

SUMMARY

In the search for new antibiotic substances and food biopreservatives, antimicrobial peptides produced by Lactic Acid Bacteria (LAB), named bacteriocins have been identified as promising new candidates and have thus received much attention. They kill spoilage and food-borne pathogenic bacteria with high efficiency, and they constitute a good model system for structure-function analyses of antimicrobial peptides in general.

The aims of this Thesis were to investigate structural features and antimicrobial activities of natural and synthetic Plantaricins, and also to design a hybrid Pediocin-Plantaricin bacteriocin, in order to evaluate their potential applications as food biopreservatives.

Lactobacillus plantarum LP31 strain, isolated from Argentinian dry-fermented sausages by Simonetta et al. (1997), produced an antimicrobial substance active against some spoilage and foodborne pathogenic bacteria including *Pseudomonas* sp., *Staphylococcus aureus*, *Bacillus cereus*, and *Listeria monocytogenes*, but had no action against some strains of lactic acid bacteria belonging to the collection of the Cátedras de Microbiología y Biotecnología (FIQ-UNL). This antimicrobial substance was inactivated by proteolytic enzymes, it was stable to heat and catalase, and exhibited maximum activity in the pH range 5-6. Consequently it was preliminarily characterized as a bacteriocin.

The maximal bacteriocin production was obtained during the exponential growth phase of the producer organism. In the present work, the bacteriocin was sequentially purified by C₁₈ solid phase extraction, gel filtration chromatography and reverse phase HPLC. We have found that Plantaricin produced by *L. plantarum* LP31 is a peptide with a molecular weight of 1558.85 Da, as determined by MALDI-TOF-MS, and has bactericidal effect against the indicator organisms mentioned above. The amino acid analysis indicated the presence of Ala (2), Arg (1), Asn(1), Glu (4), Gly (1), Lys (2), Ser (1) y Thr (1).

Plantaricin-149 is a bacteriocin produced by *Lactobacillus plantarum* NRIC that was isolated from pineapple, and consist of a peptidic chain of 22 amino acid residues, (YSLQMGATAIKQVKKLFKKKGG) [Kato, T. et al. (1994)]. In this work, a synthetic C-terminal amidated peptide analog denoted as Pln149a was prepared by solid phase Fmoc chemistry and the antagonistic activity against gram-positive and gram-negative bacteria was tested.

The secondary structure was studied by Circular Dichroism (CD) and the vicinity of Tyr₁ by Fluorescence Spectroscopy, under several conditions. We report the results about interaction of Plantaricin 149 with reverse micelles prepared from the amphiphilic sodium bis (2-ethylhexyl)-sulfosuccinate (AOT) in cyclohexane. Synthetic Plantaricin was active against one strain of *Staphylococcus aureus* and four strains of *Listeria* at pH 5.5 and 7.4, and like the natural variant it was inhibitory against *Lactobacillus plantarum* ATCC 8014. CD experiments in samples containing Pln149a and reverse micelles of AOT suggest the stabilization of the amphipathic helical structure by electrostatic interactions between positive charged residues of Lys (peptide net charge +7) and the anionic surfactant. Fluorescence experiments in phosphate buffer pH 7.4 suggested that the N-terminal Tyr residue is closed to the polar heads of AOT molecules.

Considering that Pln149a was shown to be active against *Listeria* strains, a hybrid peptide was designed combining the N-terminal amino acid sequence of pediocin PA-1 (1-18), and Pln149a (6-22) (KYYGNGVTCGKHSCSVDWGATAIKQVKKLFKKKGG), denoted as HP (HPL, linear; HPC, cyclic). The complete hybrid molecule and the two joined sequences were also synthesized by Fmoc chemistry.

The sequence corresponding to the C-terminal part of HP, denoted as C-T (residues 19-35) was active against *Listeria* strains, in accordance with the results found for HP and Pln149a. Nevertheless, it was found that C-T was less active than Pln149a. This result suggests that the pentapeptide sequence YSLQM, which is present in Pln149a but absent in C-T, facilitates the interaction of the peptide with the bacterial membrane, particularly through the hydrophobic residues of Leu₃ and Met₅.

Inhibition assays performed on the sequence corresponding to the N-terminal part of the hybrid (1-18, denoted as N-T) did not show antimicrobial activity, both in the linear and cyclic forms. According with this result it appears that the N-terminal half of the hybrid mediates the initial binding of this bacteriocin to target cells through electrostatic interactions but it is not involved in the antilisterial activity.

Additionally, the results confirmed that the disulphide bridge between Cys₉ and Cys₁₄ is not essential for the hybrid antimicrobial activity, because similar results were obtained with HPL and HPC.

In comparison to Pediocin PA-1, the hybrid bacteriocin has some physicochemical and biological properties that may emphasize the potential technological interest of this

molecule: the absence of Met residues, the presence of only one disulphide bridge, a reduced number of His residues, and an increased positive net charge at pH 7 (HP=+8, Pediocin PA-1=+4), due to the high content of Lys residues. Furthermore, changing the pH from 5.5 to 7.4 increased the antilisterial activity, suggesting that electrostatic interactions govern hybrid binding to the target membrane.

Considering our results about secondary structure of the cyclic hybrid peptide studied by CD and the similar ones reported for Pediocin AcH in water, it is important to remark the high similarity found in the secondary structure content of both peptides (Hybrid-H20: 4% helix, 16.9% β -strand, 9.2% turn, 69.8% unordered; Pediocin AcH-H20: 8% helix, 20% β -strand, 15 % turn, 56 % unordered).

The Stern-Volmer constants found for iodure quenching of the fluorescence of HP in buffer and in the presence of AOT reverse micelles suggested that the Trp₁₈ is inserted into the hydrophobic core of the micelle.

All the peptides synthesized in this work have shown to be selectively active against bacterial cell membranes, since none of them have demonstrated a significant ability to lysate red blood cells.

Concluding remarks: The bactericidal activity of Pln149a against strains of Gram-positive foodborne pathogens *Listeria monocytogenes* and *Staphylococcus aureus* found in this work is a promissory result that may be explored more deeply in order to evaluate the potential biotechnological application of this bacteriocin for food preservation.

According to the results obtained with the hybrid bacteriocin we conclude that the molecule contains two domains: a cationic, N-terminal β -sheet domain that mediates binding of the bacteriocin to the target cell surface and an amphipathic C-terminal α -helical domain that possibly may be involved in the penetration into the hydrophobic part of the target cell membrane

Consequently with these results, we are exploring new research areas about the design of new modified analogs and hybrid bacteriocins that may be actives against bacteria recognized as foodborne illness agents.