Toxicity of bovicin HC5 against mammalian cell lines and the role of cholesterol in bacteriocin activity

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Bacteriocins are ribosomally synthesized antimicrobial peptides produced by Bacteria and some Archaea. The assessment of the toxic potential of antimicrobial peptides is important in order to apply these peptides on an industrial scale. The aim of the present study was to investigate the in vitro cytotoxic and haemolytic potential of bovicin HC5, as well as to determine whether cholesterol influences bacteriocin activity on model membranes. Nisin, for which the mechanism of action is well described, was used as a reference peptide in our assays. The viability of three distinct eukaryotic cell lines treated with bovicin HC5 or nisin was analysed by using the MTT assay and cellular morphological changes were determined by light microscopy. The haemolytic potential was evaluated by using the haemoglobin liberation assay and the role of cholesterol on bacteriocin activity was examined by using model membranes composed of DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) and DPoPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine). The IC_{50} of bovicin HC5 and nisin against Vero cells was 65.42 and 13.48 μM, respectively. When the MTT assay was performed with MCF-7 and HepG2 cells, the IC_{50} obtained for bovicin HC5 was 279.39 and 289.30 μM, respectively, while for nisin these values were 105.46 and 112.25 μM. The haemolytic activity of bovicin HC5 against eukaryotic cells was always lower than that determined for nisin. The presence of cholesterol did not influence the activity of either bacteriocin on DOPC model membranes, but nisin showed reduced carboxyfluorescein leakage in DPoPC membranes containing cholesterol. In conclusion, bovicin HC5 only exerted cytotoxic effects at concentrations that were greater than the concentration needed for its biological activity, and the presence of cholesterol did not affect its interaction with model membranes.

INTRODUCTION

Ribosomally synthesized antimicrobial peptides are widely distributed in nature, and are produced by a variety of micro-organisms, plants, insects and animals, including humans (Thomson et al., 2004). Bacteriocins are antimicrobial peptides produced by many species of bacteria and often differ in their biochemical properties and mode of action (Cleveland et al., 2001). Peptides from lactic acid bacteria, such as the type A lantibiotics, are the most studied bacteriocins due to their potential to inhibit pathogens in food and to prevent infections in humans and animals.

Bovicin HC5, produced by Streptococcus bovis HC5 (Mantovani et al., 2002), is a lantibiotic that shares structural and functional similarities with nisin, the most well known bacteriocin (Fig. 1). Bovicin HC5 has a broad spectrum of activity and previous work indicated that it was effective against spoilage bacteria and also inhibited growth of bacterial species that are detrimental to silage fermentation and protein utilization in the bovine rumen (Mantovani & Russell, 2003; Lima et al., 2009).

The cell membrane is the target of many antimicrobial peptides, particularly the type A lantibiotics. As already

Abbreviations: CF, carboxyfluorescein; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DPoPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide.
demonstrated for nisin (Breukink et al., 1999, 2003), the mode of action of bovicin HC5 is based on its interaction with lipid II (Paiva et al., 2011, 2012), an essential molecule in peptidoglycan synthesis, present in bacterial cell membranes but absent in eukaryotic organisms. Nevertheless, in high concentrations, both bovicin HC5 and nisin are able to directly interact with the lipid bilayer in vitro, disrupting the integrity of model membranes composed of fatty acids with 16 carbons, even in the absence of lipid II (Paiva et al., 2011). This is an important feature for the mode of action of bovicin HC5 and nisin, since the fatty acid chains in phospholipids and glycolipids of cell membranes usually contain an even number of carbon atoms; the 16- and 18-carbon acyl chains are the most abundant (Zachowski, 1993).

Another characteristic that influences the activity of antimicrobial peptides is the presence of cholesterol, a molecule that is exclusively present in eukaryotic cell membranes. In animal cells, cholesterol has specific effects on the phase behaviour of the membrane lipids, generally resulting in greater packing of the lipid acyl chains and increased rigidity of the lipid bilayer structure (Mouritsen & Zuckermann, 2004). Studies on model membranes have demonstrated that the presence of cholesterol reduces the adsorption and disruption of membranes caused by some antimicrobial peptides (Prenner et al., 2001; Raghuraman & Chattopadhyay, 2004; Verly et al., 2008; Wu et al., 2010).

Very few bacteriocins produced by S. bovis strains have been characterized biochemically, but some of these antimicrobial peptides could be useful to prevent food spoilage or reduce the dependence on classical antibiotics commonly added to cattle food as feed additives (Joerger, 2003). However, in order to be considered for practical applications, particularly in food safety and animal health, the inherent risks of the administration of antimicrobial agents should be addressed, which includes the determination of cytotoxicity towards animal cells.

The aim of this study was to investigate the in vitro cytotoxicity and haemolytic activity of bovicin HC5 against different eukaryotic (animal) cell lines. We also used model membranes to determine the role of cholesterol on bacteriocin activity. Nisin, a well-studied bacteriocin that has GRAS (“generally recognised as safe”) status and is currently used as a food additive, was used for comparison of the results obtained with bovicin HC5.

METHODS

Micro-organisms and growth conditions. S. bovis HC5 was routinely cultivated under anaerobic conditions (39 °C, 18 h) in basal medium containing (l−1): 22 mmol glucose, 1.7 mmol K2HPO4, 2.1 mmol KH2PO4, 3.6 mmol (NH4)2SO4, 8.3 mmol NaCl, 0.75 mmol MgSO4.7H2O, 0.43 mmol CaCl2.2H2O, 2.8 mmol cysteine hydrochloride, 38 mmol Na2CO3, 5 g Casamino acids, 0.1 g Trypticase (BBL), 5 g yeast extract, vitamins and minerals (Mantovani et al., 2002). Lactococcus lactis ATCC 19435 was cultivated under aerobic conditions (37 °C, 18 h) in MRS medium.

Bacteriocin preparation. Semi-purified extracts of bovicin HC5 were prepared as described previously (Mantovani et al., 2002). Bovicin HC5 was purified by HPLC using a reverse phase column [Shimadzu C18; 5 μm; 150 by 6 mm (inner diameter)]. The column was equilibrated with buffer A (0.1 % trifluoroacetic acid in water) and the peptide was eluted using a linear gradient of 35–50 % buffer B (80 % acetonitrile, 0.1 % TFA in water), 22 °C, and at a flow rate of 1 ml min−1. The absorbance was monitored at 214 and 280 nm. Eluted fractions corresponding to pure bovicin HC5 were lyophilized and stored at −20 °C until further use.

Nisin solution was prepared by adding 1 g Chrissin C (2.5 % w/w nisin, balanced sodium chloride and denatured milk solids, 1000 IU mg−1; Christian Hansen) to 10 ml sodium phosphate solution (5 mmol l−1, pH 2.0). The nisin suspension was centrifuged (1742 g, 10 min), the pellet was discarded and the supernatant (stock solution) was stored at −20 °C until use. For experiments using model membranes, nisin was purified by HPLC, following the same procedures described above for bovicin HC5.

The antimicrobial activities of the bacteriocins were determined by agar well-diffusion assay (Tagg et al., 1976). Bovicin HC5 and nisin extracts were serially diluted in phosphate solution (5 mM, pH 2.0) and assayed for antimicrobial activity using L. lactis ATCC 19435 as the indicator organism (105 c.f.u. ml−1). One arbitrary unit (AU) was defined as the reciprocal of the highest dilution that showed a zone of inhibition at least 9 mm in diameter (Lewus & Montville, 1991). The antimicrobial activity obtained against L. lactis ATCC 19435 was 20480 AU ml−1 for bovicin HC5 (333 μM) and 160 AU ml−1 for nisin (135 μM).
Peptide concentration was determined by HPLC using a reverse phase column [Acclaim 120 C18: 5 μm; 150 by 4.6 mm (inner diameter)]. Samples were eluted with a flow rate of 1 ml min\(^{-1}\) at 22 °C. For bovicin HC5 quantification, the column was equilibrated with buffer A (0.1% trifluoroacetic acid in water) and the peptide was eluted using a linear multi-step gradient starting with 35% buffer B (80% acetonitrile, 0.1% TFA in water) for 9 min, increasing to 50% buffer B for 11 min and then increasing to 100% buffer B for 5 min, then returning to initial conditions. For nisin quantification, the peptide was eluted using an isocratic gradient of 35% buffer A and 65% buffer B. The absorbance was monitored at 214 and 280 nm using a Dionex 3000 Diode Array Detector (DAD). HPLC-purified nisin and bovicin HC5 were used as standards. Working solutions of bovicin HC5 and nisin were diluted in minimal essential medium (MEM, Sigma Aldrich) for the cytotoxic assays, in PBS (35 mmol phosphate l\(^{-1}\), 150 mmol NaCl l\(^{-1}\), pH 7.0) for the haemolytic assays or in acetic acid (0.05% in MilliQ water) for the model membrane experiments. 

The purity of bovicin HC5 and nisin (95% purity) was confirmed using analytical HPLC and electrospray mass spectrometry.

**Cytotoxic effect.** The in vitro effect of bovicin HC5 and nisin on the viability of eukaryotic cells was evaluated using the colorimetric method of MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; Sigma Aldrich] (Mosmann, 1983). The eukaryotic cell lines Vero (monkey kidney epithelial cell line), MCF-7 (human breast adenocarcinoma cell line) and HepG2 (human liver hepatocellular carcinoma cell line) used in this study were obtained from the Universidade de Campinas (UNICAMP, São Paulo, Brazil). The cells were pre-cultured in tissue culture flasks containing MEM (pH 7.2) until the formation of a monolayer at the bottom of the flasks. The adherent cells were transferred to a 96-well plate at a density of 1×10\(^4\) cells per well and allowed to attach overnight. Monolayers of cells were treated with 250 μl working solutions of bovicin HC5 (0–333 μM) or nisin (0–135 μM) diluted in MEM, and maintained for 24 h at 37 °C in a humidified incubator with 5% CO\(_2\) atmosphere. The culture supernatants were removed and MTT stock solution (0.5 mg ml\(^{-1}\)) was added to each well. The plates were then incubated for 4 h at 37 °C and the dark blue formazan crystals were dissolved by adding 100 μl 0.04 M 2-propanol/HCl to each well followed by quick mixing. Absorbance at 516 nm was determined after 1 h with a Microplate Reader (Qiagen, TiterTek Multiskan PLUS MK II). Cells incubated with fresh MEM were used as negative control.

The percentage of viable cells after treatment with the peptides was calculated as \(A_t/A_c \times 100\), where \(A_t\) and \(A_c\) are the absorbance of treated and control cells (100% survival), respectively. The IC\(_{50}\), which refers to the peptide concentration that inhibits cell viability by 50% after 24 h exposure time, was calculated based on regression analysis of the concentration–response curves obtained for MTT assays (Todorov et al., 2005).

Vero cells treated with bovicin HC5 and nisin were also monitored on an inverted optical microscope (Olympus IX70) to determine the effects on cell morphology (loss of monolayer, granulation and vacuolization of cytoplasm) compared with untreated cells. All incubations were carried out in triplicate and the results represent means ± SD of three independent experiments. Variance analysis was determined with mean percentages of viability, and significance differences were determined by \(t^2\) test with 5% probability (\(P<0.05\)).

**Haemolytic activity.** Haemolytic activity of bovicin HC5 and nisin was determined by a haemoglobin release assay (Shin et al., 2001; Maher & McClean, 2006). Fresh defibrinated sheep erythrocytes were rinsed three times with PBS (35 mM phosphate buffer, 150 mM NaCl, pH 7.0), centrifuged for 15 min at 900 g, and suspended at 4% (v/v) in PBS. Samples (100 μl) of the suspensions were treated with 100 μl bovicin HC5 (0–184 μM) or nisin (0–33.75 μM) and incubated for 1 h at 37 °C before being centrifuged at 1000 g for 5 min. Aliquots (100 μl) of the supernatant were transferred to a 96-well plate, where haemoglobin release was monitored spectrophotometrically at 414 nm with a Microplate Reader (Qiagen, TiterTek Multiskan PLUS MK II).

The proportion of haemolysis was calculated by using the formula: % haemolysis = \(\frac{A_{414} \, \text{in treated supernatant} - A_{414} \, \text{in PBS}}{A_{414} \, \text{in 0.1% Triton X-100} - A_{414} \, \text{in PBS}} \times 100\). The zero values and 100% haemolysis were determined in PBS and 0.1% Triton X-100, respectively. All experiments were performed in triplicate and the results represent means ± SD of three independent experiments.

**Carboxyfluorescein leakage experiments.** Phospholipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (C\(_{18:1}\), DOPC) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (C\(_{16:1}\), DPOPC), and cholesterol were purchased from Avanti Polar Lipids. Carboxyfluorescein (CF) was purchased from Invitrogen.

Stock solutions of cholesterol and phospholipids (C\(_{18:1}\); C\(_{16:1}\)) were prepared (10 mM final concentration) in chloroform : methanol (1:1) and stored at −20 °C until use. Large unilamellar vesicles (LUVs) of the desired lipid composition, with and without cholesterol (30%), were prepared by the extrusion technique (Hope et al., 1985), and the dried lipid films were hydrated by the addition of buffer containing 10 mM Tris, 150 mM NaCl and 50 mM CF (pH 7.5). The LUVs were prepared by repeated extrusion through polycarbonate filters (0.2 μm pore size, Isopore membrane filters, Millipore), and the non-enclosed probe was removed through a Sephadex G-50 spin column (equilibrated with 10 mM Tris, 150 mM NaCl, pH 7.5).

The concentrations of the lipid stock solution and the LUVs were determined by inorganic phosphate determination (Rouser et al., 1970). The final vesicle concentration used was 25 μM (final lipid-P) and the final concentration of cholesterol was 7.5 μM.

Bovicin HC5 (1.5 μM) or nisin (0.45 μM) was added to buffer containing 10 mM Tris and 150 mM NaCl (pH 7.5) and the desired concentration of CF loaded vesicles. The leakage of CF from the vesicles was monitored by measuring the fluorescence intensity at 516 nm (excitation wavelength at 492 nm), using an SLM Aminco Spectrofluorometer (SPF-500). The samples were continuously stirred in a 10×4 mm quartz cuvette and kept at 20 °C using a circulation water bath. Triton X-100 (final concentration 0.2%) was added 2 min after the addition of the desired peptide, to disrupt the vesicles, and the resulting fluorescence was taken as 100% leakage value.

The percentage of CF leakage was calculated by the equation: \(\frac{[F_t - F_0]/(F_0 - F_t) \times 100]}\), where \(F_t\) was the fluorescence at time \(t\), \(F_0\) was the fluorescence at time \(0\), and \(F_t\) was the maximum fluorescence after addition of Triton X-100. The mean percentage of fluorescence obtained in the presence and absence of cholesterol was compared by Student’s \(t\)-test. A probability value of less than 0.05 was considered statistically significant (\(P<0.05\)).

**RESULTS**

**Cytotoxic effect**

In this study, the cytotoxic effect of bovicin HC5 and nisin was determined against the following eukaryotic cell lines: Vero cells, a monkey kidney epithelial cell line, MCF-7, a human breast adenocarcinoma cell line, and HepG2, a human liver hepatocellular carcinoma cell line.
The addition of bovicin HC5 or nisin decreased the cell viability in a concentration-dependent manner, but the concentration of bacteriocin needed to achieve the IC$_{50}$ was remarkably distinct. Nisin displayed an IC$_{50}$ of 13.48 μM towards Vero cells, while the bovicin HC5-treated Vero cells only reached 50% reduction of formazan crystals formation when the concentration was 65.42 μM (Fig. 2).

The IC$_{50}$ values obtained with MCF-7 and HepG2 cells were higher than the values obtained with Vero cells. For bovicin HC5, the IC$_{50}$ was 279.39 and 289.30 μM for MCF-7 and HepG2 cells, respectively (Figs 3a and 4a). When the cells were treated with nisin, the IC$_{50}$ values obtained were 105.46 and 112.25 μM for MCF-7 and HepG2 cells, respectively (Figs 3b and 4b).

Observation of the cells treated with bovicin HC5 or nisin on an inverted optical microscope indicated a decrease in cell number at peptide concentrations equivalent to the IC$_{50}$ (data not shown). When compared with untreated controls, the most typical morphological changes caused by the peptides above the IC$_{50}$ values were shrinkage and vacuolization of the cytoplasm, condensation and lateralization of the nucleus and detachment of the cell mat. The greatest detachment of the cell mat was observed at the highest concentrations analysed (virtually 100% of cell death). Cells treated with peptide concentrations below the IC$_{50}$ did not show noticeable changes in morphology (data not shown).

**Haemolytic activity**

The haemolytic activity of purified bovicin HC5 and commercial nisin was determined as the percentage of lysis of sheep erythrocytes. The bacteriocins showed little haemolytic activity, regardless of the concentration tested. However, the haemolysis caused by bovicin HC5 was always lower than the values determined for nisin, even though the concentration of bovicin HC5 was up to 5.45-fold greater than nisin. At the highest concentrations tested, the relative haemolysis was less than 5% for bovicin HC5 and 6.6% for nisin compared with 0.1% (v/v) Triton X-100 (100% of haemolysis) (Fig. 5).

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**Fig. 2.** Cytotoxic effect of bovicin HC5 (a) and nisin (b) on Vero cells using the MTT assay. The cells were initially maintained in a humidified incubator at 37 °C for 48 h, and bovicin HC5 or nisin was added at different concentrations. The percentage of viable cells was calculated as described in Methods. The results are shown as means ± SD of two independent experiments performed in triplicate.

**Fig. 3.** Cytotoxic effect of bovicin HC5 (a) and nisin (b) on MCF-7 cells using the MTT assay. The cells were initially maintained in a humidified incubator at 37 °C for 48 h, and bovicin HC5 or nisin was added at different concentrations. The percentage of viable cells was calculated as described in Methods. The results are shown as means ± SD of two independent experiments performed in triplicate.
Since bovicin HC5 and commercial nisin can inhibit target bacteria at concentrations much lower than the maximum concentration used in our in vitro assays, it appears that both bacteriocins have low haemolytic activity in red blood cells.

**Effect of cholesterol**

To evaluate whether the presence of cholesterol could influence the ability of bovicin HC5 or nisin to permeabilize synthetic membranes, liposomes containing cholesterol and different lipid compositions were used. The model membrane systems lack lipid II, the target used for both bacteriocins, because our aim was to mimic the eukaryotic cell membrane, where the specific target for bovicin HC5 and nisin is absent.

Because of technical limitations due to the low detection limit of the SLM Aminco Spectrofluorometer, the concentrations of the bacteriocins analysed against the model membranes were lower than those used in the experiments to measure cytotoxic and haemolytic effects. If higher concentrations of the peptides were used, the equipment would go out of range.

For the two model membranes tested, the concentration of bovicin HC5 required to cause comparable CF efflux was 3.33-fold greater than nisin. In liposomes made of pure DOPC, bovicin HC5 or nisin caused minor efflux of CF, and no more than 2.5% of the probe was released from liposomes composed of DOPC. The presence of 30% cholesterol on DOPC vesicles did not influence the CF efflux obtained upon addition of the bacteriocins ($P>0.05$) (Fig. 6).

In CF-loaded DPOPC vesicles, nisin and bovicin HC5 caused significant efflux of CF from the liposomes. In these model membranes, the release of CF caused by bovicin HC5 was not influenced by the presence of cholesterol ($P>0.05$), while the CF leakage caused by nisin was reduced in the presence of cholesterol (CF leakage of 25.1% in DPOPC liposomes containing cholesterol versus 30.2% in pure DPOPC liposomes, Fig. 6) ($P<0.05$).
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DISCUSSION

An increased interest in antimicrobial peptides has been 

observed particularly because of their potential applications 
in the food industry, and human and veterinary 

medicine (Reddy et al., 2004). However, limited information 
regarding the cytotoxicity and haemolytic potential of 
cationic antimicrobial peptides, including bacteriocins, is 
currently available. In the present study, the in vitro 
cytotoxicity and haemolytic activity of bovicin HC5 were 
assessed against mammalian cell lines as an initial screening 
to determine the safety of this peptide.

In vitro cytotoxicity assays are useful to define basal 
cytotoxicity, indicating the intrinsic ability of a compound 
to cause cell death or loss of viability as a consequence of 
damage to several cellular functions (Fotakis & Timbrell,
2006). The MTT assay is a commonly used assay to detect 
early toxicity (Fotakis & Timbrell, 2006), and it is based on 
a reduction of a water-soluble tetrazolium salt (MTT) to an 
insoluble purple formazan by cleavage of the tetrazolium 
ring by mitochondrial dehydrogenases in metabolically 
active cells (Mosmann, 1983). According to Berridge & Tan 
(1993), the reduction of MTT could also be mediated by 
NADH or NADPH within the cells but outside of 
mitochondria.

The MTT assays indicated that both bacteriocins decreased 
viability of Vero cells in a concentration-dependent 
manner. However, typical characteristics of cytotoxic 
effect, such as vacuolization of the cell cytoplasm and 
detachment of the confluent cell monolayer, were observed 
with different concentrations of the bacteriocins. The toxic 
effect caused by bovicin HC5 on Vero, MCF-7 and HepG2 
cells was obtained only at high concentrations of the 
peptide, while nisin showed cytotoxic activity at concentra-
tions much lower than those determined with bovicin 
HC5, regardless of the cell lines evaluated.

Microcin E492, a bacteriocin produced by Klebsiella pneumoniae RYC492, showed cytotoxic effects against 
several human cell lines, including HeLa, Jurkat and 
RJ2.25 (Hetz et al., 2002). Vaucher et al. (2010) 
investigated the cytotoxicity of the antimicrobial peptides 
P40 and nisin, by MTT assays using Vero cells, and the IC50 
was 0.3 and 0.35 µg ml\(^{-1}\) for the purified peptide P40 and 
commercial nisin, respectively. The IC50 determined for 
nisin against the human intestinal epithelial cell lines HT29 
and Caco-2 was 89.9 and 115 µM, respectively (Maher & 
McClean, 2006), while the IC50 determined for the same 
bacteriocin against Jurkat and Moul-4 cells was 225 µM 
(Begde et al., 2011). Those values are much higher than the 
IC50 determined for nisin against the cell lines used in the 
present study.

The differences in toxic concentrations observed for 
bovicin HC5 and nisin against the mammalian cells tested 
in this study may be related to differences regarding the 
mechanism of action of the peptides, the composition of 
the cell membrane, the metabolic activity of the cells and 
the assay used. According to Maher & McClean (2006), 
differential cytotoxicity of bacteriocins and other anti-
microbial peptides may also be related to differences in the 
preparation of peptides, exposure time to the peptide and 
the medium used to cultivate the cells. Differences in the 
cell surface hydrophobicity may also influence the effective 
binding and cytotoxic action of antimicrobial peptides, but 
the exact mechanism by which toxicity differs among 
different cell types is not completely elucidated (Vaucher 
et al., 2010).

We previously demonstrated that even though bovicin 
HC5 and nisin share the same membrane target, the ability 
to form pores in model membranes is quite distinct 
between the two peptides (Paiva et al., 2011). Nisin is much 
more efficient in membrane permeabilization than bovicin 
HC5. These effects might be explained by differences in size 
and in the mechanism of pore formation of the two 
peptides. Nisin is approximately 12 amino acids longer 
than bovicin HC5 and can easily permeabilize model 
membranes containing acyl chains with 16 or 18 carbons. 
The pore-forming activity of bovicin HC5 is clearly 
dependent on membrane thickness and very little leakage 
of intracellular compounds was observed in membranes 
with C\(_{18}\) lipids, even if the bacteriocin concentration was 
10-fold greater than nisin (Paiva et al., 2011).

The assessment of haemolytic activity is also used as a 
measure for cytotoxicity, as well as to estimate the 
therapeutic index of antimicrobial peptides (Maher & 
McCLean, 2006). In this study, both bacteriocins exhibited 
low toxicity against sheep erythrocytes; however, the 
relative haemolysis observed for bovicin HC5 was always 
lower than the effect observed for nisin, even if the
concentration of bovicin HC5 was 5.45-fold higher than the concentration of nisin.

Vaucher et al. (2010) demonstrated a haemolysis level of 6% in fresh defibrinated human erythrocytes when 3.35 μg commercial nisin ml⁻¹ was used, which was not considered a toxic effect by the authors. Maher & McClean (2006) reported a relative haemolysis of 12.14% ± 10.10 (compared with 0.1% Triton X-100) when 230 μM nisin was assayed. In another study, 750 μM nisin caused approximately 10% relative haemolysis against human red blood cells (Begde et al., 2011).

Some antimicrobial peptides have shown a cell-line-specific cytotoxic effect, interacting with components of the target cell surface (Hetz et al., 2002), and the association or activity of the peptide can also be affected by membrane lipid composition (Wessman et al., 2008) or by the presence of cholesterol (Benachir et al., 1997; Prenner et al., 2001; Verly et al., 2008). The presence of cholesterol in eukaryotic cell membranes particularly enhances the rigidity of lipid bilayers, reducing or inhibiting the membrane disruption by antimicrobial peptides (Brender et al., 2012). Therefore, the antimicrobial peptide concentration necessary to permeabilize cholesterol-containing membranes should be higher than the concentration necessary to span bacterial membranes, which are cholesterol-free (Prenner et al., 2001).

In this study, we have investigated the role of cholesterol on the activity of bovicin HC5 and nisin against artificial model membranes, composed of phospholipids with 16 and 18 carbon atoms, which are the most common lipids encountered on eukaryotic cell membranes. We used unsaturated lipids (C₁₈:1 and C₁₆:1), that are able to create a kink, preventing the fatty acids from tightly packing together, and increasing the fluidity of the membrane.

The presence of cholesterol did not influence the ability of bovicin HC5 or nisin to permeabilize the thicker membranes, composed of DOPC, and no differences in CF leakage were observed. These results contrasted with the findings obtained for other antimicrobial peptides, such as melittin (Mouritsen & Zuckermann, 2004; Raghuraman & Chattopadhyay, 2004), the amphibian skin antimicrobial peptide DD K (Verly et al., 2008) and gramicidin S (Prenner et al., 2001), which penetrates deeper into pure bilayers when compared with liposomes containing cholesterol.

According to Raghuraman & Chattopadhyay (2005, 2007), the melittin-induced leakage from erythrocytes and from DOPC/cholesterol liposomes might be partly induced by major morphological changes of the membrane bilayer caused by melittin, and not by pore formation. This could be the case for the leakage caused by nisin and high concentrations of bovicin HC5 in DPOPC liposomes, since these membranes are composed of shorter-chain lipids, and the bacteriocins can more easily span these membranes when compared with the DOPC membranes.

As observed for DOPC membranes, the presence of cholesterol in DPOPC membranes had no significant effect on the rate of CF release caused by bovicin HC5. However, the binding of nisin to DPOPC liposomes was significantly affected by cholesterol (P<0.05), and the CF leakage induced by nisin was reduced by 11.2% when cholesterol was present on DPOPC membranes. These results suggest that the presence of cholesterol attenuates but does not inhibit the permeabilization of the phospholipid bilayer by nisin.

In conclusion, only high concentrations of purified bovicin HC5 are able to exert toxic effects on mammalian cells and haemolytic activity on sheep erythrocytes. The quantities required for in vitro antimicrobial activity for bovicin HC5 are in the nanomolar range, thus much lower than the concentrations needed to cause some effect on mammalian cells. This discrepancy is due to the presence of lipid II, used as a specific receptor by bovicin HC5 in sensitive bacterial cells. However, in the micromolar range, bovicin HC5 is able to destabilize the bilayer integrity in sensitive cells, in an unspecific way (independent of lipid II), causing leakage of intracellular compounds by interacting directly with the membrane phospholipids (Paiva et al., 2011). In addition, the presence of physiologically relevant concentrations of cholesterol does not affect the interaction of bovicin HC5 with model membranes, independent of the membrane composition and the bacteriocin concentration tested. These results confirm that bovicin HC5 has little cytotoxicity against eukaryotic cells and strengthen its potential for future use in controlling undesirable bacteria in mammalian hosts.

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