Disruption of *Saccharomyces cerevisiae* by Plantaricin 149 and investigation of its mechanism of action with biomembrane model systems

José Luiz S. Lopes, Thathyane M. Nobre, Álvaro Siano, Verónica Humpola, Nelma R.S. Bossolana, Maria. E.D. Zaniquelli, Georgina Tonarelli, Leila M. Beltramini

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**A B S T R A C T**

The action of a synthetic antimicrobial peptide analog of Plantaricin 149 (Pln149a) against *Saccharomyces cerevisiae* and its interaction with biomembrane model systems were investigated. Pln149a was shown to inhibit *S. cerevisiae* growth by more than 80% in YPD medium, causing morphological changes in the yeast wall and remaining active and resistant to the yeast proteases even after 24 h of incubation. Different membrane model systems and carbohydrates were employed to better describe the Pln149a interaction with cellular components using circular dichroism and fluorescence spectroscopies, adsorption kinetics and surface elasticity in Langmuir monolayers. These assays showed that Pln149a does not interact with either mono/polysaccharides or zwitterionic LUVs, but is strongly adsorbed to and incorporated into negatively charged surfaces, causing a conformational change in its secondary structure from random-coil to helix upon adsorption. From the concurrent analysis of Pln149a adsorption kinetics and dilatational surface elasticity data, we determined that 2.5 μM is the critical concentration at which Pln149a will disrupt a negative DPPG membrane. Furthermore, Pln149a exhibited a carpet-like mechanism of action, in which the peptide initially binds to the membrane, covering its surface and acquiring a helical structure that remains associated to the negatively charged phospholipids. After this electrostatic interaction, another peptide region causes a strain in the membrane, promoting its disruption.

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1. Introduction

The huge increase in the number of antibiotic-resistant bacteria calls for the development of new compounds and classes of antimicrobial agents. Antimicrobial peptides (AMPs) are natural antimicrobial compounds that have been extensively studied and suggested as a potential tool for overcoming microbial resistance to conventional antibiotics. Particular attention has been given to their application in both the food industry and pharmaceuticals, since AMPs seem to play important roles as natural food preservatives and as the mechanism of action with biomembrane model systems.

Previous studies have described the inhibitory activity of Pln149a against *Listeria* and *Staphylococcus* strains and its interaction with AOT (sodium-bis-(2-ethylhexyl)-sulfosuccinate) reverse micelles as a model for cell membranes [9]. In the present work, these studies have been extended to elucidate the mechanism by which Pln149a...
2. Materials and methods

2.1. Materials

Reagents and solvents used in synthesis, purification and measurements were P.A. grade. All phospholipids were purchased from Avanti Polar Lipids.

2.2. Peptide synthesis and purification

Plantaricin 149 analog (Pln149a) was manually synthesized on solid phase by Fmoc chemistry, using Rink Amide (4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl phenoxy) resin to prepare the C-terminal peptide amide. N-Fmoc-protected amino acids were from NovaBiochem. Couplings were performed by TBTU ([N,N,N′-dimethoxyphenyl-Fmoc-aminomethyl phenoxo) resin] and the preparation of animal ration. Generally, the lysis is done with enzymes and/or mechanical rupture of the cells. Neither process is very economical, since they demand high amounts of enzymes (lipolytic and glycolytic) and/or mechanical energy [12]. The possibility of using an antimicrobial peptide like Pln149a to increase the efficiency and to reduce the cost of this process offers new opportunities for the application of this peptide that was once expected to be a food-borne pathogenic antibacterial agent [9].

In this sense, this study also focuses on the growth inhibition promoted by Pln149a on S. cerevisiae cultures, using scanning electronic microscopy to check the peptide’s action on the yeast. In parallel, a series of model systems were used to simulate the outer leaflet of cell membranes to investigate the interaction of Pln149a with different phospholipids in both monolayers and vesicles. Additionally, interactions with different membrane and cell wall components were also investigated with circular dichroism and fluorescence spectrosocopies in order to observe the surface properties of the lipid–water interface and the structural changes of the peptide.

2.4. Far-UV circular dichroism (CD) and secondary structural content of Pln149a

The CD spectrum of Pln149a (0.1 mg/mL) in MilliQ water was recorded from 190 to 250 nm on a JASCO J-715 spectropolarimeter (Jasco Instruments, Tokyo, Japan) as an average of 16 scans, using a 0.1-cm path length cylindrical quartz cuvette at 25 °C. The CD spectra of Pln149a incubated with each different LUV were also recorded under the conditions described above. Incubation was performed in a 1:20 peptide/lipid ratio.

The estimation of secondary structure elements in the peptide structure was performed by CD spectrum deconvolution using CDPro package [13], containing the ContinLL, Selcon3 and CDSSTR programs. Additionally, the formalism of Chen et al. [14] was used to estimate the helicoid content (fHelix) based on both the ellipticity at 222 nm (θ222 nm) and the number of amino acid residues (n), as in Eq. (1):

\[
f_{\text{Helix}} = \frac{\theta_{222\text{ nm}}}{-39,500 \cdot (1 - 2.57/n)}.
\]

2.5. Steady-state fluorescence

The fluorescence emission spectra of Pln149a were obtained in an ISS K2 spectrofluorimeter (ISS Fluorescence, Analytical and Biomedical Instruments, Illinois, USA), at 25 °C using a circulating water bath (Fisher Scientific) around a 1-cm path length rectangular quartz cuvette. Pln149a (0.1 mg/mL in MilliQ water) was excited at 274 nm and the emission spectra were recorded from 290 to 450 nm. Reference spectra were recorded and subtracted after each measurement.

2.6. Surface tension and dilatational surface elasticity measurements

The kinetics of Pln149a adsorption on DPPG monolayers were examined with surface tension measurements using the axisymmetric drop shape analysis method (OCA-20 from Dataphysics Instruments GmbH, Germany), with the oscillating drop accessory ODG-20, as previously described [15,16]. DPPG solution (0.1 mM) was prepared in chloroform and methanol (4:1, v/v) and was gently spread on the surface of a Pln149a solution drop (from 0.125 to 8.25 μM) to generate a lipid monolayer. The drop was expanded up to 30 mN m\(^{-1}\) corresponding to biomembrane surface packing [17]. Subsequent changes in surface pressure due to peptide adsorption were plotted against time. The dynamic dilatational surface elasticity data were obtained after the surface tension reached equilibrium, using a periodic drop oscillation of 0.1 mm amplitude (relative area variation ΔA/A of 5.5%) and 1.0 Hz frequency. The viscous effect (imaginary elasticity) of the surface elasticity was estimated from the phase angle.
2.7. Yeast growth inhibition assays

Cultures of \textit{S. cerevisiae} were grown overnight in yeast extract peptone dextrose medium (YPED). Ten microliters of this saturated culture was used to inoculate 5 mL of YEPD medium in the presence of Pln149a (80 \(\mu\)M). A control culture was grown in the absence of the peptide. Cultures were grown at 30 °C and 200 rpm; turbidity at 600 nm was monitored at 16 and 24 h.

2.8. Scanning electron microscopy (SEM)

Cultures of \textit{S. cerevisiae} were grown in YEPD culture medium in the presence and in the absence of Pln149a (1 mg/mL). Different aliquots of these cultures were taken at 6, 12 and 24 h of incubation and observed by SEM. Aliquots of the culture (200 \(\mu\)L) were filtered through a cellulose-acetate membrane (0.45 \(\mu\)m) that was washed with 0.9% NaCl to remove sample excess, and further fixed in 2.5% glutaraldehyde for 12 h, followed by washes with 100-mM sodium phosphate (pH 7.4) and dehydration in a series of 50, 70, 80, 90, 95 and 100% ethanol. Critical point drying was promoted with HMDS (hexamethyldisilazane). Specimens on stubs were coated with gold and examined at 25 kV in a Zeiss-DSM 960 scanning electron microscope (Oberkochen, Germany).

3. Results and discussion

3.1. Pln149a synthesis and purification

Peptide synthesis produces enough amounts of peptide to be used in the investigation of its mechanism of action and activity against microorganisms and biomembrane model systems.

After manual synthesis, the peptidyl-resin cleavage had an 85% yield of the crude peptide, according to the resin capacity. On the \(\text{C}_{18}\) purification, the major peptide fraction (78% of the crude peptide) eluted at 38% of the acetonitrile gradient, as in Muller et al. [9], corresponding to purified Pln149a that was further used for structural characterization.

3.2. Pln149a action against \textit{S. cerevisiae}

Pln149a was shown to inhibit \textit{S. cerevisiae} growth by 82% in YPD medium for up to 16 h, according to the turbidity decrease at 600 nm in comparison to control culture. Even after incubation for 24 h, a 47% growth reduction was observed, showing that the peptide remained active and suggesting its probable resistance to the proteolytic action of yeast proteases present in the culture medium after 24 h of incubation.

\textbf{Fig. 1.} SEM of \textit{S. cerevisiae} cells in the presence and absence (controls) of Pln149a (400 \(\mu\)M). Controls: A, D and G, at 6, 12 and 24 h of growth, respectively. The images in B and C correspond to two aliquots of cells incubated in the presence of Pln149a taken after 6 h of incubation. As previous, the images in E and F correspond to 12 h of incubation with Pln149a, and images H and I correspond to 24 h of incubation. Bars = 5 \(\mu\)m.
Fig. 1 presents SEM images of *S. cerevisiae* cultures exposed to Pln149a and also control cultures. The action on *S. cerevisiae* cells was observed over a period ranging from 6 to 24 h of incubation. Morphological changes in the yeast cell wall could be observed in the culture with Pln149a even after 6 h of exposure. Additionally, intact cells were rarely observed after 12 h of incubation, suggesting a strong action of the peptide against the yeast cells. However, although many antimicrobial peptides act at the cell membrane level [18], it remains unclear if this is the case for Pln149a. Also, it is not known with which cellular component it interacts, since the mechanism through which many of the α-helical antimicrobial peptides (AMPs) cause cell death does not involve binding to specific receptors [19].

The observed inhibition of *S. cerevisiae* growth created interest in studying the Pln149a mechanism of action, since the mechanism proposed for Pln149a on bacteria (carpet mechanism) [9] does not explain how Pln149a would act on yeast cell walls. The *S. cerevisiae* cell wall has external and internal layers constituted of three principal components: glucan, mannan and chitin [12], and the bacterial cell surface is composed of negatively charged components, such as lipopolysaccharide and teichoic acids. Thus, because the electrostatic interaction between cationic AMPs and the negatively charged bacterial cell surface should play an important role in their antibacterial activity [20], we first investigated Pln149a interaction with some mono- and polysaccharides that present similar properties to those of the *S. cerevisiae* cell wall.

### 3.3. Circular dichroism

No conformational changes in Pln149a CD spectra were observed when incubated with a neutrally charged galactomannan, a negatively charged carrageenan, or the neutral monosaccharide N-acetylgalactosamine, which suggests that Pln149a does not interact with these polysaccharides or simple neutral monosaccharides (data not shown). Therefore, the peptide passes through the *S. cerevisiae* cell wall in a permeable way and interacts with the phospholipids from the plasma membrane, thus promoting a subsequent disruption of the yeast cell wall. Using different membrane models (constructed with the lipids DPPG, DPPC, DLPC, DMPC, DPPE, DSPE and DPPS), the interaction between Pln149a and the model systems has been investigated by CD and fluorescence spectrosopies.

Pln149a in aqueous medium shows an unordered structure in its CD spectrum (Fig. 2), with a minimum at 197 nm and a maximum at 196 nm. This result can be attributed to the negative surface of the DPPG vesicles, which provided a region where all the six basic Lys residues could be electrostatically stabilized, resulting in a strong interaction between them.

The interaction of Pln149a with the DPPS liposomes also produced no alterations in the peptide CD spectrum, and it can be assumed that this phospholipid did not provide a suitable environment for the interaction of this cationic peptide. The negative regions of the serine group (the carboxylate-end and phosphate groups) had a negative surface density, decreased by the presence of a helical element, assigned to the two negative minima at 222 nm and 208 nm and the positive maximum at 196 nm. This result can be attributed to the negative surface of the DPPG vesicles, which provided a region where all the six basic Lys residues could be electrostatically stabilized, resulting in a strong interaction between them.

A coil-to-helix conformational change can also be seen in the structure of other antimicrobial peptides, either from amphibians [22, 24] or from bacteria, like Plantaricin A [10]. These are all positively charged peptides that undergo a helical induction when interacting with anionic species.
The cationic character of Pln149a and the formation of an amphipathic α-helix structure when binding to negative phospholipids (like DPPG) have been shown to be compatible with the carpet mechanism, proposed by Shai [25], for linear amphipathic peptides. The amphipathic helix generates a polar side with a positive net charge (due to Pln149a basic residues) that permits interactions with negative species, and a more apolar environment on the other helix side, where the hydrophobic residues of Pln149a can interact favorably with the membrane aliphatic chains.

The static fluorescence spectra of Pln149a measured after excitation at 275 nm, in aqueous solution and in the presence of the vesicles, revealed the same λ_{max} at 303 nm seen for Tyr residue emission when exposed to the aqueous phase [26], which can be attributed to the N-terminal position of this aromatic residue in the peptide. A small decrease in fluorescence intensity was observed in the presence of the phospholipid vesicles. Nevertheless, it was more likely due to quenching caused by changes in the chemical environment (from water to high density) than to the peptide interaction, since it was noted for all liposomes tested (Fig. 2, inserted box).

### 3.4. Surface activity

From Fig. 3 it is apparent that Pln149a caused no surface tension (γ) variation when spread on pure water subphase, even for the highest concentration studied, 8.12 μM. This means that the peptide did not present surface activity at the bare air–water interface in the concentration range studied here. However, the kinetics of adsorption of the peptide in the monolayer showed an induced surface activity promoted by the presence of DPPG (Fig. 3). Furthermore, the curves indicated a behavior dependent on the peptide concentration. From 0.125 to 2.06 μM, the adsorption kinetics presented similar behavior, accentuated by the increasing peptide concentration. The curves are characterized by a continuous decrease in the surface tension, but the trend expected for a diffusion mechanism and the equilibrium of adsorption are not reached in 500 s. On the other hand, the curves corresponding to 4.12 and 8.25 μM of Pln149a showed different behavior. Although the surface tension variation in the initial stages was faster than the result for smaller concentrations, a steady state (γ_{eq}) was apparently reached in these cases. Moreover, the γ_{eq} values were increased as much as the concentration. This behavior was not the one expected for a simple increase in the surface density with spread concentration, which should promote a higher decrease in surface tension. Thus, to better understand the interaction of Pln149a with the biomembrane model system, the DPPG monolayer, dilational surface elasticity (E) measurements were performed.

![Fig. 4. Dilational surface elasticity for Pln149a/DPPG mixed monolayers. Measurements were taken at a frequency of 1 Hz, relative area deformation of 5.5% and temperature of 25 °C.](image)

Fig. 4 presents the E data for DPPG monolayers in the presence of different Pln149a concentrations in the subphase. For pure lipid, the obtained E value was 228.3 mN m^{-1}. Thus Pln149a decreased the E values, compared to DPPG monolayer formed at the air–water interface when present in concentrations below 2.06 μM. Similar results were observed for the AMP PGLa [27], where the structure and bending rigidity of DPPG monolayers formed at the liquid–liquid interface in the presence of the antimicrobial peptide were investigated and it was concluded that the peptide acts as a plasticizer for the membrane. However, the authors did not investigate the effect of peptide concentration for that system.

Coming back to Fig. 4, although for up to 1.65 μM of Pln149a, E increased only slightly and seemed to reach a plateau, a steep increase was observed above 2.5 μM. This change in behavior with concentration agrees with the adsorption kinetics data, for which a change was also reported above 2.06 μM. At 2.5 μM, a maximum was reached, surpassing the value for pure DPPG monolayer. After this point, an inversion in the behavior of the system was observed, and E values started to decrease, indicating an increase in the fluidity of the model membrane.

Different studies in the literature have reported the effect of an antimicrobial peptide on membrane fluidity. It has been observed that oleuropein [28] expands different lipid monolayers, including polar lipids extracted from E. coli, suggesting that it is incorporated into the lipid monolayer. This assumption is corroborated by the surface elasticity data that presented lower values for the peptide. Another antimicrobial peptide, dicynthaurin [29], expands the DPPG monolayer because it accumulates on the monolayer. Furthermore, the presence of cynthaurin destabilizes the membrane model even at higher surface pressure values (30 mN m^{-1}) [17], as confirmed by GIXD and epifluorescence microscopy, and the fluidization of the condensed chain lattice suggests that the peptide is able to expand the bacterial membrane, which would be relevant for the in vivo mode of action.

In Fig. 5, a model of the interaction between Pln149a and the DPPG monolayer, showing the induction of structural changes in the peptide structure, followed by the monolayer disruption is proposed. Firstly, the disordered structure-like peptide in bulk solution is electrostatic attracted to the charged phospholipids, promoting a peptide accumulation on the monolayer surface. The amphipathic peptide remains associated with the negatively phospholipids by its hydrophilic region.
and, at the same time, the N-terminal residues (its more hydrophobic region) is inserted among the acyl chains of the phospholipids, altering the surface packing of the monolayer and promoting its disruption.

4. Conclusions

The observed action of Pln149a against S. cerevisiae suggests additional potential applications for this peptide in the food industry or as a non-enzymatic/non-mechanical process for disrupting cells.

Only the negatively charged DPPG was able to induce structural changes in Pln149a, as shown by the CD spectra changes recorded for DPPG LUVs in the presence of Pln149a. In contrast, the spectra recorded for the unordered Pln149a in solution and for liposomes prepared with DPPS, DPPE and DPPC were quite similar. Therefore, stabilization of the helix induced on Pln149a structure appears to be an important step of the “binding” that occurs by electrostatic interactions between the positively charged residues of the peptide and the anionic phospholipid heads. In addition, it can be assumed that the helical form imposed on the Pln149a structure is proportional to the driving forces that generate the attraction between these two species. That is, the interaction depends on both the electrical charge density of the phospholipids and the special features of Pln149a segments, such as the positive charge distribution and likelihood of hydrophobic portions being hidden inside the loops of the helix.

Analyzing the surface data, some important observations can be made about the mechanism of interaction between the peptide and the monolayer. Pln149a initially interacts with DPPG, adsorbing and accumulating at the lipid–air interface, as can be seen from the adsorption kinetics. This process becomes more effective as the concentration of peptide increases. On the other hand, the surface elasticity decreases, most likely because small amounts of peptide change the surface packing of the monolayer, resulting in a mixed and more fluid monolayer than that of pure DPPG. In fact, insertions of the peptide within the polar head group region should require space, disturbing the acyl chain packing.

However, increasing the peptide concentration produces a system with an elevated dilatational elasticity modulus. This finding can only be explained if we assume that the manner in which the peptide interacts with the monolayer changes. If we examine the CD data for DPPG liposomes in the presence of Pln149a, we might assume that above a certain concentration the electrostatic interaction operates cooperatively, inducing a helix structure in the peptide that remains below the head groups, shielding the electrical charge of the negatively charged head groups and bringing them closer. This picture explains the elevated η values measured for higher Pln149a concentrations. However, there is a limit for this kind of interaction, because above 2.5 μM the steep decrease in η is attributed to a disruption in the monolayer by a solubilization mechanism.

A similar mechanism probably takes place on the yeast cells (as observed in Fig. 1), in which the yeast membrane cell disruption leads away the wall cell disorganization, generating the aggregated (probably membrane and wall cells) and/or deformed cells (as in boxes C, F and G).

In summary, all the results taken together have led us to conclude that Pln149a is an amphipathic α-helical antimicrobial peptide that initially binds to the surface of negatively charged membranes, ultimately covering it in a carpet-like manner. Once the critical concentration of Pln149a is reached (close to 2.5 μM), the lipid layer is disrupted.

To better describe the mechanism of action of Pln149 in biologic membranes, its action against different classes of microorganisms, as well studies of other Pln149-derived peptides, with the aim of obtaining a peptide with potential activity are in current investigation in our group.

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References


