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Effect of Enterocin CRL35 on *Listeria monocytogenes* cell membrane

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Abstract

The antimicrobial peptide Enterocin CRL35, a class II bacteriocin, produces at high concentrations (8 μg ml^{-1}) localized holes in the wall and cellular membrane of *Listeria monocytogenes*, reflected in the efflux of macromolecules such as proteins and other ultraviolet-absorbing materials. At lower concentrations (0.5 μg ml^{-1}), neither ultra structural changes nor macromolecules efflux were observed, however potassium and phosphate ions were released, dissipating the proton motive force. As a result the bacteria were killed.

Keywords: Enterocin; Antimicrobial peptide; Bacteriocin

1. Introduction

Bacteriocins are antimicrobial peptides lethal to closely related bacteriocin-producing species. According to Moll et al. [1], bacteriocins can be classified in three groups: (I) lantibiotics, small peptides (< 5 kDa) that undergo post-translational modifications, (II) small heat-stable peptides (< 10 kDa) and (III) large heat labile bacteriocins (> 30 kDa). The group II is subdivided in four subgroups: (IIa) *Listeria* active peptides, which have an N-terminal consensus sequence, YGNGVXC. (IIb) Bacteriocins that require two different peptides for activity. (IIc) Sec dependent bacteriocins and (IId) class II bacteriocins that do not belong to the other groups. Class IIa bacteriocins, which seem to have a common mechanism of action, would act by dissipating the membrane proton motive gradient [2].

Enterococcus faecium CRL35, a strain isolated from regional Argentinean cheese (Tafi cheese) produces a bacteriocin called Enterocin CRL35, which was recently described [3]. Enterocin CRL35 possess activity against the food borne pathogen *Listeria monocytogenes*, and because of its efficiency this peptide has potential as antimicrobial agent in food. The partial N-terminal sequence was obtained and showed that it can be categorized in the group II [4].

To use bacteriocins in the most effective way, it is important to determine their mode of action against food-spoilage and food-borne bacteria. The purpose of this study was to obtain a general view of the mechanism of action of Enterocin CRL 35.

The investigations were performed on whole *L. monocytogenes* cells, examining the effect on the microscopic structure and the efflux of different molecules.

2. Materials and methods

2.1. Bacterial strains and media

E. faecium CRL35 belongs to the CERELA Stock collection and the sensitive strain *L. monocytogenes* LS01 was provided by Cátedra de Bacteriología, Facultad de Bioquímica, Química y Farmacia (UNT). Both strains were grown at 30°C in Laptg broth, without Tween 80 [5].

2.2. Purification of Enterocin CRL35

E. faecium CRL35 was grown in 1 l of media for 18 h. The cells were removed by centrifugation and the peptide present in the supernatant was precipitated by adding $(NH_4)_2SO_4$ to a final concentration of 60% (w/v). The pellet, obtained by centrifugation at 12 000 × *g*, was dis-

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Abbreviations: cfu, colony forming units; $DiSC_3(5)$, 3,3′-dipropylthiadicarbocyanine iodide

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solved in 100 ml of distilled water and passed through a C_{18} cartridge (Impaq RG1080 C_{18}) which was washed with several volumes of distilled water and then eluted with increasing concentrations of acetonitrile. The fractions showing antibiotic activity were pooled, concentrated and applied to a CM cellulose cation exchanger column equilibrated with 10 mM acetate buffer pH 5.2. The column was washed with the same buffer until the OD at 280 nm decreased to zero. The bacteriocin was eluted with 300 mM of NaCl in the same buffer. The active fractions were concentrated and loaded on a HPLC system utilizing a 300×4.6 mm Bonda Pack C18 reversed-phase column (Waters). The column was equilibrated with 0.1% trifluoroacetic acid (TFA) in water and eluted with a linear gradient of 0–100% acetonitrile containing 0.1% TFA, at a flow rate of 1 ml min^{-1}. This procedure yields approximately 0.2 mg of Enterocin CRL35 per liter of culture, which appeared homogeneous on analytical RP-HPLC and SDS-PAGE [6].

2.3. Effects of Enterocin CRL35 on sensitive cells

Sensitive cells were grown 4 h, harvested, washed twice and suspended to approximately 10^7 cells per ml in 10 mM HEPES–Na buffer pH 7.2. The bacteriocin was added at different concentrations and samples were taken at appropriate times to determine the colony forming units (cfu).

2.4. Efflux of different cellular materials

L. monocytogenes LS01 harvested by centrifugation were washed three times and suspended in 5 mM HEPES–Na buffer pH 7.2 at a concentration of 10^8 cells ml^{-1}. After incubation with a determinate concentration of Enterocin CRL35, the samples were subjected to filtration through a Millipore filter (Millipore 0.22 μm pore size). The extracellular K^+, phosphate and UV absorbing materials in each filtrated were measured. Two controls were carried out; one of them consisted of cells incubated in the absence of Enterocin CRL35 and the other a complete cellular lysis by sonication after incubation.

2.5. Measurement of the transmembrane electrical potential

The transmembrane electrical potential, $\Delta\psi$, was determined as described by Bennik et al. [7]. Briefly: cells were grown until mid-exponential phase, harvested by centrifugation, washed twice and resuspended in 50 mM HEPES–K buffer pH 7.4 (0.05 DO, 1.5×10^8 cells ml^{-1}) containing 10 mM glucose and 0.5 μM of 3,3′-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$), a potential sensitive fluorescent probe (Molecular Probes). Fluorescence was measured with a SLM 4048c spectrofluorometer at 30°C, excitation and emission wavelength were 622 and 674 nm respectively. A completely dissipated $\Delta\psi$ was achieved with 1 μM of valinomycin. ΔpH was dissipated with nigericin.

2.6. Electron microscopy

Mid-exponential phase cultures of *L. monocytogenes* were inoculated with different amounts of Enterocin CRL35 to 30 μg ml^{-1}. Non-inoculated samples were used as a control. After 30 min of incubation at 30°C, cells were pelleted, and fixed for 3 h with 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, 1 mM $CaCl_2$. The fixed material washed with the same buffer was included in 2% agar and subjected to an overnight post fixation with 1% osmium tetroxide in the same phosphate buffer, and then to a 40-min fixation with 2% uranyl acetate. Dehydration was carried out in a graded ethanol series, exchanged through acetone and embedded in Spurr resin (Pelco Inc.). Blocks were sectioned on a Sorvall Porter Blum MT1 ultramicrotome. Silver gray sections were stained with uranyl-acetate and lead citrate and examined with a Zeiss EM 109 transmission electron microscope.

2.7. Other determinations

UV absorbing materials were determined in a Gilford spectrophotometer measuring OD at 260 and 280 nm. Potassium was measured by flame photometry [8]. The inorganic phosphorus was determined by the method of Ames [9] and proteins with the Bio-Rad protein assay.

3. Results

3.1. Effects of bacteriocin CRL35 on the viability of sensitive cells

Experiments in which *L. monocytogenes* were incubated

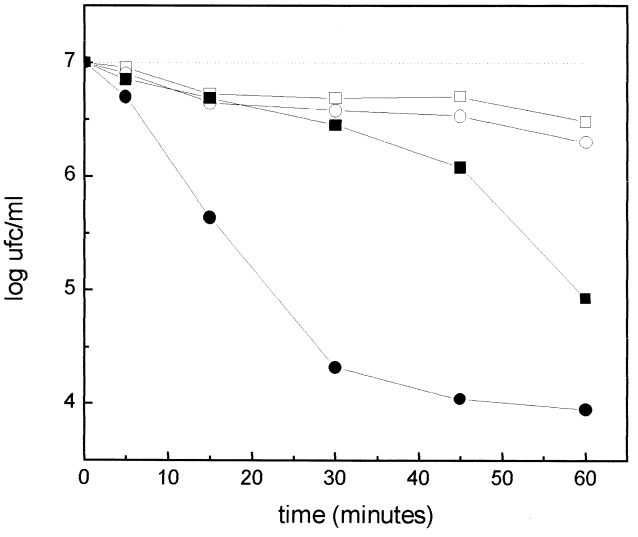

Fig. 1. Effect of Enterocin CRL35 on the viability of *L. monocytogenes*. Viability of a 4-h culture of *L. monocytogenes* after exposure to 0.16 μg ml^{-1} (squares) or 0.3 μg ml^{-1} (circles). Open symbols represent cells suspended in 10 mM HEPES buffer pH 7.2. Closed symbols represent cells suspended in the same buffer supplemented with 10 mM glucose.

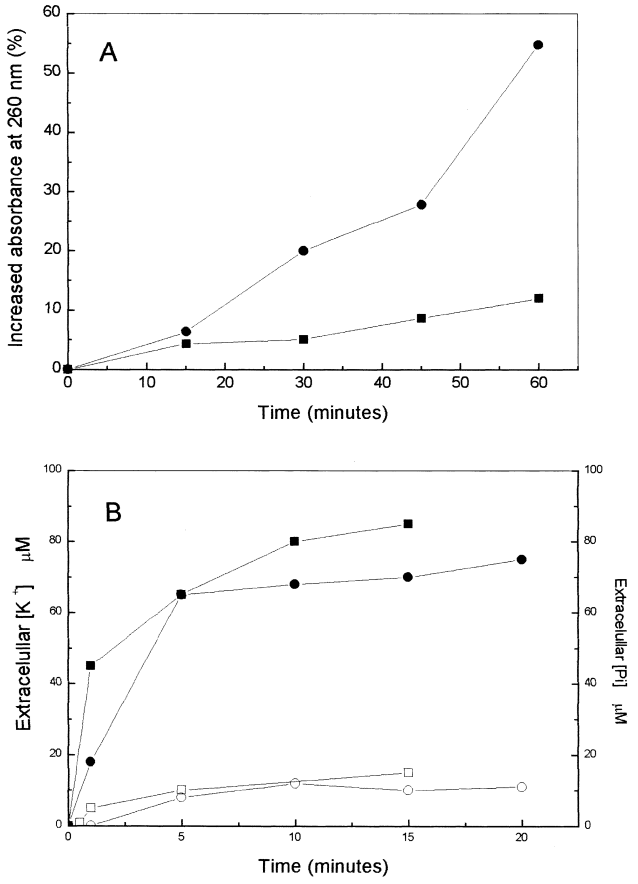

Fig. 2. Effect of Enterocin CRL35 on the release of cellular materials from glucose-energized *L. monocytogenes*. A: Release of UV-absorbing materials induced by 0.5 μg ml^{-1} (■) and 8 μg ml^{-1} (●) of bacteriocin. B: Efflux of potassium (■) and phosphate (●) ions induced by 0.25 μg ml^{-1} bacteriocin. Open symbols represent controls in the absence of peptide.

with different concentrations of Enterocin CRL35 resulted in a decrease of the bacteria viability. Fig. 1 shows how the survival cells diminish as a function of time, reaching very low values after 30 min of incubation. As can be seen, cells suspended in an energizing medium containing glucose were more sensitive than cells in a non-energized one. In this case, a few percent of cells remain alive even after a long time of exposure.

3.2. Effects of Enterocin CRL35 on the release of cellular material

The ultraviolet absorbance of the supernatant of sensitive cell suspensions is important as lysis indicator, and should reflect the efflux of macromolecules as proteins and other substances [10]. *L. monocytogenes* treated with pure Enterocin CRL35 resulted in the leakage of ultraviolet-absorbing materials, compared with controls (Fig. 2A). The release was time and peptide concentration dependent. Cells treated with 8 μg ml^{-1} of bacteriocin released a high percentage of ultraviolet-absorbing materials. It is interesting to note that the values of absorbance began to rise when less than 10% of cells was viable (compare Fig. 1 and Fig. 2A). On the other hand low concentrations of Enterocin CRL35 (0.5–0.25 μg ml^{-1}) provoked imperceptible changes in the absorbance at 280 nm, however potassium and phosphate ions were released as a function of time in a high extension, reaching a maximum leakage after 10 min of incubation (Fig. 2B).

3.3. Microscopy studies

Fig. 3 illustrates the morphology of the *L. monocytogenes* grown in the absence of Enterocin CRL35 and the cells treated with a crude polypeptide extract (12 μg ml^{-1}), added in the same culture medium. Fig. 2A shows normal cells in different stages of division. After treatment the cells change their morphology drastically becoming more spherical and refringent (Fig. 2B). A magnification study shows that the damage was localized on the cell surface (Fig. 2C). The membrane and cell wall were broken and

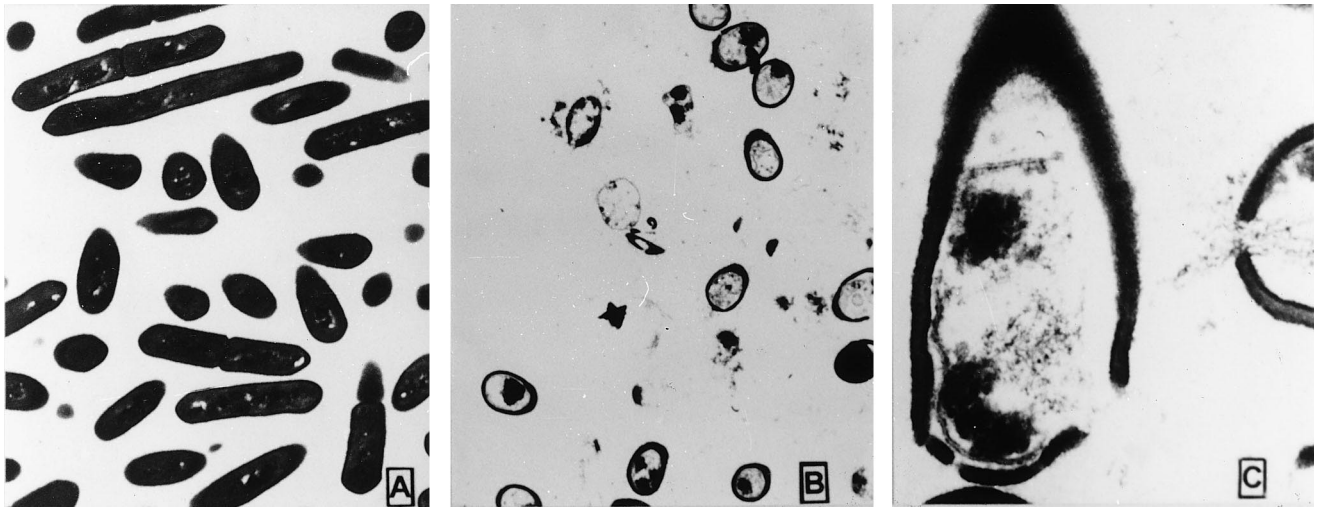

Fig. 3. Electron microscopy analysis of the effect of Enterocin CRL35 on *L. monocytogenes*. A: Normal bacterial culture. B and C: Cells treated with Enterocin CRL35 57 μg ml^{-1}. A,B = ×18 700; C = ×82 600.

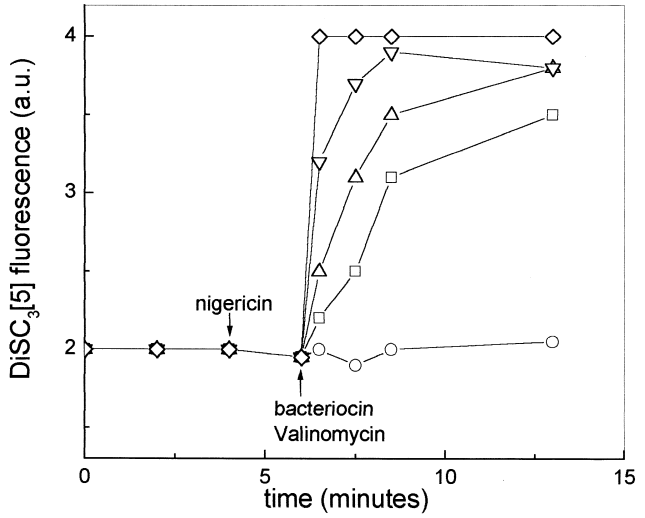

Fig. 4. Effect of Enterocin CRL35 on the membrane potential of glucose-energized *L. monocytogenes*. Cells (1.5×10^8 cfu ml^{-1}) labelled with $DiSC_3(5)$ and suspended in 10 mM glucose were treated for indicated times with 1 μM nigericin, 1 μM valinomycin (◇), and Enterocin CRL35 0 μg ml^{-1} (○), 0.25 μg ml^{-1} (□), 0.5 μg ml^{-1} (△), and 1 μg ml^{-1} (▽).

the cellular content spread in the external medium. Additionally, a separation between membrane and cell wall could be observed. When we used relatively low concentrations of bacteriocin (0.25–2 μg ml^{-1}), the treated cells did not show any morphological difference compared with control cells, although they were also killed.

3.4. Effect of Enterocin CRL35 on membrane ion gradient dissipation

To determine if potassium and phosphate ions release, induced by Enterocin CRL35, dissipate the cell transmembrane electrical potential ($\Delta\psi$), we studied the fluorescence of $DiSC_3(5)$ a sensitive probe to $\Delta\psi$. Addition of 1 μM nigericin did not modify the $DiSC_3(5)$ fluorescence of a *L. monocytogenes* suspension. The $\Delta\psi$ was dissipated upon addition of bacteriocin to the cell suspension which was reflected for an increase of the fluorescence (Fig. 4). The effect was concentration dependent, and obtained at concentrations that were able to kill the bacteria. As controls we used a sample without Enterocin CRL35 and another one with 1 μM valinomycin, which provoked the entire gradient dissipation.

4. Discussion

In the present study we showed that the activity of Enterocin CRL35 on *L. monocytogenes* is bactericidal rather than bacteriostatic, causing a decrease of nearly 100% in viable cfu ml^{-1} within 30 min. The bactericidal effect is enhanced when the peptide acts on glucose-energized cells. The microscopic studies and the viability determinations taken together indicate that Enterocin CRL35's effect depends on the peptide concentration. High concentrations of bacteriocin induce a localized damage on the wall and cellular membrane, and the cytoplasmic content was spread in the external medium indicating cellular lysis. On the other hand, low concentrations did not modify the cell structure, although the K^+ and phosphate ions were spilled out and the bacteria were killed. Kinetics studies of the release of ions and ultraviolet-absorbing materials indicate that K^+ and phosphate leakage occur simultaneously with cell death, in contrast to ultraviolet-absorbing materials that are released after cell death.

The imposition of a membrane electrical potential ($\Delta\psi$) promotes the penetration of some lantibiotics into the hydrophobic region of the lipid bilayer [11]. Less information on the role of an energized membrane on the insertion of class II bacteriocins is available. The results presented here shows that Enterocin CRL35 at relatively low concentration kills sensitive bacteria by making cell membrane permeable to the efflux of potassium and phosphate ions, processes that lead to depletion of $\Delta\psi$.

In conclusion, the results of this study indicate that the primary site of action of Enterocin CRL35 appears to be the cytoplasmic membrane. At low concentrations the killing effect could be achieved by dissipating the ionic potential gradient in cell membrane. At high concentrations the peptide displays, in addition, a non-specific lytic activity against *L. monocytogenes*. More research, including receptor characterization and ion specificity involved in the gradient dissipation, are needed to determine the implicated mechanism more precisely.

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References

[1] Moll, G.N., Konings, W. and Driessen, A. (1999) Bacteriocins: mechanism of membrane insertion and pore formation. Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiol. 76, 185–198.
[2] Bruno, M. and Montville, T. (1993) Common mechanistic actino of bacteriocins from lactic acid bacteria. Appl. Environ. Microbiol. 59, 3003–3010.
[3] Farías, M.E., Ruiz Holgado, A. and Sesma, F. (1994) Bacteriocin production by lactic acid bacteria isolated from regional cheeses: inhibition of foodborne pathogens. J. Food Prot. 57, 1013–1015.

[4] Farías, M.E., Farías, R.N., Ruiz Holgado, A. and Sesma, F. (1996) Purification and N-terminal amino acid sequence of Enterocin CRL 35, a 'pediocin-like' bacteriocin produced by *Enterococcus faecium* CRL 35. Lett. Appl. Microbiol. 22, 417–419.

[5] Raibaud, P., Caulet, M., Galpin, J. and Mocquot, G. (1961) Studies in bacterial flora of the alimentary tract of pigs. II Streptococci: selective enumeration and differentiation of dominant group. J. Appl. Bacteriol. 24, 285–291.

[6] Shagger, H. and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. 166, 368–379.

[7] Bennik, M., Verheul, A., Abee, T., Naaktgeboren-Stoffels, G., Gorris, L. and Smid, E. (1997) Interactions of nisin and pediocin PA-1 with closely related lactic acid bacteria that manifest over 100-fold differences in bacteriocin sensitivity. Appl. Environ. Microbiol. 64, 3628.

[8] Mc Auliffe, O., Ryan, M.P., Ross, R.P., Hill, C., Breeuwer, P. and Abee, T. (1998) Lacticin 3147, a broad-spectrum bacteriocin which selectively dissipates the membrane potential. Appl. Environ. Microbiol. 64, 439–445.

[9] Ames, B.N. (1966) Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol. 8, 115–116.

[10] Yildirim, Z., Winters, D.K. and Johnson, M.G. (1999) Purification, amino acid sequence and mode of action of bifidocin B produced by *Bifidobacterium bifidum* NCFB 1454. J. Appl. Microbiol. 86, 45–54.

[11] Driessen, A.J.M., van den Hooven, H.W., Kuiper, W., van de Kamp, M., Sahl, H.-G., Konings, R.N.H. and Konings, W.N. (1995) Mechanistic studies of lantibiotic-induced permeabilization of phospholipid vesicles. Biochemistry 34, 1606–1614.