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Esculentin 1–21: a linear antimicrobial peptide from frog skin with inhibitory effect on bovine mastitis-causing bacteria‡

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Mastitis, or inflammation of the mammary gland, is the most common and expensive illness of dairy cows throughout the world. Although stress and physical injuries may give rise to inflammation of the udders, infections by bacteria or other microorganisms remain the major cause, and infusion of antibiotics is the main treatment approach. However, the increased emergence of multidrug-resistant pathogens and the production of milk contaminated with antibiotics has become a serious threat in the livestock. Hence, there is an urgent need for the discovery of new therapeutic agents with a new mode of action. Gene-encoded AMPs, which represent the first line of defence in all living organisms, are considered as promising candidates for the development of new anti-infective agents. This paper reports on the antibacterial activities *in vitro* and in an animal model, of the frog skin AMP esculentin 1–21 [Esc(1–21)], along with a plausible mode of action. Our data revealed that this peptide (i) is highly potent against the most common mastitis-causing microbes (e.g. *Streptococcus agalactiae)*; and (ii) is active *in vivo*, causing a visible regression of the clinical stage of mastitis in dairy cows, after 1 week of peptide treatment. Biophysical characterisation revealed that the peptide adopts an α-helical structure in microbial mimicking membranes and is able to permeate the membrane of *S. agalactiae* in a dose-dependent manner. Overall, these data suggest Esc(1–21) as an attractive AMP for the future design of new antibiotics to cure mastitis in cattle. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: frog skin; antimicrobial peptide; multidrug-resistant strains; mastitis; anti-infective agent; esculentin; peptide-membrane interaction; mode of action

Introduction

The widespread and often empirical use of conventional antibiotics to defeat microbial infections has led to a drastic reduction in their therapeutic efficacy and to the emergence of multidrug-resistant (MDR) strains. This represents a worldwide concern in human and veterinary practice.

In the latter case, bovine mastitis remains one of the most costly sicknesses in dairy industry with economic losses estimated at 1.2–1.7 billion dollars per year in the United States alone [1,2]. Note that clinical and subclinical mastitis are the two major forms of inflammation of the mammary glands in response to bacterial invasion, in dairy cows. Clinical mastitis is characterised by a decreased production of milk, with considerable alterations of its composition (e.g. decrease of protein, fat, lactose contents, increase in leucocytes counts [3]) and appearance (e.g. formation of flakes and clots). Furthermore, the infected udder becomes swollen, red, hot, sometimes painful to touch [4], whereas in severe cases the animal shows signs of generalised reaction such as fever, loss of appetite, diarrhoea, weakness, depression and shock. In contrast, subclinical mastitis is more difficult to detect, because of the absence of visible abnormalities in milk and udders, as well as of systemic signs of illness. In this case, leucocyte count in milk, which is positively correlated to the level of infection (see Materials and Methods), represents the best diagnostic method.

Intra-mammary infusion of antibiotics is the main approach to treat mastitis on many farms. However, the increased number of MDR pathogens and the production of milk contaminated with antibiotics (not suggested during lactation) have become a serious threat in the livestock [5].

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Abbreviations used: *AMP, antimicrobial peptide; Esc(1–21), esculentin-1a(1–21); CFU, colony-forming units; LB, Luria-Bertani; LC, lethal concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NaPB, sodium phosphate buffer; PBS, phosphate buffered saline; PG, phosphatidylglycerol; PE, phosphatidylethanolamine.*

Therefore, in this context, there is an urgent need for new therapeutic agents with a new mode of action. Ribosomally synthesised cationic AMPs which are produced by uni- and multicellular organisms, where they act as the first line of defence against noxious microorganisms, provide potential templates for the generation of new antimicrobials [6–9].

They kill microbes rapidly, synergise with classical antibiotics, and in some cases counteract harmful inflammatory/septic reactions induced by bacterial endotoxins [10]. The latter effect is extremely important to avoid the development of potentially fatal immune disregulations [11–15].

There are hundreds of AMPs in nature with a wide variety of size (10–50 amino acids), sequence, structure and spectrum of activity [7]. Despite such diversity, all of them have an overall positive charge at neutral pH (generally +2 to +9) and a substantial proportion of hydrophobic residues (≥30%). These properties allow the peptides to bind the membranes of microbes, which are negatively charged, and to fold into an amphiphilic structure, which is a crucial feature to assist the peptide's insertion into the membrane and to accomplish its antimicrobial effect [12,16–19].

Among the natural sources for peptide-antibiotics, the skin of Amphibia of Rana genus is one of the richest storages [20,21]. Esculentins-1 are a family of frog skin AMPs endowed with the longest chain (46 amino-acids) and a broad range of antimicrobial activity [22,23]. Mode of action studies of amphibian AMPs, using artificial and biological systems, have pointed out the bacterial membranes as the major target for their killing process [24–26]. Interestingly, recent reports on esculentin-1b, isolated from the skin of the green eatable frog *Phelophylax lessonae/ridibundus* (formerly named *R. esculenta* [27]) have shown that its *N*-terminal 1–18 region [Esc(1–18)] preserves the antimicrobial activity of the full-length peptide (Table 1) [28]. Lately, it was demonstrated that the analogue peptide corresponding to the first 21 amino acids of the native esculentin-1a (Table 1) was able to inhibit the growth of the yeast *Saccharomyces cerevisiae* and to modulate the synthesis of those proteins which are involved in the metabolism of cell membranes [29]. As depicted in Table 1, unlike Esc(1–18), Esc(1–21) carries the substitution Leu 11 → Ile and three additional *C*-terminal residues (Leu–Lys–Gly) which give it a higher net positive charge.

Overall, the finding that (i) the *N*-terminal 1–18 fragment of esculentin-1b preserved the antimicrobial activity of the full-length peptide esculentin-1b, and (ii) the analogue Esc(1–21) was active against yeasts, which are among the most representative fungal genera causing clinical and subclinical mastitis in cows [30,31] (although the incidence of mycotic mastitis is lower than that induced by bacterial pathogens), prompted us to focus our attention on the *N*-terminal portion of esculentins-1, rather then the entire peptide sequence.

The choice of Esc(1–21) and not Esc(1–18) was based on the consideration of a higher cationicity of the former, due to an additional basic residue, at its *C*-terminal region (Lys 20). This should favour the peptide's interaction with the negatively charged components of the microbial membrane.

Note that the amidation at the carboxyl-end of both Esc(1–18) and Esc(1–21) (Table 1), which is a very common post-translational modification in linear AMPs from frog skin [24,32,33], was chemically produced in order to neutralise the negative charge of the carboxyl group of the *C*-terminal amino acid, and hence to increase the overall net positive charge of the peptide.

The aim of this study was to get insight into the antibacterial activities of Esc(1–21), *in vitro* and in an animal model, as well as a plausible mode of action, by studying: (i) its activity against those bacteria which are mainly responsible for bovine mastitis; (ii) its bactericidal effect in the presence of biological fluids, such as bovine serum; (iii) its toxicity on mammalian erythrocytes and keratinocytes; (iv) its *in vivo* effects on mastitis-infected cows; (v) its secondary structure in buffer and in membrane–mimetic environments; and (vi) its ability to permeate the bacterial plasma-membrane.

Noteworthy, although mastitis represents the most common and expensive disease of dairy cattle throughout the world, only a few studies of AMPs on mastitis-causing microbes have been conducted until now.

Our data have highlighted that Esc(1–21) has a potent and fast killing activity against the most diffused causative microorganism of this illness (e.g. *Streptococcus agalactiae*). Most importantly, this peptide also exerts a remarkable *in vivo* antimicrobial activity.

Materials and Methods

Materials

Synthetic Esc(1–21) was purchased from GENEPEP (Prades le Lez, France). SYTOX™ Green was from Molecular Probes (Invitrogen, Carlsbad, CA, USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and the antibiotics cephalosporin C, ampicillin and tetracycline were all purchased from Sigma (St. Louis, MO, USA). Egg yolk L-a-PG and PE from *E. coli* were purchased from Avanti Polar Lipids (Alabaster, AL, USA). All other chemicals were of reagent-grade.

Peptide Synthesis

Esc(1–21) was assembled by step-wise solid-phase synthesis on an ACT 396 peptide synthesiser (Advanced Chemtech) using

Table 1. Amino acid sequences and net charge of esculentin peptides

Peptide	Sequence[a]	Net charge at neutral pH
Esculentin-1a[b]	H_2N-GIFSKLAGKK**I**KNLLISGLKNVGKEVGMDVVRTGIDIAGCKIKGEC-COOH	+5
Esculentin-1a(1–21)	H_2N-GIFSKLAGKK**I**KNLLISGLKG-$CONH_2$	+6
Esculentin-1b[c]	H_2N-GIFSKLAGKK**L**KNLLISGLKNVGKEVGMDVVRTGIDIAGCKIKGEC-COOH	+5
Esculentin-1b(1–18)	H_2N-GIFSKLAGKK**L**KNLLISG-$CONH_2$	+5

[a] Amino acids are indicated using the single-letter code. The terminal amino and carboxyl groups are also indicated, as well as the amide group at the *C*-terminal of Esc(1–21) and Esc(1–18). Amino acid substitutions are boldfaced.
[b] Peptide sequence taken from Ref. 22.
[c] Putative peptide derived from cDNA [22].

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a standard F-moc strategy. The N^{α}-Fmoc protecting groups were removed by treating the protected peptide resin with a 20% solution of piperidine in *N*,*N*-dimethylformamide. The peptide resin was washed with *N*,*N*-dimethylformamide (2×) and dichloromethane (1×) and the deprotection protocol was repeated after each coupling step. The *N*-terminal F-moc group was removed as described above, and the peptide was released from the resin with TFA/triisopropyl-silane/water (95 : 2.5 : 2.5, v : v : v) for 3 h. The resin was removed by filtration, and the crude peptide was recovered by precipitation with cold anhydrousethyl ether to give a white powder which was purified by RP-HPLC on a semipreparative C18-bonded silica column (Kromasyl, 5 µm, 100 Å, 2 cm × 25 cm) using a gradient of CH3CN in 0.1% acqueous TFA (from 3 to 100% in 70 min) at a flow rate of 2.0 ml/min. The product was obtained by lyophilisation of the appropriate fraction. Analytical RP-HPLC indicated a purity >98%. The correctness of the sequence was assessed by MALDI-TOF Voyager DE (Applied Biosystems). The peptide concentration was determined by quantitative amino acid analysis using a Beckman System Gold instrument equipped with an ion-exchange column and ninhydrin derivatisation.

Microorganisms

Bacterial strains used in this study consisted of the following reference strains obtained from the American Type Culture Collection (ATCC): the Gram-negatives *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 15692 and *Klebsiella pneumoniae* ATCC 13883; the Gram-positives *Staphylococcus aureus* ATCC 6938; *Staphylococcus epidermidis* ATCC 12228; *Streptococcus agalactiae* ATCC 13813, *Streptococcus dysgalactiae* ATCC 27957.

Antibacterial Activity

Susceptibility testing was performed by the inhibition zone assay on LB-agarose plates, as described previously [34]. The bactericidal activity of Esc(1–21) against *S. agalactiae* was evaluated in diluted LB medium (5 g/l tryptone; 2.5 g/l yeast extract; 2.5 g/l NaCl, pH 7.4) as well as in the presence of different concentrations (33 and 50%) of heat-inactivated bovine serum. Briefly, 100 µl of exponentially growing bacteria (1×10^6 cells/ml) were incubated with the peptide at 37 °C for 30 min. Following incubation, the samples were plated onto LB-agar plates. The number of surviving bacteria, expressed as CFU, was determined after overnight incubation at 37 °C. Controls were run without peptide and in the presence of peptide solvent (20% ethanol). Killing kinetics was also performed by counting the number of surviving microorganisms at different time-intervals.

Haemolytic Activity

The haemolytic activity of Esc(1–21) was determined using fresh bovine and human erythrocytes. The blood was centrifuged and the erythrocytes were washed three times with 0.9% NaCl. Peptide solutions were incubated with the erythrocyte suspension (1×10^7 cells/ml) at 37 °C for 40 min. Then, the extent of haemolysis was measured on the supernatant, from the optical density at 415 nm. Hypotonically lysed erythrocytes were used as a standard for 100% haemolysis.

Cytotoxic Activity

The cytotoxic effect of Esc(1–21) was determined by the inhibition of MTT reduction to insoluble formazan, by mitochondrial reductases, on the immortalised keratinocyte cell line HaCaT. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% heat-inactivated foetal bovine serum, glutamine (4 mM) and antibiotics (penicillin and streptomycin) and then plated in triplicate wells at 1×10^4 cells/well [96-well plates (Falcon) were used]. After 2 h at 37 °C in a 5% CO_2 atmosphere, the medium was replaced with 100 µl fresh DMEM supplemented with Esc(1–21) at different concentrations. The plate was then incubated for 90 min at 37 °C in a 5% CO_2 atmosphere. Afterwards, the medium was removed and replaced with fresh DMEM containing 0.5 mg/ml MTT.

After 4-h incubation, the formazan crystals were dissolved by adding 100 µl of acidified isopropanol and viability was determined by absorbance measurements at 595 nm. Cell viability was calculated with respect to control cells (cells not treated with peptide).

Experimental Cows

The study was carried out on four dairy farms located near Guadalajara, Jalisco, México, with approximately 1000 Holstein dairy cows that were milked by machine, three times daily. A cow with a milk leucocyte count above 200 000 cells/ml (see the section on 'Milk collection and examination') was considered to be suffering from mastitis.

Clinical status of mastitis was monitored by a veterinarian, through visual estimation of milk viscosity, udder appearance (size, colour, heat, pain), palpation (to detect possible fibrosis, inflammatory swellings) and physical examination (rectal temperature, heart rate, rumen contraction rate, hydration status).

Depending on the leucocyte counts and clinical inspection, animals were categorised as cows affected by subclinical (grades 1, 2, 3) or clinical mastitis.

A total of nine lactating Holstein cows with different stages of lactation (2–3 months post-calving) and with one udder quarter affected by clinical mastitis were used.

In vivo Treatment

One udder quarter of each cow was injected with 10 ml of Esc(1–21) solution, at a concentration of 10 µg/ml in phosphate buffered saline (PBS), twice per day (at 8 a.m. and 8 p.m.) for 5 days. Before peptide treatment, the diseased quarter was thoroughly milked out by hands and the teat end was cleaned using a cotton swab soaked with 70% ethanol. Cows were clinically monitored along the entire treatment. In addition, leucocyte counts and bacteriological examination of the collected milk were performed, either before or after peptide treatment.

Milk Collection and Examination

Fifty millilitres of milk from each cow was collected in sterile vials after cleaning the teat orifice with 70% ethanol and after discarding the first few streams of milk. The number of leucocytes present in the milk was estimated by the California Mastitis Test (CMT). Briefly, when CMT reagent is added to milk, it reacts with the nuclei of leucocytes, forming a gel. The reaction is then visually scored depending on the amount of gel formed. The greater the mastitis infection, the higher number of leucocytes in milk

and the more gel-like substance that forms [35]. Therefore, CMT represents a valuable system to measure the inflammation within a quarter and can be scored on the basis of degree of gel formation (Table 3).

Milk collected from cows, before and after peptide treatment was also bacteriologically examined. As standard, 10 µl of milk was streaked onto sheep-blood agar plate which was incubated for 24–48 h at 37 °C. Afterwards, the plate was screened for the primary isolation of mastitis pathogens. A milk sample was considered contaminated when three or more different types of bacterial colonies were detected.

A single colony was then inoculated into broth medium and incubated for 18–24 h at 37 °C. Identification of bacterial species was carried out by several biochemical tests, as suggested in the National Mastitis Council guide [36,37].

Statistical Analysis

Wilcoxon test for paired experiments was used for statistical analysis. Probabilities less than 0.05 were considered significant.

Peptide's Effect on Plasma-Membrane Permeation

To assess the ability of Esc(1–21) to alter the membrane permeability of *S. agalactiae*, 4×10^6 cells were mixed with 1 µM SYTOX™ Green (a probe which cannot permeate undamaged membranes) in PBS for 5 min in the dark. After adding peptide, the increase of fluorescence (due to the intracellular influx and binding of the dye to intracellular DNA) was measured at 37 °C in a microplate counter (Wallac 1420 Victor^{3}™, Perkin Elmer), using 485- and 535-nm filters for excitation and emission wavelengths, respectively. Controls used were given by bacteria without adding peptides, whereas 100% membrane permeation was obtained by treating microbial cells with 1% Triton. Fluorescence recorded after 15 min from the addition of peptide was plotted as arbitrary unit.

CD Analysis

CD experiments were performed using a JASCO J-600 spectropolarimeter with a 1-mm path length cell. The CD spectra of the peptide were recorded at 25 °C at 0.2 nm intervals from 195 to 250 nm, at a concentration of 5 µM in 10-mM NaPB pH 7.4 or in a suspension of lipid vesicles composed of PE/PG (7 : 3, w : w) to mimic the microbial cell membranes, extruded to a diameter of 50 nm at a 2-mM concentration. CD data from eight scans were averaged and expressed as per-residue molar ellipticity.

Results

In vitro Antimicrobial Activity

The antimicrobial activity of Esc(1–21) was examined on the most representative mastitis-causing bacteria (e.g. *S. aureus, S. epidermidis, S. agalactiae, S. dysgalactiae, K. pneumoniae* [38,39]) and secondary pathogens (e.g. *P. aeruginosa* and *E. coli* [40]) by the inhibition zone assay. This activity was expressed as LC, which is the minimal peptide concentration that inhibits microbial growth in LB-agarose plates. All values are reported in Table 2, where ampicillin, tetracycline and cephalosporin C, which are currently used in the treatment of bovine clinical mastitis [1], are also included as references.

With the exception of *S. aureus* that revealed to be a quite resistant strain to Esc(1–21), this peptide displayed a strong antimicrobial activity against all the other microorganisms, with LC values ranging from 0.65 to 3.2 µM.

According to the data of Table 2, ampicillin and tetracycline resulted to be more active than Esc(1–21) on *S. agalactiae* and *S. dysgalactiae*; in contrast, at least tenfold lower potency than that of Esc(1–21) was displayed by these two conventional antibiotics on *E. coli*. In addition, they were not active on both *Klebsiella* and *Pseudomonas* species, whereas a comparable efficacy to that of Esc(1–21) was manifested by them against *S. epidermidis*. Interestingly, cephalosporin C was inactive or less active than Esc(1–21) against the Gram-negative or Gram-positive bacterial strains (except *S. aureus*), respectively (Table 2).

Next, bactericidal effect and rate of killing of Esc(1–21) were investigated against *S. agalactiae*, which is one of the most common mastitis pathogens in dairy cattle [41]. As indicated in Figure 1, a peptide concentration equal to the LC (3 µM) reduced the number of surviving microorganisms to approximately 5%, in 15 min. Surprisingly, when Esc(1–21) was analysed in biological fluids, such as 33 and 50% bovine serum, it partially preserved its microbicidal activity, causing 99.6 and 65% mortality, respectively,

Table 2. Lethal concentration (LC) of Esc(1–21) and some conventional antibiotics

Microorganisms	Lethal concentration (µM)[a]			
	Esc(1–21)	Ampicillin	Tetracyclin	Cephalosporin C
Gram-negative bacteria				
Escherichia coli ATCC 25 922	0.65	>100	5.80	NA
Pseudomonas aeruginosa ATCC 15 692	3.20	NA	NA	NA
Klebsiella pneumoniae ATCC 13 883	1.60	NA	NA	NA
Gram-positive bacteria				
Staphylococcus aureus ATCC 6938	14.0	0.40	1.70	6.0
Staphylococcus epidermidis ATCC 12 228	3.0	5.80	1.44	16.0
Streptococcus agalactiae ATCC 13 813	3.20	0.50	0.50	17.0
Streptococcus dysgalactiae ATCC 27 957	3.0	0.12	0.24	4.60

NA, not active.
[a] Results are the mean of three independent experiments, each performed in duplicate, with standard deviation not exceeding 20%.

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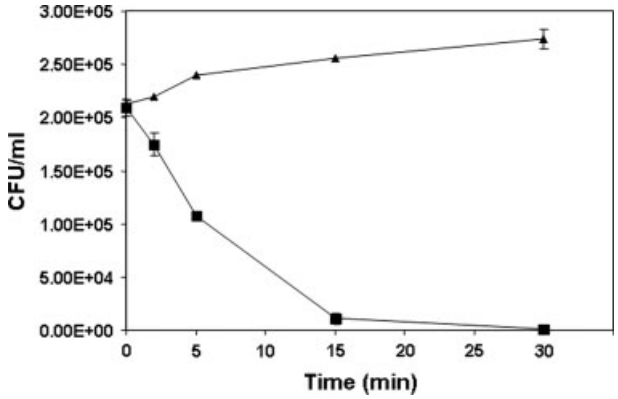

Figure 1. Killing kinetic of Esc(1–21) on *S. agalactiae*. Bacteria (1×10^6 cells/ml) were incubated with 3 μM Esc(1–21) in diluted LB medium at 37 °C. The number of surviving cells [filled square (■)], at different incubation times, is indicated as CFU/ml. The control [filled triangle (▲)] is given by bacteria without peptide. Data points represent the mean of triplicate samples ± SD from a single experiment, representative of three independent experiments.

Table 4. CMT score of milk collected from nine cows, before and after peptide treatment

		CMT score	
		Before	After
Cow designation	Infected quarter	Peptide treatment[a]	
1	FR	4	1
39	FR	4	3
79	RR	4	3
70	RL	4	1
B	FR	4	3
C	RL	4	3
L	RR	4	3
M	RR	4	3
P	RL	4	3

FR, front right; RR, rear right; RL, rear left.
[a] Differences of CMT scores before and after peptide treatment are statistically significant ($p < 0.05$).

within 30 min at 12 μM. This is in contrast with the majority of AMPs which lose almost total activity in the same conditions [42,43].

In vitro Cytotoxic Activity

The toxicity of the peptide towards mammalian cells was investigated on bovine and human erythrocytes as well as on HaCat cell line. Esc(1–21) was devoid of lethal effect on keratinocytes, up to 50 μM. Accordingly, a very slight haemolytic activity was obtained against both bovine and human erythrocytes (<3.5% at 50 μM).

Peptide's Effect on Mastitis-Infected Cows

Mastitis occurs when the udder becomes inflamed, with leucocytes moving from the bone marrow to the mammary gland in response to bacterial invasion of the teat canal. These bacteria multiply and produce toxins which damage milk-secreting process and cause injury to various ducts throughout the mammary gland [44]. When an elevated number of leucocytes (predominantly neutrophils [45]) are present at the damaged tissue, they aggregate and form clots that block small ducts preventing complete milk removal. Therefore, the determination of milk leucocytes is a widely used method to monitor udder health and milk quality. Table 3 shows how the leucocyte count affects the milk viscosity and the CMT score (see the section on Experimental cows).

Interestingly, 5-day intra-mammary administration of Esc(1–21), at a concentration of 10 μg/ml (see Materials and Methods), to cows affected by clinical mastitis regressed the udder infection to a subclinical level. Such differences in the clinical status of the disease, before and after peptide treatment, revealed to be statistically significant ($p < 0.05$). In particular, as indicated in Table 4, among nine cows with a clinical stage of the disease (score 4), seven animals reverted to subclinical mastitis grade 3, and two of them to subclinical mastitis, grade 1. Note that most of those cows (i.e. B, C, L, M and P) that, after peptide treatment, reverted to subclinical mastitis grade 3, had initially a more serious level of udder infection, with double-sized and hotter quarters than those of healthy animals.

Furthermore, four additional cows with subclinical mastitis (score 3) were injected with 10 ml peptide solution at a lower concentration (5 μg/ml). Importantly, one animal healed (stage 0) and the other three regressed to subclinical mastitis, grade 2 (results not shown). No signs of worsening were noted up to 3 months post-peptide-treatment, thus underlining a good *in vivo* antimicrobial activity of Esc(1–21).

These data suggest that a peptide dosage of 100 μg, twice per day for 1 week, should be suitable to treat cows with subclinical/clinical mastitis. However, further *in vivo* analysis is required. Samples of milk were also bacteriologically examined, before and after peptide infusion. Streptococchi, staphylococchi and coliform species were found to be the predominant microbial pathogens in all sick cows. Noteworthy, such pathogenic bacteria were no longer detected after peptide treatment (data not shown). These results are consistent with the recovery of health of inflamed udder quarters, upon injection of Esc(1–21).

Table 3. California mastitis test (CMT) scores and their relation to the average leucocyte counts in milk and to the appearance of milk-CMT reagent mixture (interpretation of CMT scores is also reported)

CMT score	Range of leucocyte counts (10^6/ml) in milk	Test appearance	Interpretation
0	0–0.2	Mixture liquid	No mastitis
1	0.2–0.4	Slight thickening	Subclinical mastitis, grade 1
2	0.4–1.2	Distinct precipitate with no tendency to form a gel	Subclinical mastitis, grade 2
3	1.2–5.0	Immediate thickening with slight gel formation	Subclinical mastitis, grade 3
4	Over 5.0	Strong gel formation	Clinical mastitis

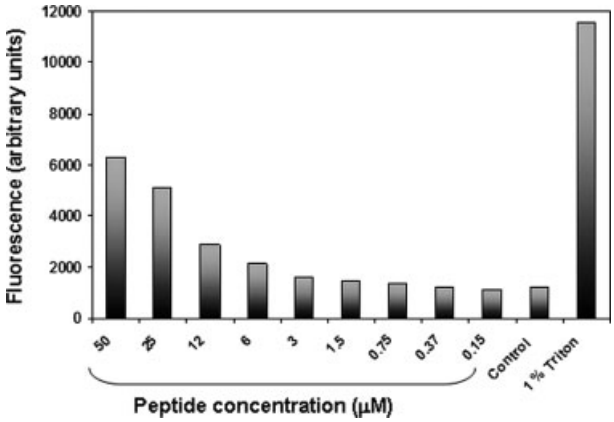

Figure 2. Membrane permeation of *S. agalactiae* by Esc(1–21). Bacteria (4×10^6 cells in 100 μl) were incubated with 1-μM SYTOX™ Green in PBS. Once basal fluorescence reached a constant value, the peptide was added at different concentrations and changes in fluorescence were monitored for 15 min ($\lambda_{exc} = 485$ nm, $\lambda_{ems} = 535$ nm), when a constant value was reached. These values are plotted as arbitrary units and represent the mean of triplicate samples from a single experiment, representative of three different experiments.

Peptide's Effect on Plasma-Membrane Permeation

To shed light into the mode of action of the peptide, we analysed the ability of Esc(1–21) to damage the plasma-membrane of *S. agalactiae*, by monitoring the intracellular influx of SYTOX™ Green, whose fluorescence dramatically raises when bound to nucleic acids (see Materials and Methods). The data showed that Esc(1–21) increased the bacterial membrane permeability, within the first 15 min and in a dose-dependent manner (Figure 2). However, it did not cause a complete alteration of the plasma-membrane's structure, up to approximately 15-fold the LC (50 μM), as manifested by the higher fluorescence that was emitted when bacteria were lysed by 1% Triton (Figure 2).

CD Spectra

The peptide's structure was evaluated by CD in NaPB as well as in the presence of PE/PG (7 : 3, w : w) lipid vesicles mimicking the microbial cell membrane. While the peptide was disordered in solution, it adopted an α-helical structure when associated with the membranes (Figure 3a). In this structure, its side chains are oriented in an amphiphilic arrangement, with all charged residues segregated to one face of the helix (Figure 3b).

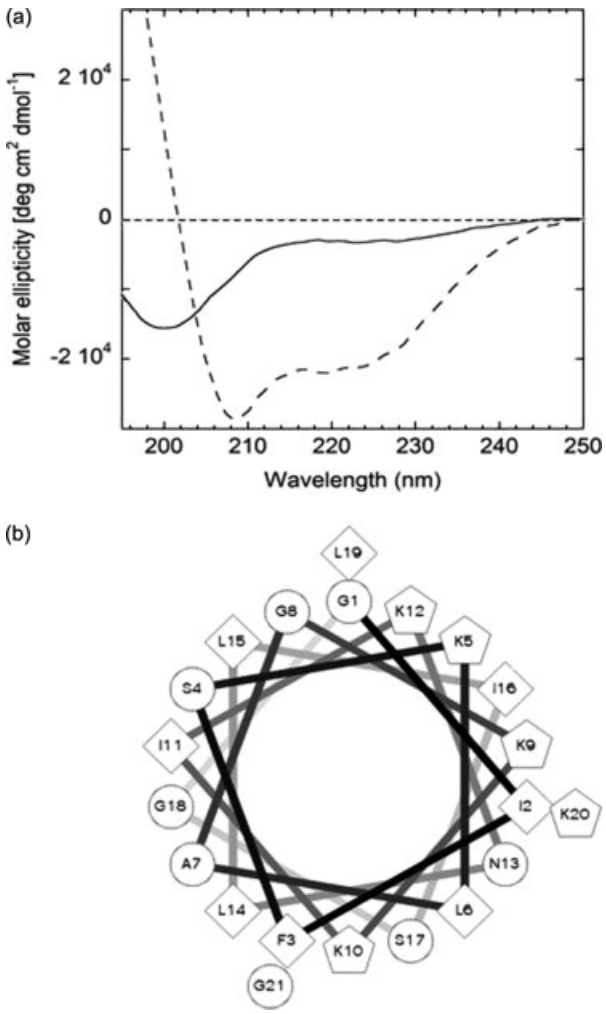

Figure 3. (a) CD spectra of Esc(1–21) in buffer (solid line) and PE/PG vesicles (broken line); (b) Helical wheel plot of Esc(1–21): hydrophilic, hydrophobic and potentially positively charged residues are represented as circles, diamonds and pentagons, respectively.

Discussion

Once an infectious agent is established, hosts need to minimise the agent's impact on their health by attacking and destroying the pathogen population. However, host defence mechanisms cannot always work in the most efficient way [46], and the therapeutic use of AMPs may be appropriate.

This paper describes the activity of the *N*-terminal 1–21 fragment of the frog skin AMP esculentin-1a on microbial pathogens which are mainly responsible for bovine mastitis. It is worthwhile noting that the majority of conventional antibiotics recognise intracellular targets and interfere with cellular processes such as DNA replication, protein and cell wall synthesis without affecting membrane permeability and morphology of the target cell. However, this mechanism makes it easier for the pathogens to acquire resistance. In contrast, because of a rapid and quite selective membranolytic effect which induces damage hard to fix, AMPs are generally considered as promising candidates for the development of new anti-infective agents to propose in both human medicine and veterinary. In animals, the annual incidence of clinical mastitis is about 56% in a commercial dairy herd [1], accounting for the highest impact of all cattle diseases. Control of mastitis involves hygienic practises such as teat dipping and infusion of antibiotics into the udder [47]. However, once present, antibiotics cannot be removed from milk; milk from antibiotic-treated cows cannot be sold for 3–5 days after treatment, and cost of discarding milk is quite significant [48]. Hence, the discovery of new compounds which are active on mastitis-causing microorganisms, without being a threat to consumer health, is extremely important. Up to now, with the exception of nisin [5], only a few AMPs have been tested on this type of microorganisms as well as on cows with infected udders. Here, we have discovered that the frog skin Esc(1–21) is a peptide endowed with a powerful antimicrobial activity against the most diffused mastitis-causing bacteria in cattle (e.g. *S. agalactiae, P. aeruginosa, E. coli*) with LC values ranging from 0.65 to 3.2 μM. In

contrast, a weak antimicrobial effect was displayed against strains of *S. aureus*.

Furthermore, the peptide could partially preserve its antibiotic activity in the presence of bovine serum and was not toxic to mammalian erythrocytes and keratinocytes.

CD spectra of Esc(1–21) in negatively charged vesicles, mimicking microbial cell membranes, pointed out an α-helical structure of the peptide in which its side chains attain an amphiphilic arrangement, a structural prerequisite for membrane-binding and perturbing activity. Accordingly, studies on the mode of action of Esc(1–21) against *S. agalactiae* ATCC 13813, which is one of the most common mastitis-causing pathogens [40], have highlighted that the peptide can rapidly kill this microorganism, at its LC, with a concomitant permeation of the microbial membrane, as demonstrated by the results of SYTOX™ Green (Figure 2).

Because the peptide binds microbial-mimicking membranes (as suggested by CD spectra analysis) and permeates the plasma-membrane of *S. agalactiae,* we can assume that the different pattern of antimicrobial activity shown by Esc(1–21) (e.g. potent activity against *E. coli* and almost no activity against *S. aureus*, see Table 1) is related to differences in the cell wall of the target microorganisms. Indeed, cell-selectivity of AMPs does not only depend on the physicochemical properties of the peptide (alpha-helicity, amphipathicity, cationicity, charge distribution, oligomeric state), but also on the composition of the target cell surface, which can serve as a barrier and makes it more difficult for a peptide to reach and permeate the cytoplasmic membrane.

In addition, changes in the composition of the membranes of various bacteria can also affect the peptide's ability to permeate them, thus endowing distinct microorganisms with different sensitivities to the same AMP. However, we cannot exclude the involvement of additional targets in the mechanism of antimicrobial activity of Esc(1–21) against different bacterial strains.

As shown by CD spectra, Esc(1–21) has the tendency to adopt an amphipathic alpha-helical structure when bound to the bacterial mimicking membrane. Taking into account the peptide ability to permeate the bacterial membrane without causing a complete alteration of its structure, even at 15-fold the LC (Figure 2), we can rule out a simple lysis mechanism for Esc(1–21) membrane permeation.

Furthermore, the high content of basic amino acids (net charge of +6 at neutral pH) and their spread distribution along the entire sequence of the peptide, would make it quite difficult for peptide monomers to aggregate and form transmembrane pores, as described by the barrel-stave mechanism [17]. Therefore, the peptide might bind to the membrane surface, in a carpet-like arrangement, inserting into the polar phospholipid headgroups. This would generate an unfavourable tension that results in the formation of local membrane breakages or pores leading to bacterial death. Nevertheless, additional experiments are required to verify this hypothesis as a possible mechanism underlying the membrane permeabilisation of Esc(1–21).

Most interestingly, Esc(1–21) was found to explicate a remarkable antimicrobial effect also *in vivo*. Indeed, the peptide-based formulation used to challenge clinical mastitis in dairy cows revealed to be highly effective, inducing a clear reduction in the count of leucocytes and pathogenic bacteria in milk. Furthermore, intra-mammary infusion of Esc(1–21) gave rise to a regression of the disease, with a clinical improvement rate of 100%, within 5-days of treatment. Importantly, no side effects were detected in all treated animals. Overall, because of a fast selective bactericidal action, ability to preserve antimicrobial activity in serum and *in vivo* efficacy in the treatment of inflamed bovine udders, without inducing irritant effects to the animals, Esc(1–21) represents a promising therapeutic agent for the development of new drugs to cure mastitis, caused by bacteria, in dairy cattle. Altogether, our results encourage further study of Esc(1–21), to clarify its global effects on a larger scale along with its underlying molecular mechanism, pharmacokinetics and half-life.

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