A 70% TFE/water solution has been used frequently in the study of α-helical peptides. Accordingly, the three-dimensional structure of esculentin-1c was calculated in a 70% TFE/water solution for a stable helical structure.

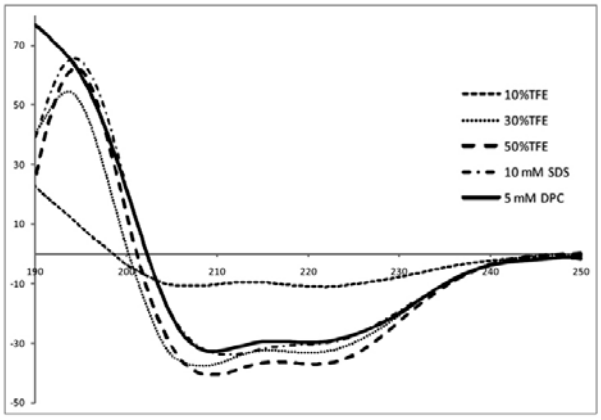

Fig. 3. CD spectra of esculentin-1c 10% TFE/water (·····), 30% TFE/water (·····), 50% TFE/water (– – –), 5 mM DPC (—·—) and 10 mM SDS (——).

The solution structure of esculentin-1c in TFE/water was characterized by NMR spectroscopy. The sequential and medium-range inter-residue NOE connectivities that are characteristics of α-helical conformations were unambiguously detected in esculentin-1c in TFE/water. The intra-residue disulfide bond between Cys 40 and Cys 46 was confirmed by unambiguous NOE cross-peaks between Cys 40-H^{β} and Cys 46-H^{N}, and Cys 40-H^{N} and Cys 46-H^{β}. As a result, the structure of esculentin-1c adopts a full helical structure, which is composed of three α-helices with a C-terminal disulfide bond. These three α-helices correspond to residues K5 to S17 (αI), G23 to R32 (αII), and A38 to K43 (αIII), as presented in Fig. 4. helix I and helix II appear to some extent to be one helix, however, they are separated into two helices connected by a short loop. Accordingly, esculentin-1c has three helices. The N-terminal helix I and helix II are located in a straight line, while the short C-terminal helix III bends by approximately 100 degrees against N-terminal helix axis. Simulated annealing (SA) calculations were run to produce structures with a common fold that were in good agreement with the experimental restraints with low total energies. The 20 structures with the lowest energies were chosen to represent the solution structure of esculentin-1c as shown in Fig. 4.

Each helix in esculentin-1c shows a typical amphipathic property, with hydrophobic residues on one side and hydrophilic residues on the other side in the helical axis, as clearly depicted in the helical wheel diagram shown in Fig. 5. The amphipathic helix of anuran-skin antimicrobial peptides is one of the most important bases for their interaction with membranes. After they bind to bacterial membranes, they act via a pore-forming mechanism or a carpet-like mechanism, which have generally been accepted as the mechanisms of the membrane permeation of amphipathic α-helical antimicrobial peptides. Therefore, the amphipathic property of helices in esculentin-1c may contribute to any interaction with bacterial membranes. Although

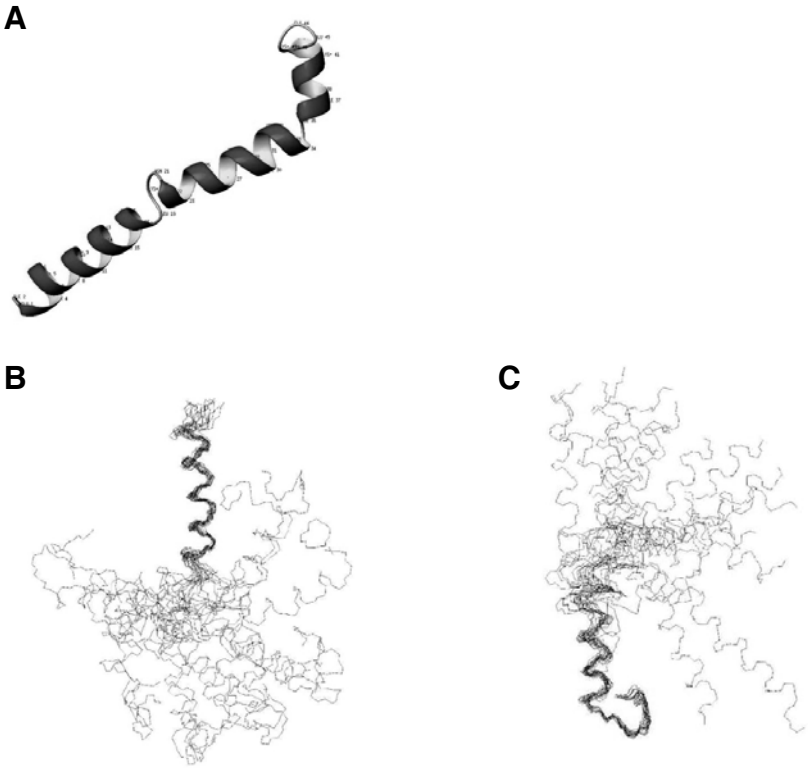

Fig. 4. NMR solution structure of esculenta-1c. (A) Ribbon drawing of esculentin-1c. (B) Backbone ensemble of the 20 best energy-minimized conformers of the N-terminal. (C) Backbone ensemble of the 20 best energy-minimized conformers of the C-terminal.

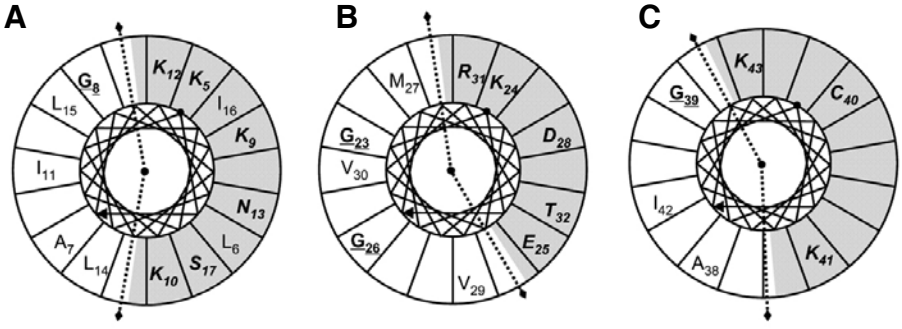

Fig. 5. Helical wheel diagrams of three helices in esculentin-1c. (A) The first α-helix of esculentin-1c (K5-S17). (B) The second α-helix of esculentin-1c (G23-R32). (C) The third α-helix of esculentin-1c (A38-K43). Hydrophobic and hydrophilic residues are represented by the thin letters and italic bold letters with a grey background, respectively. Glycine residues are underlined.

the hydrophilic face in the helix I is interrupted by the two hydrophobic residues of Leu 6 and Ile 16, this is a shared feature of all esculentin-like peptides, and the helical stretch from Lys 5 to Ser 17 is perfectly amphipathic. Thus, the structural components of esculentin-1c are identical to those of the esculentin-1-like peptide, exhibiting a broad spectrum of antimicrobial activity. The positively-charged residues would facilitate peptide binding as well as the passage of small molecules such as conventional antibiotics, whereas hydrophobic residues would be responsible for the disruption and permeabilization of the inner membrane leading to cell death (Hancock, 1997).

It was also reported that no antimicrobial effect was detected with esculentin-1 (19-46) and that the N-terminal 1-18 fragment of esculentin-1b showed antibiotic activity (Mangoni et al., 2003). This implies that at least the first 18 residues are required to display antimicrobial properties. The antibiotic activity of esculentin-like peptides is located in its N-terminal portion and its amphipathic property is important for antimicrobial activity. The first N-terminal α helix of eslculentin-1c shows an amphipatic characteristic, as well. Therefore, we can assume that the antimicrobial activity of esculentin-1c comes from this N-terminal α helix as in other esculentin-like peptides. In addition, an activity comparison of cyclic esculentin with linear esculentin showed that their antimicrobial activities had not changed, thus demonstrating that the presence of a disulfide bridge in the C-terminal regions is not essential for the molecule to have antimicrobial and cytolytic properties (Ponti et al., 1999). It was reported that this Rana box in other ranidae antimicrobial peptides serves to interact with the membrane and stabilize interactions. The Rana box in esculentin-1c as well does not appear to be related to antimicrobial activity but to structural stability.

In summary, esculentin-1c in TFE/water solution has three α-helices with each helix showing amphipathic characteristics. This structural property seems to play a crucial role for the mechanism permeating into bacterial membranes, which results in its antimicrobial activity. Especially, the N-terminal α helix of esculentin-1c is responsible for its antimicrobial activity like other esculentin-like peptide. The structure of esculentin-1c obtained by NMR spectroscopy in this study is the first solved one in esculentin-like antimicrobial peptides. In addition, it is

meaningful that 46-amino-acid of esculentin-1c is very long length among antimicrobial peptides.

ACKNOWLEDGMENTS

This work was financially supported by a grant from the National Research Foundation of Korea (NRF) funded by the Korean Government (Ministry of Education, Science and Technology) (No. 20100001707). This work was also supported by a grant of the Korea Healthcare Technology R&D project, Ministry for Health and Welfare, Republic of Korea (A092006) and by the 2009 Brain Korea 21 project for Medicine, Dentistry and Pharmacy. We thank National Center for Inter-University Research Facilities and Korea Basic Science Institute for the use of NMR machines.

REFERENCES

Ali, M.F., Lips, K.R., Knoop, F.C., Fritzsch, B., Miller, C., and Conlon, J.M. (2002). Antimicrobial peptides and protease inhibitors in the skin secretions of the crawfish frog, Rana areolata. Biochim. Biophys. Acta *1601*, 55-63.

Amblard, M., Fehrentz, J.A., Martinez, J., and Subra, G. (2006). Methods and protocols of modern solid phase Peptide synthesis. Mol. Biotechnol. *33*, 239-254.

Barra, D., and Simmaco, M. (1995). Amphibian skin: a promising resource for antimicrobial peptides. Trends Biotechnol. *13*, 205-209.

Basir, Y.J., Knoop, F.C., Dulka, J., and Conlon, J.M. (2000). Multiple antimicrobial peptides and peptides related to brady-kinin and neuromedin N isolated from skin secretions of the pickerel frog, Rana palustris. Biochim. Biophys. Acta *1543*, 95-105.

Bechinger, B. (1997). Structure and functions of channel-forming peptides: Magainins, cecropins, melittin and alamethicin. J. Membr. Biol. *156*, 197-211.

Boman, H.G. (1995). Peptide antibiotics and their role in innate immunity. Ann. Rev. Immunol. *13*, 61-92.

Brunger, A.T., Adams, P.D., Clore, G.M., DeLano, W.L., Gros, P., Grosse-Kunstleve, R.W., Jiang, J.S., Kuszewski, J., Nilges, M., Pannu, N.S., et al. (1998). Crystallography & NMR system: A new software suite for macromolecular structure determination. Acta Crystallogr. D Biol. Crystallogr. *54*, 905-921.

Delaglio, F., Grzesiek, S., Vuister, G.W., Zhu, G., Pfeifer, J., and Bax, A. (1995). NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J. Biomol. NMR *6*, 277-293.

Dennison, S.R., Wallace, J., Harris, F., and Phoenix, D.A. (2005). Amphiphilic alpha-helical antimicrobial peptides and their structure/function relationships. Protein and Pep. Lett. *12*, 31-39.

Gabay, J.E. (1994). Ubiquitous natural antibiotics. Science *264*, 373-374.

Gesell, J., Zasloff, M., and Opella, S.J. (1997). Two-dimensional 1H NMR experiments show that the 23-residue magainin antibiotic peptide is an alpha-helix in dodecylphosphocholine micelles, sodium dodecylsulfate micelles, and trifluoroethanol/water solution. J. Biomol. NMR *9*, 127-135.

Goraya, J., Knoop, F.C., and Conlon, J.M. (1999). Ranatuerin 1T: an antimicrobial peptide isolated from the skin of the frog, *Rana temporaria*. Peptides *20*, 159-163.

Hancock, R.E. (1997). Peptide antibiotics. Lancet *349*, 418-422.

Hancock, R.E.W., and Scott, M.G. (2000). The role of antimicrobial peptides in animal defenses. Proc. Natl. Acad. Sci. USA *97*, 8856-8861.

Jenssen, H., Hamill, P., and Hancock, R.E. (2006). Peptide antimicrobial agents. Clin. Microbiol. Rev. *19*, 491-511.

Johnson, B.A., and Blevins, R.A. (1994). Nmr view - a computer-program for the visualization and analysis of Nmr data. J. Biomolecular Nmr. *4*, 603-614.

Koczulla, A.R., and Bals, R. (2003). Antimicrobial peptides: current status and therapeutic potential. Drugs *63*, 389-406.

Mangoni, M.L., Fiocco, D., Mignogna, G., Barra, D., and Simmaco, M. (2003). Functional characterisation of the 1-18 fragment of esculentin-1b, an antimicrobial peptide from *Rana esculenta*. Peptides *24*, 1771-1777.

Mor, A., and Nicolas, P. (1994). The NH2-terminal alpha-helical domain 1-18 of dermaseptin is responsible for antimicrobial activity. J. Biol. Chem. *269*, 1934-1939.

Morikawa, N., Hagiwara, K., and Nakajima, T. (1992). Brevinin-1 and -2, unique antimicrobial peptides from the skin of the frog, Rana brevipoda porsa. Biochem. Biophys. Res. Commun. *189*, 184-190.

Nascimento, A.C., Fontes, W., Sebben, A., and Castro, M.S. (2003). Antimicrobial peptides from anurans skin secretions. Protein Pept. Lett. *10*, 227-238.

Nicolas, P., and Mor, A. (1995). Peptides as weapons against microorganisms in the chemical defense system of vertebrates. Annu. Rev. Microbiol. *49*, 277-304.

Ponti, D., Mignogna, G., Mangoni, M.L., De Biase, D., Simmaco, M., and Barra, D. (1999). Expression and activity of cyclic and linear analogues of esculentin-1, an anti-microbial peptide from amphibian skin. Eur. J. Biochem. *263*, 921-927.

Reed, J., and Kinzel, V. (1993). Primary structure elements responsible for the conformational switch in the envelope glycoprotein gp120 from human immunodeficiency virus type 1: LPCR is a motif governing folding. Proc. Natl. Acad. Sci. USA *90*, 6761-6765.

Rinaldi, A.C. (2002). Antimicrobial peptides from amphibian skin: an expanding scenario - Commentary. Curr. Opin. Chem. Biol. *6*, 799-804.

Rozek, T., Wegener, K.L., Bowie, J.H., Olver, I.N., Carver, J.A., Wallace, J.C., and Tyler, M.J. (2000). The antibiotic and anticancer active aurein peptides from the Australian Bell Frogs Litoria aurea and Litoria raniformis - the solution structure of aurein 1.2. Eur. J. Biochem. *267*, 5330-5341.

Shai, Y. (2002). Mode of action of membrane active antimicrobial peptides. Biopolymers *66*, 236-248.

Simmaco, M., Mignogna, G., Barra, D., and Bossa, F. (1993). Novel antimicrobial peptides from skin secretion of the European frog Rana esculenta. FEBS Lett. *324*, 159-161.

Simmaco, M., Mignogna, G., Barra, D., and Bossa, F. (1994). Antimicrobial peptides from skin secretions of Rana esculenta. Molecular cloning of cDNAs encoding esculentin and brevinins and isolation of new active peptides. J. Biol. Chem. *269*, 11956-11961.

Simmaco, M., Mignogna, G., and Barra, D. (1998). Antimicrobial peptides from amphibian skin: what do they tell us. Biopolymers *47*, 435-450.

Sivaraman, T., Kumar, T.K., and Yu, C. (1996). Destabilisation of native tertiary structural interactions is linked to helix-induction by 2,2,2-trifluoroethanol in proteins. Int. J. Biol. Macromol. *19*, 235-239.

Sonnichsen, F.D., Van Eyk, J.E., Hodges, R.S., and Sykes, B.D. (1992). Effect of trifluoroethanol on protein secondary structure: an NMR and CD study using a synthetic actin peptide. Biochemistry *31*, 8790-8798.

Vanhoye, D., Bruston, F., Nicolas, P., and Amiche, M. (2003). Antimicrobial peptides from hylid and ranin frogs originated from a 150-million-year-old ancestral precursor with a conserved signal peptide but a hypermutable antimicrobial domain. Eur. J. Biochem. *270*, 2068-2081.

Vignal, E., Chavanieu, A., Roch, P., Chiche, L., Grassy, G., Calas, B., and Aumelas, A. (1998). Solution structure of the antimicrobial peptide ranalexin and a study of its interaction with perdeuterated dodecylphosphocholine micelles. Eur. J. Biochem. *253*, 221-228.

Wellings, D.A., and Atherton, E. (1997). Standard Fmoc protocols. Methods Enzymol. *289*, 44-67.

Wishart, D.S., and Sykes, B.D. (1994a). The 13C chemical-shift index: a simple method for the identification of protein secondary structure using 13C chemical-shift data. J. Biomol. NMR *4*, 171-180.

Wishart, D.S., and Sykes, B.D. (1994b). Chemical shifts as a tool for structure determination. Methods Enzymol. *239*, 363-392.

Wishart, D.S., Bigam, C.G., Yao, J., Abildgaard, F., Dyson, H.J., Oldfield, E., Markley, J.L., and Sykes, B.D. (1995). 1H, 13C and 15N chemical shift referencing in biomolecular NMR. J. Biomol. NMR *6*, 135-140.

Won, H.S., Park, S.H., Kim, H.E., Hyun, B., Kim, M., and Lee, B.J. (2002). Effects of a tryptophanyl substitution on the structure and antimicrobial activity of C-terminally truncated gaegurin 4. Eur. J. Biochem. *269*, 4367-4374.

Won, H.S., Jung, S.J., Kim, H.E., Seo, M.D., and Lee, B.J. (2004a). Systematic peptide engineering and structural characterization

to search for the shortest antimicrobial peptide analogue of gaegurin 5. J. Biol. Chem. *279*, 14784-14791.
Won, H.S., Kim, S.S., Jung, S.J., Son, W.S., Lee, B., and Lee, B.J. (2004b). Structure activity relationships of antimicrobial peptides from the skin of Rana esculenta inhabiting in Korea. Mol. Cells *17*, 469-476.
Won, H.S., Kang, S.J., and Lee, B.J. (2009). Action mechanism and structural requirements of the antimicrobial peptides, gaegurins. Biochim. Biophys. Acta *1788*, 1620-1629.
Wüthrich, K. (1986). NMR of proteins and nucleic acids (New York, USA: John Wiley & Sons).
Zasloff, M. (1987). Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc. Natl. Acad. Sci. USA *84*, 5449-5453.
Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. Nature *415*, 389-395.
Zasloff, M. (2003). Vernix, the newborn, and innate defense. Pediatr. Res. *53*, 203-204.