A molecular view by FTIR spectroscopy of the relationship between lactocin 705 and membranes: speculations on antimicrobial mechanism

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ABSTRACT

Lactocin 705 is a bacteriocin whose activity depends upon the complementation of two peptides, termed Lac705α and Lac705β. Neither Lac705α nor Lac705β displayed bacteriocin activity by itself when the growth of sensitive cells was monitored. To obtain molecular insights into the lactocin 705 mechanism of action, Fourier transform infrared spectroscopy was used to investigate the interactions of each peptide (Lac705α and Lac705β) with dipalmitoylphosphatidylcholine liposomal membranes. Both peptides show ability to interact with zwitterionic membrane but at different bilayer levels. While Lac705α interacts with the interfacial region inducing dehydration, Lac705β peptide interacts only with the hydrophobic core. This paper presents the first experimental evidence that supports the hypothesis that Lac705α and Lac705β peptides could form a transmembrane oligomer. From the obtained results, a mechanism of action of lactocin 705 on membranes systems is proposed. The component Lac705α could induce dehydration of the bilayer interfacial region and Lac705β peptide could insert in the hydrophobic region of the membrane where the peptide has adequate conditions to achieve the oligomerization.

Running title: Interaction of lactocin 705 with membrane model system

Keywords: Antimicrobial peptide, lactocin 705, mechanism of action, FTIR, membrane model

List of abbreviation: FTIR (Fourier transform infrared spectroscopy), DPPC (dipalmitoylphosphatidylcholine), LAB (Lactic acid bacteria)
INTRODUCTION

In the last decade there has been a growing interest in the biopreservation through the use of microorganisms and/or their metabolites to prevent food spoilage and to extend the shelf life of foods (1-3). Lactic acid bacteria (LAB) are of particular interest as biopreservative organisms. The preserving effects of these organisms are due to the production of antimicrobial substances including hydrogen peroxide, organic acid and bacteriocins (4, 5). Bacteriocins are ribosomally synthetized antimicrobial peptides active against closely related bacteria. The major classes of bacteriocins produced by LAB include: (I) lantibiotics, (II) small heat stable peptides, (III) large heat labile proteins, and (IV) complex proteins. Most of the bacteriocins produced by LAB belong to class II that can be subdivided into (IIa) Listeria-active peptides, (IIb) two peptide bacteriocins, (IIc) sec-dependent bacteriocins, and (IId) bacteriocins that not belong to the other subgroups (6). The potential application of LAB bacteriocins as food preservatives requires an in-depth knowledge of how they exert their bactericidal effect. Many bacteriocins appear to elicit their lethal effects by permeabilizing the cell membrane of target organisms, in certain cases by targeting intermediates of cell wall biosynthesis (7, 8) or possibly proteins of the sugar phosphotransferase systems (9, 10). The meat isolate Lactobacillus curvatus CRL705 (formerly identified as Lactobacillus casei CRL705) produce lactocin 705, a small antimicrobial substance that belongs to the class IIb bacteriocin whose activity depends upon the complementation of two peptides, termed Lac705α (GMSGYIQGIPDFLKGYLGISAANKHKKGRLGY; pI=9.87) and Lac705β (GFWGGLGYIAGRVAAYGHAQASANNHHSPING, pI=8.61) (11, 12). Lactocin 705 exerted an inhibitory effect on the indicator strain Lactobacillus plantarum CRL691 with an optimal Lac705α/Lac705β peptide ratio of 1 to 4. Neither Lac705α nor Lac705β displayed
bacteriocin activity by itself when the growth of sensitive cells was monitored (12). Both peptides were required to dissipate the proton motive force of energized cells of *Lactobacillus plantarum* CRL691 (13). However, the mechanism by which lactocin 705 interacts with bacterial membrane is not clearly established. A way to provide insight into this research area is to study the interactions of each peptide Lac705α and Lac705β with membrane model systems using FTIR spectroscopy. This technique is known to be a versatile and powerful tool to investigate protein and lipid structure and their interactions (14). In this work some evidences that could help to understand the mechanism of action of the two component peptides of lactocin 705 are provided.

**MATERIALS AND METHODS**

**Bacteriocin synthesis.** The synthesis of the 33-amino acid Lac705α peptide and of the 33-amino acid Lac705β peptide was performed according to Palacios *et al.* (15) and Cuozzo *et al.* (11) by Geminys Biotech (Alachua, FL, USA) and Bio-synthesis (Lewisville, TX, USA), respectively. Peptides stock solutions were prepared in 20 mM HEPES/D₂O pD 7.4.

**Vesicles preparation.** The zwitterionic phospholipid dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids (Birmingham, AL, USA). An appropriate amount of DPPC was dissolved in chloroform:methanol (2:1, v/v) and dried under nitrogen onto the wall of a Corex glass tube and then placed in a vacuum oven to completely remove any remaining solvent. The lipid was then rehydrated in 20 mM HEPES/D₂O pD 7.4 and the large multilamellar vesicles formed were sonicated on ice under nitrogen with probe-type sonifier. Cycles of sonication (1-min pulse) and cooling (1 min) were repeated up to 15 times until the initially cloudy lipid dispersion became translucent. To remove titanium
debris the suspension was centrifuged for 15 min at 1100 x g to obtain a pure DPPC small
unilamellar vesicle (SU) suspension (16).

**Samples for FTIR spectra preparation.** For solution spectra, peptides were dissolved to a
final concentration of 10 mg ml\(^{-1}\) in 20 mM HEPES/D\(_2\)O pH 7.4. For samples in the
presence of lipids, each peptide was added to liposomes to give a molar ratio of 20:1 (lipid
to protein). The peptide-vesicle mixtures were incubated for 10 min at room temperature
(20-22ºC) before data acquisition. Phospholipids and peptide concentration was measured
by standard methods (17, 18).

**FTIR spectroscopy.** The samples were recorded in a Nicolet Magna II spectrometer
equipped with a MCT detector (Thermo Nicolet Wisconsin) using a demountable liquid cell
(Harrick Scientific, Ossining, NY) with calcium fluoride windows and 50-µm spacers. A
tungsten-copper thermocouple was placed directly onto the window and the cell placed in a
thermostatized cell mount. Thermal analysis was performed by heating continuously in the
range of 20-80°C with a heating rate of 1°C/min. Spectra were collected by using rapid
scan software running under OMNIC (Nicolet). Usually, 310 scans/sample were taken,
averaged, apodized with a Happ–Genzel function, and Fourier transformed to give a final
resolution of 2 cm\(^{-1}\). Contribution of D\(_2\)O in the Amide I’ region was eliminated by
subtracting the buffer spectra from that of the solution at the same temperature to obtain a
flat baseline between 2000 and 1700 cm\(^{-1}\). Liposomes either in the absence or in the
presence of the peptides were repeated three times with fresh new samples to test the
reproducibility of the measurements. In all cases, the differences among the three
experiments were lower than 5%. The error in estimation of the percentage of secondary
structure depends mainly on the removal of spectral noise, and it was estimated to be 2%
(19). For the measurement of the tyrosine ring vibration wavenumber, an exponential
baseline was subtracted. The wavenumber of the symmetric C-H stretching mode ($v_s[\text{CH}_2]$) of the acyl chain methylene groups of the lipids was determined after a linear baseline was subtracted in the 3050 to 2750 cm$^{-1}$ region. Deconvolution, band-position determination, bandwidth and curve fitting of the original Amide I’ band were performed as reported previously (20-21). Briefly, band component positions were obtained from deconvolution and derivation. Since the results obtained after iterations may not be unique, the following restrictions were applied: i) the band position cannot diverge from initial guesses more than the distance between data points; and ii) the width of the bands should be less than one-half of the Amide I’ bandwidth. The use of several spectra recorded at different temperatures below the thermal denaturation reduces the error of the quantification procedure to around 3% (22).

RESULTS

Effect of Lac705$\alpha$ and Lac705$\beta$ peptides on the CH$_2$ stretching vibration of DPPC.

The impact of each peptide component of lactocin 705 (Lac705$\alpha$ and Lac705$\beta$) on DPPC bilayers was investigated. The methylene stretching vibration of the lipid chains is shown in Figure 1. Two main bands around 2800 and 3100 cm$^{-1}$ dominate the spectra in this region and are assigned to the antisymmetric ($v_{as}[\text{CH}_2]$) and symmetric ($v_s[\text{CH}_2]$) methylene stretching mode, respectively. The thermotropism of lipids is characterized by the shift of the wavenumber of these modes that have shown to be sensitive to the presence of gauche conformers (23- 24). Thus, they are useful probes for monitoring the lipid phase transitions (25). Figure 1 presents the evolution of the $v_s[\text{CH}_2]$ wavenumber of DPPC acyl chains as function of temperature in the absence and presence of Lac705$\alpha$ and Lac705$\beta$ peptides.
The $\nu_{\text{as}} [\text{CH}_2]$ wavenumber showed just the same behavior with the peptide addition (data not shown). For pure DPPC, the transition temperature $Tm$ was 42ºC, as deduced from the curve, which is in agreement with the literature (26). In presence of Lac705$\alpha$, $Tm$ is slightly increased to about 2ºC. However, there are no significant changes in the onset of the $\nu_{\text{as}} [\text{CH}_2]$ and $\nu_{\text{s}} [\text{CH}_2]$ above or below the phase transition indicating that this peptide does not alter the conformation of the acyl chains. On the contrary, Lac705$\beta$ peptide modified the $Tm$ from 42 to 33ºC. This shift to lower temperatures may be taken as evidence of an interaction of the peptide with the hydrocarbon lipid chains (27). The more rapid increase (compared to the curve of pure DPPC) of the wavenumber with the change in temperature in the range preceding the abrupt increase due to the phase transition, may also indicate a disturbance of the gel phase organization. Furthermore, the wavenumbers of methylene stretching band are higher in the presence of Lac705$\beta$ peptide than those observed for pure DPPC bilayers. These observations show that the presence of this peptide results in a decrease of the conformational order of the lipid acyl chains (27).

**Effect of Lac705$\alpha$ and Lac705$\beta$ peptides on the hydration of the polar groups of DPPC.** The stretching vibration of the lipid carbonyl groups ($\nu (\text{C}=\text{O})$) can provide details concerning the hydration, the environment and the conformation of the membrane interfacial region. The ester band of DPPC is known to be composed of two overlapped components located near 1745 and 1730 cm$^{-1}$, which can be evidenced by Fourier self deconvolution. These are attributed to free (anhydrous) and hydrogen-bonded (hydrated) carbonyl groups, respectively (28). Thus, the study of this band as a function of temperature provides insights into the hydration level of the polar-apolar interface of DPPC bilayers. Table 1 presents an analysis for DPPC bilayers at different phase states in the absence and...
presence of Lac705α and Lac705β peptides. In the absence of peptides, there is a well-
known intensity increase in the lower wavenumber component upon increasing
temperature, interpreted as a rise in the amount of water molecules bound to the ester
groups due to a higher penetration of water into the interfacial region above the phase
transition. In the presence of Lac705β peptide, the thermal behavior of the $\nu$ (C=O) band is
did not reflect changes in the water accessibility regarding to the observed in the absence of
the peptide. However, after addition of Lac705α peptide an increase in the higher
wavenumber component in either the gel or in the liquid crystalline lipid phase was
observed.

**Effect of DPPC bilayer on Lac705β Amide I´ vibration.** Figure 2 presents a representative
spectrum of the Lac705β Amide I´ band in the absence and in the presence of DPPC
liposomes. The peptide spectrum in aqueous solution at 25 ºC as well as 50ºC is centered at
about 1650 cm$^{-1}$ which is typical of α-helix structure. Furthermore, the peptide could be
heated up to 50ºC without observing aggregation in the Amide I´ band shape. In the presence
of DPPC liposomes, the maximum of the spectrum shifts to 1628 cm$^{-1}$ at both temperatures
indicating that structural modification occurs. It is important to notice that the bilayer acyl
chain conformational order influences the magnitude of the peptide structural changes,
because the shift from 1650 to 1628 is increased in the liquid-crystalline phase.

To observe the underlying components of the broad Amide I´ band, Fourier self
deconvolution and derivative methods have been applied to the original envelope (29-30).
We have identified six different components bands at frequencies about 1687, 1678, 1670,
1655, 1639 and 1620 cm$^{-1}$ for Lact705β in solution at pD 7.0, for both, 25 and 50º C (Table
2). To assign the components bands to specific structural features and estimate the
percentage of each component, the guides given in Materials and Methods were followed and compared with literature values (30). Still, some of the bands can be unambiguously assigned, while for others, reasonable approximations can be made by comparison with data from other techniques (21). The band around 1655 cm\(^{-1}\) in D\(_2\)O corresponds to canonical \(\alpha\)-helix (21). The bands located around 1666 to 1688 cm\(^{-1}\) arises from \(\beta\) turns (31-32). The band at 1677 cm\(^{-1}\) may also arise from a small contribution of the high-frequency vibration of the antiparallel \(\beta\)-strand (32). Bands appearing at about 1639 cm\(^{-1}\) are assigned in D\(_2\)O to unordered conformation (21, 32). The band located at 1620 cm\(^{-1}\) could be attributed to intermolecular hydrogen bond resulting from aggregation of protein and peptides (33). These results, taken together, indicate that in deuterated solution and at 25°C Lac705\(\beta\) consists of 29% \(\alpha\)-helix, 35% unordered and 14% \(\beta\)-structure (Table 2). Even though the Amide II region, between 1600 and 1500 cm\(^{-1}\) is not as well documented as the Amide I region, it also provides valuable information on the secondary structure of proteins. In our D\(_2\)O condition and in the range of 20 to 80°C, the Amide II band completely disappears indicating a full deuteration of the amide nitrogen (data not shown). This H/D exchange between Lac705\(\beta\) and the aqueous medium is rapid, occurring during the sample preparation and suggests that the solvent can easily access the NH groups of the protein. These results, together with the broad contour of the Amide I spectrum, is characteristic of proteins or peptides with a loose secondary structure (33). In the presence of DPPC liposomes in liquid crystalline phase, the band located at 1621 cm\(^{-1}\) shift to 1628 cm\(^{-1}\) increasing its area significantly. It has already been attributed to extended antiparallel \(\beta\)-sheet (34). However, this band increases together with the aggregation band located at 1688 cm\(^{-1}\) suggesting the formation of weaker intermolecular hydrogen bonds (35). The
formation of such aggregation has already been observed for magainin-2 and related peptides and it is expected to occur for polypeptide chains that retain some secondary structure, preventing a close alignment of chains (35). Another noticeable change observed in the presence of DPPC liposomes, is the shifting of the band located at 1639 cm\(^{-1}\) to 1642 cm\(^{-1}\) with a significant decrease in its intensity. The conformational changes of Lac705\(\beta\) described above, depends on the lipid phase of the phospholipids because in liquid crystalline it is greater than in gel phase, suggesting the influence of the hydrophobic region packing in the membrane-peptide interaction. Moreover, the full width at 0.75 height diminishes from 41 to 32 cm\(^{-1}\) when the lipids goes from gel to liquid crystalline phase.

**Effect of DPPC bilayer on Lac705\(\alpha\) Amide I\' vibration.** The Amide I\' band decomposition of Lac705\(\alpha\) shows six different component bands at frequencies about 1688, 1677, 1656, 1640 and 1625 cm\(^{-1}\). For the assignment and quantification of each band the same criteria described above was used. Table 3 shows that the Amide I\' of Lac705\(\alpha\) in D\(_2\)O solution at 50ºC is composed of 44% \(\alpha\)-helix, 26% unordered and 19% of \(\beta\) structure. It is important to point out that the Lac705\(\alpha\) Amide I\' band did not show significant differences in the presence or in the absence of DPPC liposome in the temperature range from 20 to 80ºC (data not shown).

**Effect of DPPC on the peptides tyrosine vibration.** Lac705\(\alpha\) and Lac705\(\beta\) contain two and three tyrosine residues, respectively. Tyr is a relatively strong infrared absorber due to its polar character and its most intense band is due to the aromatic ring (\(\nu\) C-C) located around 1515 cm\(^{-1}\) (36). However, this wavenumber is affected by the tyrosine´s local environment, being different according to whether it is exposed to water or to a hydrophobic environment. For example, it increases upon peptide unfolding (37-38). Therefore, this vibration mode may
represent a marker providing information relative to the peptide surroundings (36). The tyrosine wavenumber of pure Lac705β peptide in D2O solution show its maximum at 1515.14 cm\(^{-1}\) without any change when the temperature increases from 20ºC to 80ºC. When the Lac705β peptide was incubated with DPPC vesicles, a decrease in the wavenumber to 1511.5 cm\(^{-1}\) was observed. This decrease could be related to a more hydrophobic environment (36) induced by peptide folding or by the peptide insertion to the hydrophobic region of the membrane. The Lac705α peptide shows its maximum at 1514 cm\(^{-1}\) without any change in the presence of DPPC liposomes in the range of 20 to 80ºC (data not shown).

**DISCUSSION**

The present work provides evidences of the mechanism of action of the two-component bacteriocin lactocin 705, whose activity relies upon the complementation of Lac705α and Lac705β peptides. The FTIR studies presented in this paper show that Lac705α and Lac705β peptides can interact with zwitterionic DPPC bilayers and that each peptide exerts its effect on different regions of the membrane. Lac705β peptide interacts only with the hydrophobic core. This fact is proved by the decrease of the conformational order of the DPPC acyl chains without significant change in the membrane interfacial region upon Lac705β peptide addition (27). The peptide tyrosine wavenumber shifting from 1515.14 cm\(^{-1}\) to 1511.5 cm\(^{-1}\) is also another evidence that the peptide change to a more hydrophobic environment (36-37). These results support the hypothesis that this peptide could insert into DPPC bilayer (38). As a consequence, the FTIR spectrum of Lac705β in the Amide I’ region reveals an important conformational reorganization upon the interaction with DPPC vesicles (Fig. 2). The shifting of the band located at 1620 cm\(^{-1}\) to 1628 cm\(^{-1}\) is notable (Table 2). Therefore, it is possible
that two types of Lac705β aggregate exist, one in aqueous medium with a band at 1620 cm\(^{-1}\) and the other one at 1628 cm\(^{-1}\) in a hydrophobic environment due to different hydrogen-bond patterns (35). Bands around 1625 cm\(^{-1}\) have been associated with intra-molecular interactions, such as monomer-monomer contacts (39). It has also been described in membrane proteins that helix-helix interaction can occur through a surface similar to an intramolecular beta-sheet (40), which would agree with the observed increase in the 1628 cm\(^{-1}\) band. Even though Lac705β peptide is relatively short and may not be able to completely span a bilayer, its aggregation into the membrane can form oligomers involved in the membrane permeabilization (35).

On the other hand, in aqueous medium the main component of the Amide I´ suggests that Lac705β adopts a mainly unordered structure. However, bands corresponding to \(\alpha\)-helix have been observed. These results are in good agreement with previous circular dicroism studies on plantaricin E/F and J/K and lactococcin G, bacteriocins which share the same subclass of lactocin 705. The above peptides showed an unstructured conformation under aqueous conditions but, in the presence of trifluoroethanol and micelles of dodecylphosphocholine, they adopted some \(\alpha\)-helical structure (41-42). Regarding Lac705α, the peptide was not able to induce any modification in the hydrophobic region of the membrane or tyrosine wavenumber shift. However, it induces strong dehydration of the carbonyl region, suggesting that Lac705α can play interfacial interactions. Moreover, the spectrum of Lac705α in the Amide I´ region did not show any conformational reorganization upon the interaction with DPPC vesicles (Fig. 2). Thus, we suggest that this peptide could interact with the surface without penetration into zwitterionic bilayer. Lac705α as well as Lac705β peptide remains essentially in random conformation upon heating up to 80°C. This property has direct
implications on the potential use of these antimicrobial peptides as food preservatives. It is interesting to note that purified lactocin 705 and the synthetic peptides, Lac705α and Lac705β, have the same minimal inhibitory concentration (40 nM) suggesting that the peptides are correctly ensemble *in vitro*.

There are three models to explain the antimicrobial activity of peptides: the toroidal pores, the barrel stave and the carpet-like structures. Bacteriocins class II may function by creating barrel staves-like pores or a carpet mechanism (43). In the barrel stave model, the attached peptides aggregate and insert into the hydrophobic core of the bilayer. On the contrary, in the carpet mechanism, the peptides do not aggregate but bind to the surface of the lipid bilayer to form a closely packet layer or “carpet” of peptide, which renders the membranes permeable (44). Regarding Lac705α and Lac705β peptides, interfacial and hydrophobic interaction occurs, thus the carpet-like mechanism does not explain our results. Taking together the results presented in this work, it is possible to hypothesize the differential roles of the two component peptides of lactocin 705 in the interaction with membrane model. Lac705α would dehydrate the interfacial region of the bilayer while Lac705β would insert in the hydrophobic core.

This paper show the first experimental evidence that support the hypothesis that Lac705α and Lac705β peptides could form transmembrane oligomer (pore?) responsible partway of the previously reported bactericidal effects induced by this bacteriocin in sensitive cells, such as increase in the permeability, efflux of ions and changes in the membrane potential and pH gradient (13).
ACKNOWLEDGEMENTS. The authors are grateful to the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) PICT2003 Nº 09-13499, Argentina, for financial support of this work and to Miss Amelia Campos for the English revision of the manuscript. Rosana Chehín acknowledges a visiting fellowship from the Basque Government.
REFERENCES


Table 1. DPPC carbonyl stretching vibration in the presence and in the absence of lactocin 705

Taber n y (C=O) stretching vibration bands of DPPC at pH 7.0 for a pure DPPC SUV liposomes in the presence and in the absence of lactocin 705 in gel phase (25°C) and in the liquid crystalline phase (50°C)

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<th>DPPC + Lac705β</th>
<th>DPPC + Lac705α</th>
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<td></td>
<td>Band Position</td>
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<td>66</td>
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<td>50°C (liquid crystalline phase)</td>
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Table 2. Lac705β Amide I’ band components

Amide I’ band decomposition parameters corresponding to Lac705β peptide in D_2O buffer in the presence and in the absence of DPPC liposomes

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Table 3. Lac705α Amide I' band decomposition

Amide I’ band decomposition parameters corresponding to Lac705α peptide in D₂O buffer in the presence and in the absence of DPPC liposomes.

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FIGURE LEGENDS

**Figure 1:** Temperature dependence of the wavenumber of symmetric methylene stretching \( \nu_s [\text{CH}_2] \) vibration bands of DPPC at pH 7.0 for a pure DPPC SUV liposomes (○) and in the presence of Lac705β (●) or Lac705α (▲)

**Figure 2:** FTIR absorption spectra in the Amide I’ region of Lac705β peptide (solid line) and Lac705β peptide with DPPC liposomes (dashed lines) at 25°C (A) and 50°C (B)
FIGURES

Figure 1

![Figure 1](image1)

Figure 2

![Figure 2](image2)