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Short peptides derived from the NH_2-terminus of subclass IIa bacteriocin enterocin CRL35 show antimicrobial activity

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***Objectives*: Subclass IIa bacteriocins are characterized by a hydrophilic N-terminal domain that shares a YGNGVxCxxxxC consensus and a variable hydrophobic C-terminus. Enterocin CRL35 is a 43-amino-acid heat stable peptide with antilisterial activity. Short synthetic peptides derived from the N-terminal half of enterocin CRL35 and other subclass IIa bacteriocins were evaluated for antimicrobial properties.**

***Methods*: *In vitro* activities of synthetic peptides were evaluated in complex, chemically defined and minimal media. MIC assays were performed by the agar well-diffusion method. Fluorescence assays to evaluate the dissipation of membrane potentials in intact cells were carried out. Time–kill kinetics of *Listeria innocua* cells with the active peptide were performed.**

***Results and conclusions*: A 15-mer peptide derived from enterocin CRL35 inhibited the growth of *L. innocua* and *Listeria monocytogenes* in synthetic/minimal media and dissipated the membrane potential of sensitive cells, with MICs of 10 and 50 μM, respectively. 15-mer derivatives from other class IIa bacteriocins (mesentericin Y105, pediocin PA-1 and piscicolin 126) also showed antimicrobial activities.**

Keywords: enterococci, pediocin, liposomes

Introduction

Bacteriocins are antimicrobial proteinaceous compounds synthesized ribosomally by bacteria. The ecological function of these peptides is not yet fully understood.[1] Most food-grade lactic acid bacteria (LAB) produce bacteriocins. These peptides permeabilize the membrane and deplete the proton motive force of sensitive cells and artificial liposomes or induce lysis through the activation of autolysins.[2–4] Subclass IIa bacteriocins are heat stable short peptides with antilisterial activity.[3,5,6] These compounds share a YGNGVxCxxxxC consensus in their cationic and hydrophilic N-terminal domain; however, the C-terminal domains are somewhat more diverse, which allows subclass IIa antimicrobials to be divided into three subgroups.[7,8] It has been postulated that the C-terminal half is implicated in cell specificity.[9]

As subclass IIa bacteriocins have strong antilisterial activity, these peptides constitute a novel approach to control *Listeria monocytogenes*, the causative agent of listeriosis, in food and make them attractive candidates as next-generation therapeutic agents.[10]

The fact that they are ribosomally synthesized peptides opens the possibility of improving the characteristics of each peptide in order to enhance their spectra of activity. As a preliminary approach, numerous studies about the relationship between primary structure and function have been performed.[5,9,11,12] Fleury *et al.*[12] showed that truncated analogues of mesentericin Y105 are not active against *Listeria*. Fimland *et al.*[13] have demonstrated that a peptide derived from the C-terminal domain could increase the MIC of different bacteriocins. Alternatively, Johnsen *et al.*[9] demonstrated that hybrid bacteriocins remain active and suggested that the C-terminal hairpin domain is implicated in the specificity-determining step that seems to involve interactions with lipids and/or proteins of the cell membrane. Yan *et al.*[14] reported that the interaction with a chiral receptor is a critical feature of the mode of action of subclass IIa bacteriocins and a non-specific mode of action at high concentrations.

Enterocin CRL35 is a subclass IIa bacteriocin with antilisterial and antiviral activities.[15] It is a 43-amino-acid heat stable peptide produced by *Enterococcus mundtii* CRL35 isolated from artisanal cheese from Tafí del Valle (Tucumán, Argentina). It has demonstrated synergism with some antibiotics even at sublethal

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concentrations.[8] Saavedra *et al.*[15] showed that peptides derived from the C-terminal domain of enterocin CRL35 were able to inhibit the action of the bacteriocin and those derived from the N-terminal half were able to enhance the antimicrobial activity.

In the present work, we analyse the antimicrobial properties of short synthetic peptides derived from the N-terminal sequence of enterocin CRL35 and other subclass IIa bacteriocins in complex and chemically defined media.

Materials and methods

Strains and media

Complex media brain heart infusion (BHI), LAPTg (1.5% peptone, 1% tryptone, 1% glucose, 1% yeast extract, 0.1% Tween 80, pH 6.5) and Mueller–Hinton (M–H), the minimal medium HTM (Hsiang-Ning Tsai medium)[16] and the chemically defined Bacto B12 vitamin assay medium (Difco; supplemented with tryptone, peptone or yeast extract at 10 g/L for each) were used for the assays. Agarized media were prepared with 1.5% agar. Susceptible and resistant target cells were *Lactococcus lactis* subsp. *cremoris* MG1363,[17] *Lactobacillus plantarum* CRL691 (CERELA culture collection), *L. lactis* subsp. *lactis* IL1403[18] (INRA-Jouy-en-Josas, France), *Enterococcus faecium* DPC1143 (Dairy Products Research Center, Teagasc, Moorepark, Fermoy, County Cork, Ireland), *Escherichia coli* BL21(DE3), *L. monocytogenes* FBUNT (Bacteriology Department, Faculty of Biochemistry, Universidad Nacional de Tucumán) and *Listeria innocua* 7 (INRA-Jouy-en-Josas). All bacterial stock cultures were maintained in their appropriate broths containing 20% glycerol at −80°C. *L. innocua* 7 and *L. monocytogenes* FBUNT were grown in BHI broth, HTM broth and B12 assay bacto agar (Difco), supplemented with 24 pM lipoic acid. *L. plantarum* CRL961 was cultured in *Lactobacillus* MRS broth. *L. lactis* MG1363 was cultured in LAPT with 0.5% (w/v) glucose (LAPTg). All cultures were grown overnight without aeration at 30°C.

Synthetic peptides and bacteriocins

Enterocin CRL35 and short truncated peptides derived from N-terminal sequences of enterocin A, divercin V41, piscicolin, pediocin PA-1, Listeriocin 743A, sakacin P, mesentericin and enterocin CRL35 were designed and synthesized (Table 1). Freeze-dried peptides were reconstituted in sterile distilled water and stored at −20°C until used. Different parameters of the truncated peptides were calculated using the online ProtParam tool from Expasy (www.expasy.org/tools/protparam.html). Mature divercin V41 and enterocin A were purified from cell-free supernatants from producer strains *Carnobacterium divergens* V41[19] and *E. faecium* CRL988,[20] respectively.[21] The active fractions were pooled and concentrated by N_2 flux using a TurboVap evaporator (Caliper Life Sciences, Hopkinton, MA, USA) and resuspended in distilled water.

MIC assays

The inhibitory activity of truncated derivatives and their corresponding mature bacteriocins as models of subclass IIa was studied using the well-diffusion method described previously.[12] MICs were determined by successive dilutions of antimicrobials on agar plates and defined as the lowest dilution of the bacteriocin/peptide that forms a clear inhibition zone of 1 mm, or following growth inhibition at OD_{560} in a Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA). In this case, MIC was defined as the concentration of bacteriocin/peptide that inhibited growth of the indicator strain by 50% after 12 h.

Determination of membrane potential

The ability of the peptides to depolarize the cytoplasmic membrane of *L. innocua* 7 was examined using the membrane potential-sensitive fluorescent cyanine dye 3,5-dipropylthiadicarbocyanine iodide [$DiSC_3(5)$; Molecular Probes, Eugene, OR, USA].[22] The dye concentrates in the cytoplasmic membrane of energized cells, resulting in the autoquenching of fluorescence. If the peptide forms a channel or disrupts the membrane, the membrane potential will be dissipated and the dye will be released into the medium, increasing the fluorescence. Mid-log phase *L. innocua* cells were washed twice with cold 50 mM HEPES buffer (pH 7.4) containing 12.5 mM glucose and resuspended in the same buffer up to 1×10^8 cells/mL at 4°C and immediately placed on ice for fluorescence measurements. The cell suspension was incubated with 0.5 μM $DiSC_3(5)$ in a fluorescence cuvette until the uptake was maximal, as indicated by a stable reduction in fluorescence. Different concentrations of enterocin CRL35, divercin V41 and enterocin A and their truncated peptides were added to the suspension and the fluorescence was monitored at 30ºC in a fluorescence spectrophotometer (Cary Eclipse, Varian) with the excitation monochromator set at 632 nm and the emission at 670 nm. Complete dissipation was obtained in the presence of 1 μM valinomycin.

Leakage measurement

The release of liposomal content was measured by following the fluorescence quenching of pre-encapsulated terbium-dipicolinic acid (Tb/DPA) complex upon its release into external medium containing 0.1 mM EDTA.[23] To prepare vesicles containing Tb/DPA complex, a chloroformic solution of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG) was mixed (9:1, final concentration 0.5 μmol) and exhaustively dried under nitrogen stream and suspended in 50 mM Tris–HCl buffer with Tb/DPA, pH 6.5. The large multilamellar vesicles formed were sonicated for 20 min with a probe-type sonicator. Non-encapsulated material was eliminated by incubating liposomes for 1 h at 4°C and by gel filtration using a Sephadex-50 column with 50 mM Tris–HCl/10 mM EDTA as the mobile phase. Liposomes were collected in the void volume. Total phospholipid concentration was determined by phosphate analysis. Excitation and emission wavelengths were set at 280 and 545 nm. The assays were carried out at 15ºC.

Viability assays

These assays were carried out as described by McAuliffe *et al.*[24] Briefly, cells of *L. innocua* in the exponential growth phase were collected and resuspended in 50 mM HEPES plus 12.5 mM glucose; then, 1×10^8 cells were taken and resuspended in 1 mL of the same buffer. Different concentrations of the peptides to evaluate were added and cells were quantified (as cfu/mL) at different times.

Results and discussion

As shown in Table 1, 15- and 16-mer N-terminal derivative peptides of enterocin CRL35 (called S and V peptide,

Table 1. Bacteriocins and short truncated N-terminal peptides[a] used in this work

	Length (amino acids)	pI[b]	MW[c]	GRAVY[d]	Antilisterial activity in B12 synthetic medium[e,f]	MIC[e]
Subclass IIa bacteriocins						
enterocin A	44	9.07	4832.50	−0.030	++	ND
divercin V41	44	8.82	4513.81	−0.119	++	ND
enterocin CRL35[g]	43	9.45	4289.80	−0.253	+++	300 pM
Enterocin CRL35 truncated peptides						
NH_2-KYYGNGVSCNKKGCSV	16	9.24	1706.60	−0.669	+	ND
NH_2-KYYGNGVSCNKKGCS	15	9.24	1606.84	−0.993	+	10 μM
NH_2-KYYGNGVSCNKKGC	14	9.24	1520.75	−1.007	−	−
NH_2-KYYGNGVSCNKKG	13	9.54	1417.61	−1.227	−	−
NH_2-KYYGNGVSCNKK	12	9.52	1360.56	−1.350	−	−
NH_2-KYYGNGVSCNK	11	9.11	1232.38	−1.118	−	−
NH_2-KYYGNGVSCN	10	8.18	1103.16	−0.840	−	−
Divercin V41 truncated peptide						
NH_2-TKYYGNGVYCNSKKCWV	17	9.18	2012.34	−0.729	−	−
Enterocin A truncated peptide						
NH_2-TTHSGKYYGNGVYCTKNKCT	20	9.18	2224.51	−1.070	−	−
Sakacin P truncated peptide						
NH_2-KYYGNGVHCGKHSCT	15	8.82	1650.7	−0.920	+	50 μM
Piscicolin 126 truncated peptide						
NH_2-KYYGNGVSCNKNGCT	15	8.82	1604.67	−0.960	+	50 μM
Mesentericin Y105 truncated derivative						
NH_2-KYYGNGVHCTKSGCS	15	8.82	1603.7	− 0.760	+	50 μM
Listeriocin 743A truncated peptide						
NH_2-KYYGNGVSCNKNGCT	15	8.82	1607.7	−0.960	+	50 μM
Pediocin PA1 truncated peptide						
NH_2-KYYGNGVTCGKHSCS	15	8.82	1600.68	−0.760	+	50 μM

ND, not determined.
[a]Peptides were synthesized by Biosynthesis Inc. (Lewisville, TX, USA) and GenScript (Piscataway, NJ, USA).
[b]Isoelectric point.
[c]Molecular weight.
[d]Grand average of hydropathicity.
[e]Sensitive strain: *L. innocua* 7.
[f]Symbols represent relative activity by diffusion in agar. +++, halo ≥ 5 mm; ++, halo 3 mm ≤ x <5 mm; +, halo <3 mm; −, no inhibition.
[g]Synthetic peptide.

respectively) showed antimicrobial activity (Figure 1a). The S peptide was active against *L. innocua* 7 at concentrations of 10 μM, whereas *L. monocytogenes* FBUNT was more resistant and was inhibited with concentrations of at least 50 μM in HTM or Bacto B12 vitamin assay medium (Table 2 and Figure 1b). When assayed in complex media (M−H, BHI and LAPTg) the antimicrobial activity was lower and variable (with frequent negative well-diffusion assays) when compared with that obtained in chemically defined medium (Bacto B12 vitamin assay medium) and minimal medium (HTM) (Table 3). The increased antimicrobial activity in chemically defined medium suggests that some components of the complex media might interfere with detection or activity of antimicrobial peptides. To test this hypothesis, we added different components (one by one) to the base minimal medium and analysed the activity. After 16 h, the sensitive strain forms a lawn on agar and we found a decreased antimicrobial activity in Bacto B12 vitamin assay medium supplemented with tryptone, yeast extract or peptone when compared with the activity displayed in medium without the addition of supplements (Table 3). These results demonstrate that S and V peptides derived from the N-terminal domain of enterocin CRL35 displayed antimicrobial activity in minimal medium and confirm the importance of using chemically defined medium to study the spectrum of action of bacteriocins. Besides, these results might explain in part the apparent contradictory data observed in our previous work, because antimicrobial assays done with peptide S in complex media such as LAPTg showed negative results.[15]

In contrast, shorter (10- to 14-mer) derivatives of enterocin CRL35 did not show antimicrobial activity (Table 1) even at

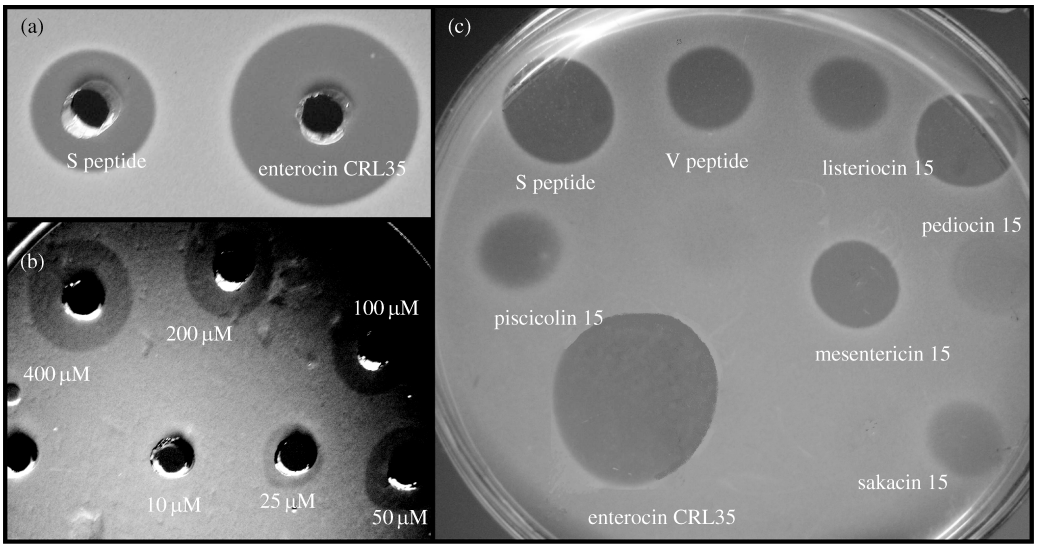

Figure 1. (a) Comparative activity by the well-diffusion agar method between S peptide (10 μM) and enterocin CRL35 (10 μM) in an overlay of *L.innocua* 7. (b) MIC, the highest dilution of peptide that could inhibit the growth of the susceptible strain forming a halo of at least 1 mm, of S peptide on a lawn of *L. innocua* 7 in Bacto B12 vitamin assay medium. (c) Inhibition halos by peptide derivatives (labelled with the name of the entire bacteriocin with the number of amino acids) on an overlay of *L. innocua* cells in BHI (600 μM) (spot-on-lawn).

high concentrations (1000 μM), indicating that 15 amino acids would be the smallest size for the peptide to retain its activity.

To evaluate the effect of peptides on membrane potential, fluorescence assays were carried out with S and V derivatives. These peptides were able to dissipate the transmembrane electrical potential ($\Delta\Psi$) of sensitive cells in a buffer system (Figure 2). In agreement with the well-diffusion assays, the S derivative was more active at dissipating $\Delta\Psi$ than peptide V. However, the more active peptide (S) displayed an activity 100-fold lower than parental bacteriocin. Furthermore, the dissipating action of S peptide occurred more slowly than the complete bacteriocin, suggesting that though lacking the C-terminal domain, the peptide derivatives could destabilize the cell membrane by forming pores in the membrane at concentrations in the order of micromolar. This could be due to the accumulation of major quantities of short peptides that auto-enhance the pore formation or activate another mechanism. Although the lack of the C-terminal domain causes an important loss of activity, the peptide at elevated concentrations retains the ability to form pores or to destabilize the cell membrane.

In order to extend our knowledge, we analysed short peptide derivatives from other class IIa bacteriocins. As the length of 15 amino acids appears to be critical for the activity, we evaluated 15-mer peptides derived from the following bacteriocins: sakacin P, mesentericin Y105, piscicolin 126, pediocin PA-1 and listeriocin 743A. Besides these 15-amino-acid class IIa derivatives, we also assayed derivatives from divercin 41 (17 amino acids) and enterocin A (20 amino acids). These last two derivatives are also derived from class IIa bacteriocins but present other amino acids in the NH_2 terminus: T in divercin V41 and TTHSG in enterocin A. Like peptides S and V, the derivatives from mesentericin Y105, pediocin PA-1, sakacin P, piscicolin 126 and listeriocin 743A were also able to inhibit a lawn of *Listeria* (Figure 1c). The peptidic derivatives from enterocin A and divercin V41 were not active against *L. innocua*, which is in agreement with previously reported data.[11] In the case of mesentericin Y105, Fleury *et al.*[12] have shown that the N-terminus is also essential for bacteriocin activity and that

Table 2. MIC of S peptide in complex and chemically defined media (μM)

	Media		
Strain	B12[a]	HTM	M–H
Listeria monocytogenes FBUNT	50	50	100
Listeria innocua 7	10	10	50

Results are expressed as means. Standard deviation < 10%.
[a]Bacto B12 vitamin assay medium (Difco).

Table 3. Sensitivity of *Listeria* in different media to S peptide

Strain	B12[a]	B12[a] plus yeast extract	B12[a] plus tryptone	B12[a] plus peptone	BHI	LAPTg	HTM	M–H
Listeria innocua	+++	++	++	++	+/−	+/−	+++	+
Listeria monocytogenes	+++	++	++	++	+/−	+/−	+++	+

+++, halo ≥ 5 mm; ++, halo 3 mm ≤ x < 5 mm; +, halo < 3 mm; +/−, variable results.
[a]Bacto B12 vitamin assay medium (Difco).

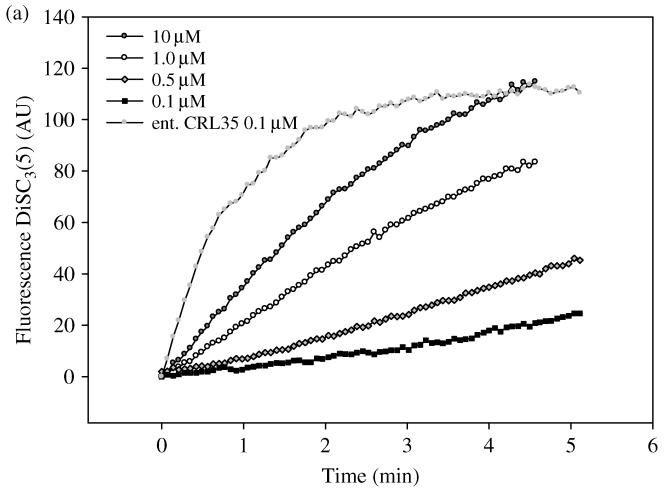

Figure 2. (a) Dissipation of $\Delta\Psi$ induced by enterocin CRL35 15-mer peptide derivative. *L. innocua* cells (1×10^8 cfu/mL) were charged with 3,5-dipropylthiadicarbocyanine iodide in 50 mM HEPES, pH 7.4. (b) Comparative dissipation of $\Delta\Psi$ induced by S and V peptide derivatives when compared with enterocin CRL35. Results are the mean values of three independent experiments. Standard deviation (<10%) was omitted to facilitate visualization. ent., enterocin.

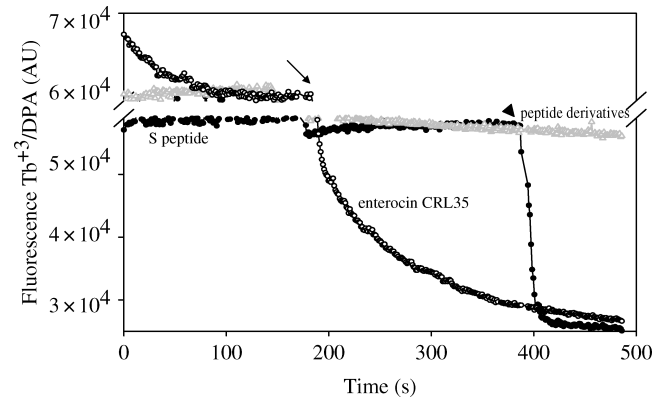

Figure 3. Leakage of Tb^{+3}/DPA from liposomes by the action of peptide derivatives. At concentrations of 10 μM, peptides could deplete the signal of fluorescence (arrow). Total extrusion was obtained with 0.1% Triton X-100 (arrowhead). Results are the mean values of three independent experiments. Standard deviation (<10%) was omitted to facilitate visualization.

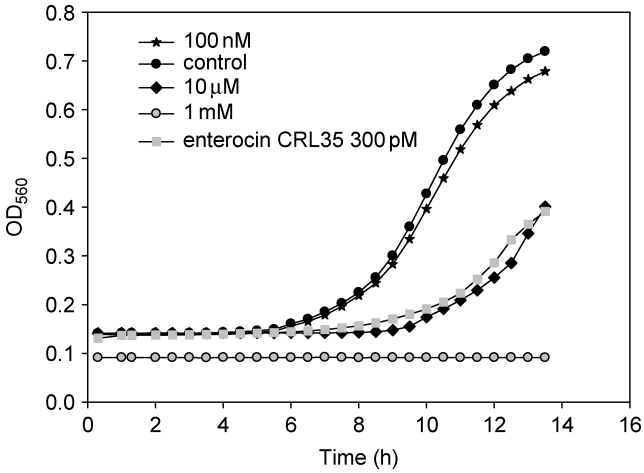

Figure 4. Microplate MIC assays: S peptide has an MIC of 10 μM. Peptide at millimolar concentrations inhibits growth totally.

the C-terminal domain is involved in determining the target cell specificity of these bacteriocins; it could also play a role in electrostatic interactions with anionic lipids of the target membrane.

All peptides that showed antimicrobial activity were also evaluated for their ability to disrupt DMPC/DMPG liposomes (Figure 3). All of them were active at 10 μM.

S and V peptides were also capable of dissipating the membrane potential of *L. plantarum* CRL691 (data not shown). S peptide was used for MIC assays using *L. innocua* 7 as a susceptible strain in broth (Figure 4); 10 μM of the peptide was able to inhibit the growth to half of the control after 14 h. Therefore, it is necessary for a great amount of peptides to autoenhance their affinity to the membranes and disrupt them.

It is known that sublethal concentrations of bacteriocins could dissipate $\Delta\Psi$ without altering the viability of *Listeria* cells.[8] In order to evaluate the bactericidal effect of S peptide on *Listeria*, viability assays were carried out as described by

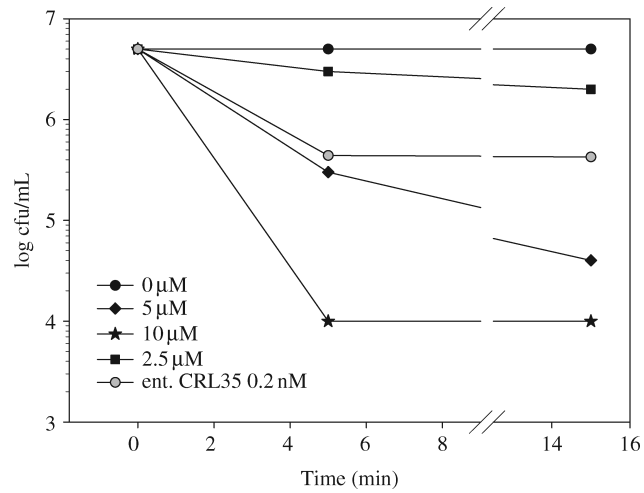

Figure 5. Time–kill kinetics of membrane-active S peptide against *L. innocua* 7. Cell viability, expressed as cfu/mL, was determined at the indicated times. Results represent the average of three independent experiments using different peptide concentrations. Circles, 0 μM; squares, 2.5 μM; diamonds, 5 μM; stars, 10 μM. ent., enterocin.

McAuliffe *et al*.[24] After 5 min of treatment with peptide concentrations equal to MIC, the viable cell count was 250-fold lower than the control without treatment (Figure 5). This effect was observed with the parental bacteriocin at nanomolar concentrations. Thus, the antimicrobial activity was reduced by more than 1000-fold.

In contrast, truncated peptides S and V were not active against *E. coli* BL21(DE3), *L. lactis* MG1363, *L. lactis* IL1403 or *E. faecium* DPC1143 (data not shown). However, the 15-mer peptide did show activity against *L. plantarum* CRL691, displaying a relatively narrow spectrum of action as was previously observed with the entire bacteriocin.[7,12] Although S and V peptides showed a similar biological activity when compared with the entire enterocin CRL35, further studies are necessary to elucidate the mechanism of action of these peptides that lack the amphiphilic domain but remain active in synthetic or minimal media. Although the elucidation of mode of action of these active peptides will be analysed in future works, it is possible to speculate that these peptides could form pores in the membrane at elevated concentrations in order to compensate for their lower activity (and size) when compared with parental bacteriocin.

As noted earlier, we reported in a previous work that the 15-mer derivative (peptide S) of enterocin CRL35 was unable to inhibit *L. innocua*.[15] Although that result appears to be contradictory with the present data, one important difference has been the implementation of synthetic or minimal media. In contrast, Fimland *et al*.[13] showed that an N-terminal derivative of pediocin PA-1 did not show antimicrobial activity. However, the peptide design in that work was different as the N-terminal amino acids were modified and the Cys was replaced with α-aminobutyric acid. Probably, the Cys is needed to form intra- and intermolecular disulphide bridges that could be crucial for the antimicrobial activity of these short peptides. Furthermore, Yan *et al*.[14] reported that a 22-mer N-terminal derivative of carnobacteriocin B2 did not show antimicrobial activity. In this experiment, the length and the sequence of the peptide (VNYGNGV in carnobacteriocin B12) were different from those of the peptides used in the present work.

It is known that many peptide derivatives from antimicrobial proteins of vertebrates have antimicrobial activity, i.e. human β-defensins[25] and mucins[26] or frog skin antimicrobial peptides,[27,28] and peptides derived from human proteins or antibiotics;[29–31] however, to the best of our knowledge, this is the first report of antimicrobial activity of short N-terminal peptidic derivatives from LAB bacteriocins.[32] It would be interesting to evaluate their use as biopreservatives in fermented food,[33] or for topical use in veterinary or medical applications.

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Transparency declarations

None to declare.

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