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ORIGINAL ARTICLE

Labaditin, a cyclic peptide with rich biotechnological potential: preliminary toxicological studies and structural changes in water and lipid membrane environment

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Abstract Cyclic peptides isolated from the plants of the *Euphorbiaceae* family have been largely studied due to their rigid conformation, which is considered significant for biologic activity. The peptide Labaditin (L_0) and its open chain analogs (L_1) were synthesized by the solid-phase peptide synthesis technique (Fmoc/tBu), and purified to elucidate its interaction with membrane models. A shift in λ_{max} emission and Stern–Volmer constants values indicate that both tryptophans migrate to a more apolar environment, with L_1 decreasing less than L_0. A circular dichroism (CD) study revealed that L_0 was kept unstructured in aqueous media as much as in the presence of dipalmitoilphosphatidylcholine liposomes. The thermodynamic studies by differential calorimetry (DSC) show a ΔH increase (50 and 18 kcal/mol, for L_0 and L_1, respectively) with peptide concentrations, which is indicative of lipids associating with peptides, resulting in the inability of the lipids to participate in the main transition. Therefore, all CD, DSC, and fluorescence data suggest a greater L_0 membrane insertion. A probable mechanism for Labaditin interaction is based initially on the hydrophobic interaction of the peptide with the lipid membrane, conformational change, peptide adsorption on the lipid surface, and internalization process. Peptide's antibacterial effect was also evaluated and revealed that only L_0 showed reduction in viability in Gram-positive bacteria while no effects to the Gram-negative.

Keywords Labaditin · Peptide synthesis · Liposome · Fluorescence · Antibacterial activity · Membrane interaction

Introduction

Labaditin (VWTVWGTIAG) is a cyclic peptide (CP) found in superior plants of the *jatropha* genus of the *Euphorbiaceae* family, popularly known as *Jarak gurita* (Indonesia) and *Mana* (Philippines). Phytochemically, this genus is known for having numerous classes of secondary metabolites such as phytosterols, flavonoids, alkaloids, terpenes, lignins, and, more recently, the above-mentioned cyclic peptides (Tan and Zhou 2006; Zhang et al. 2009). These molecules isolated from the vegetal biomass present antimicrobial, antitumor, cytotoxic effects, and anti-HIV activities (Craik et al. 1999; Daly et al. 1999; Menezes and Jared 2002; Aké et al. 2004; Reddy et al. 2004).

The cyclic peptides belong to the *Caryophyllaceae* group (Tan and Zhou 2006; Zhang et al. 2009). One of the structural characteristics of these CP is the presence of 7–10 groups with a high proportion of hydrophobic amino acids (Auvin et al. 1997). Biobollein (9 residues) and Labaditin (10 residues), isolated from *Jatropha Multifida*,

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were the first CPs of this group to be described in literature (Kosasi et al. 1989; Tan and Zhou 2006).

Another important characteristic of the Labaditin peptide is the amino acid composition since it has two tryptophan residues in its structure. According to Ridder et al. (2005), tryptophan has a fundamental role in the molecule's interaction with the biological membranes. According to these authors, the substitution of this residue for another one, also hydrophobic, significantly decreases the interaction of the peptide with the membrane.

Therefore, due to their great interaction ability with the membrane (Xiao and Pei 2007), these peptides have attracted a lot of biological and biotechnological interest, although their therapeutical application can be associated with some problems such as low water solubility leading to low absorption, bio-availability, and high sample aggregation. In order to overcome the low solubility of some drugs, some organic solvents are used in these compounds' formulation, such as ethanol or yet compounds derived from polyethoxylated ricinus oil (Torchilin 2004).

The nanotechnological insights for the use of Labaditin as a pharmaceutical model is its encapsulating into liposomes structures, which will guarantee preservation of the solubility mediated for biological systems and drug delivery when associated with some biomarkers, such as tumoral antibodies for example. But in order to have this development, it is necessary to know this CP's action mechanism, toxicity, preferential target, etc.

The other important biotechnological characteristic is the cyclic chain of these peptides which guarantees more resistance to the proteolytic degradation than its linear analogous, due to the lack of N or C-terminal groups and reduced conformational freedom (Xiao and Pei 2007). Besides that, the lower entropy associated with this greater rigidity makes these molecules have a higher affinity to and specificity with receptors (Rezai et al. 2006a, b; Xiao and Pei 2007).

Based on the biological activities exhibited, Labaditin can be considered as an interesting prototype in the development of new drugs. Allied to the peptides synthesis techniques in solid phase, the study of small cyclic peptides has shown to be a powerful approach, mainly due to the easiness of obtention and to the diverse structural modifications possible in the original peptide chain, allowing the development of more bioactive molecules. One of the feasible strategies in order to know more details on the action mechanisms and the peptide compounds interactions is the study with natural and artificial membrane systems (Nakamura et al. 1998; Anderluh et al. 1999). The knowledge of the structure–activity relationship of Labaditin and its analogous is an important factor to unveil the most common form the molecule assumes while exercising its activity (Auvin-Guette et al. 1999), because its action mode has not been elucidated yet. This is possible due to the high sensibility of the indol fluorophore to the environment surrounding it (Chattopadhyay and London 1987; Chen and Barkley 1998; Powl et al. 2003).

Differential scanning calorimetry (DSC) has also been applied to probe the thermodynamic profiles of the lipid assemblies and the consequences of peptide interactions in terms of ordering and cooperative properties of the bilayers (Lohner et al. 1999). These studies will be done and evaluated in different conditions, including interactions with natural (erythrocyte) and artificial (liposomes) membrane systems in order to understand the possible action mechanism of these peptides.

Besides the interaction and structural modification systems, the antibacterial activity in Gram-positive and -negative microorganisms was evaluated, objectifying new possibilities of therapeutical applications through a nano-biotechnological approach.

Materials and methods

Peptide synthesis, cyclization, and purification

The L_1 peptide (VWTVWGTIAG) was manually prepared by solid-phase peptide synthesis (SPPS) as previously described by Merrifield (1963) according to the standard Nα-Fmoc protecting-group strategy (Atherton and Shepard 1989) using the experimental steps described in Castro et al. (2009). After cleavage, L_0 was obtained by L_1's cyclization (Baraguey et al. 1998). The peptides L_0 and L_1 were purified by semi-preparative HPLC using a reverse phase C_{18} column and identified by electrospray mass spectrometry. All the details of this section are available in the Supplementary Material.

Liposome preparation

Large unilamellar vesicles (LUV) constituted by 1 mg/mL of dipalmitoilphosphatidylcholine (DPPC) were prepared by the extrusion method, using the experimental procedure described by Simão et al. (2010).

Dynamic light scattering measurements

Determination of size distributions (liposomes or aggregates) were carried out by dynamic light scattering (DLS), using a N5 Submicron Particle Size Analyzer (Beckman Coulter, Inc., Fullerton, CA, USA). The average value diameters were obtained from the unimodal distribution.

Circular dichroism (CD) spectroscopy

The CD spectra were recorded at 25°C with a Jasco 810 spectropolarimeter, purged with nitrogen gas, and with a 0.1 cm quartz cuvette. Initially, the L_0 and L_1 peptides concentration effect was evaluated by varying the peptide concentration from 5 to 270 μM, in aqueous media, pH 7.0. Each Peptide solution (L_0 and L_1), in different concentrations and separately, were incubated for 2 h with DPPC constituted liposomes up to 14 lipids for each peptide (mol ratio). The spectra were recorded using a 1-nm spectral bandwidth, 10 nm/min scan speed, 2 s response time with six accumulations, and a wavelength range of 250–190 nm was chosen to minimize the noise and error during the measurement. Typically, the CD spectra showed in the figures correspond to the average after buffer (or LUV) spectra subtraction and baseline correction.

Tryptophan fluorescence spectroscopy assays

Fluorescence measurements were made with a Spectronic SLM 8100 spectrofluorometer equipped with a single-grating emission monochromator and a double-grating excitation monochromator. Excitation was carried out at 280 nm to irradiate tryptophan groups, and emission spectra were measured from 300 to 500 nm, at 25°C and pH 7.0. Quartz cells with optical paths of 0.5 cm were used.

Fluorescence intensity and maximum wavelengths (λ_{max}) were obtained for each peptide (L_0 and L_1) varying the concentration from 2 to 73 μM in aqueous media and also in the presence of DPPC liposomes (100 μL). The corresponding base line obtained in the same conditions but without the peptide was always previously subtracted from the peptide spectrum.

Structural alterations in peptides due to their binding or interaction with liposomes were monitored by quenching of tryptophan fluorescence with acrylamide in the presence and absence of liposomes according to the Stern–Volmer equation:

$$F_0/F = 1 + K_{sv} \times [Q],$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, $[Q]$ is the concentration of quencher, and K_{sv} is the Stern–Volmer quenching constant. Fluorescence quenching measurements of tryptophan with acrylamide quencher were made by serial addition of small aliquots (5 μL) of concentrated acrylamide stock solution (6 M) in a cuvette containing a fixed concentration of 50 μM of the peptide solution, alone or in the presence of liposome, in a final molar ratio about 14 lipid/peptide (L/P), up to a final concentration of 1 M acrylamide.

Differential scanning calorimetry assay

Transition phase temperatures (T_c) of the DPPC-LUV membranes prepared in the absence or presence of peptide (L_0 and L_1 in a 0–50-μM range concentration) were studied by DSC. All LUV suspensions and reference buffer were degassed under vacuum (140 mbar) for 30 min prior to use. The samples were scanned from 20 to 65°C at an average heating rate of 1°C/min, and the recorded thermograms were analyzed using Nano-DSC II software (Calorimetry Sciences Corporation, CSC 6100, UT, USA).

Samples containing the peptide alone (100 μM), dissolved in the same buffer, exhibited no thermal events over the temperature range of 0–100°C. This indicates that the endothermic events observed in this study arise solely from phase transitions of the DPPC vesicles. A minimum of at least three heating and cooling scans were performed for each analysis, and all thermograms was reproducible. In order to ensure homogeneity in the analysis of the effect of the selected peptides on the lipid phase transitions, we have chosen the simplest baseline correction to introduce the least amount of variability when comparing thermograms from different sets of experiments.

Peptide antibacterial effect

The antibacterial test was done with both L_1 and L_0 peptides using the following bacteria lineages: Gram-positive (*Streptococcus mutans*) and Gram-negative (*Aggregatibacter actinomycetemcomitans*) obtained and cultivated according to Paulino et al. (2005) and Goulart et al. (2009), respectively.

Before the incubation with different concentrations of peptide (0.5 and 100 μM), the cells ($A_{600nm} = 0.5$) were serially diluted in Tryptic Soy Broad (TSB) medium to obtain 10^3 Colony Forming Units (CFU)/mL and distributed (1 mL) in assay tubes (125 × 15 mm). One set of tubes was submitted to a control cell experiment without any peptide addition.

Cells were grown by dropping and spreading 50 μL of previously diluted cell suspension directly onto Tryptic Soy Agar (TSA), and incubating it in a candle jar for 72 h at 37°C. After this period, the CFU/mL was calculated (Goulart et al. 2009). All experiments were done in triplicate.

Peptide hemolytic activity

Erythrocytes were isolated from human freshly collected blood. Blood was centrifuged (4,730×*g* by 15 min at 4°C) in sodium citrate 3.8% (1:4 v/v), and the sediment was washed over three times in this same solution. It was then resuspended in saline solution PBS (10 mM Na_2HPO_4,

2 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.4). Erythrocytes (2×10^7, determined by Neubauer chamber) were incubated in PBS buffer and with different peptide concentrations (previously determined) for 30 min at 37°C. The solution was then centrifuged (4,730×*g* for 10 min, 4°C) to remove the lysed cells. Hemoglobin release, determined through supernatant spectrophotometer absorbance at 414 nm, was correlated with the lysis of erythrocytes. Cells' maximum lysis (100%) was attained by the incubation of an erythrocyte suspension with 0.1% (v/v) of Triton X-100. All experiments were done in triplicate.

Results and discussion

Study of peptide interaction with lipid interface

Peptides were found to have limited solubility in water (maximum about 270 μM), and this has also raised the possibility that the peptide is in an aggregated state in solution at the low concentrations employed for the CD measurements (Yang et al. 1994). The L_0 and L_1 peptide solutions were also analyzed with the light scattering technique. It was possible to evidence the presence of structures as high as 100 nm, with high polydispersion index, for both peptides, indicating the formation of aggregates in these conditions. In addition, it should be noted that up to 50 μM no scattering was observed for both peptides, but the formation of complex by peptide–peptide interaction was not discarded (data not shown).

The concentration dependence of the far-UV CD spectrum of the peptides L_0 and L_1, pH 7.0, were investigated, and essentially, the concentration range was from 30 to 270 μM (Fig. 1). The spectrum obtained presented increase of ellipticity intensity [θ] according to the increase in the concentration of L_0 peptide in 205 nm. No band is attributed to the presence of interaction between tryptophan's side chains, in part, that can be attributed to the cyclic conformation which significantly restricts changes between chains (Fig. 1a). For L_1, a minimum at approximately 197 nm was observed, with increase of ellipticity intensity according to the increase in the concentration of peptide, and a maximum at 224 nm, with decrease of ellipticity intensity according to the increase in the concentration of peptide (Fig. 1b). By increasing the peptide concentration, the band which refers to the side chain interactions of tryptophan becomes less intense, evidencing the decrease of intermolecular associations verified by the positive band around 220–320 nm (Grishina and Woody 1994; Woody 1996; Andersson et al. 2001). This CD spectrum obtained for L_1 suggests that structuring of the peptide involves at least some degree of intermolecular association (Yang et al. 1994). A possible explanation for this behavior can be given analyzing the

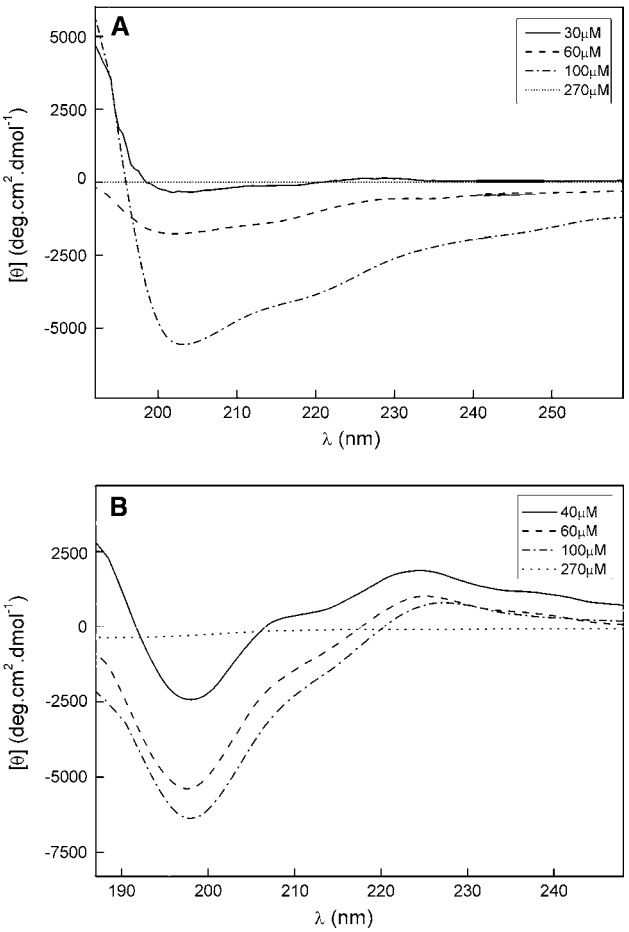

Fig. 1 Circular dichroism spectrum of L_0 (**a**) and L_1 (**b**) peptides in concentrations from 5 to 270 μM, in aqueous media, pH 7.0, as described in "Materials and methods"

peptide's isoelectric point (pI). The theoretical pI of L_1 is 6.0, close to the pH studied, which justifies the observed behavior, because with zero charges and in high concentrations, these molecules are more susceptible to intermolecular aggregations due to less repulsion. The same applies to L_0 since it does not present charged groups in its structure, as well as C- and N-terminal groups, which are compromised in cyclization.

The structural changes due to peptide–membrane interactions are essential to understand the action and regulation mechanism of the biological activity of molecules (Kelly et al. 2005). The effect of lipids on the backbone conformation of L_0 and L_1 peptides was studied by far-UV CD spectroscopy. The CD spectrum of the peptide L_0 in water, pH 7.0, has a minimum at approximately 207 nm and maximum at 190 nm, characteristic of an unordered structure (Yang et al. 1994). Addition of DPPC liposome to the solution of L_0 resulted in change in the peptide CD spectrum (Fig. 2a). The effect was concentration dependent, and the spectrum of L_0 in the presence of DPPC, in different peptide/lipids molar ratios (*P*/*L*), has a minimum

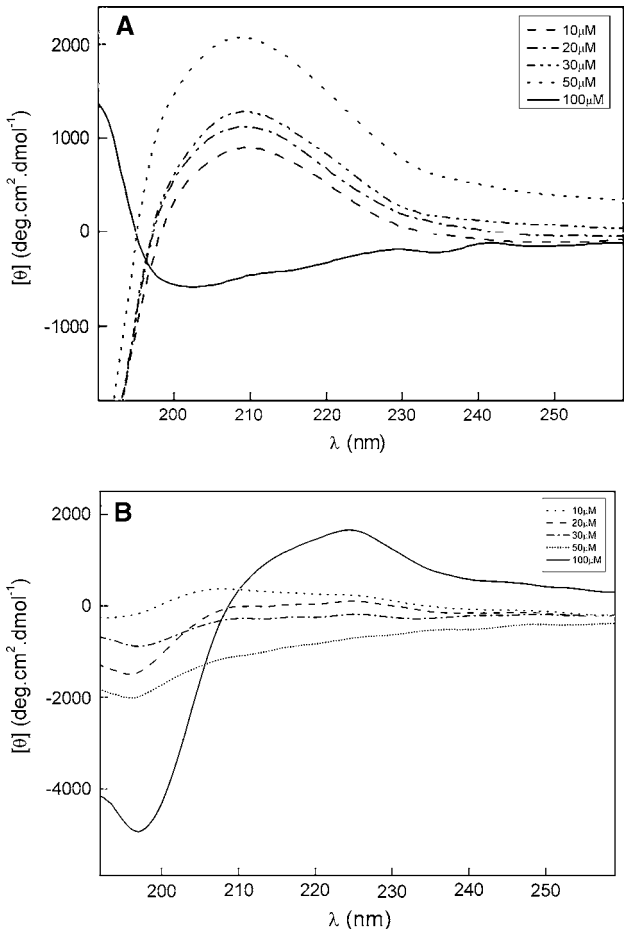

Fig. 2 Circular dichroism spectrum of the L_0 (**a**) and L_1 (**b**) in different concentrations (from 5 to 100 μM) and separately, in aqueous media, pH 7.0, incubated for 2 h with DPPC liposomes up to 14 lipids for each peptide (mol ratio), and the CD spectrum was recorded as described in "Materials and methods"

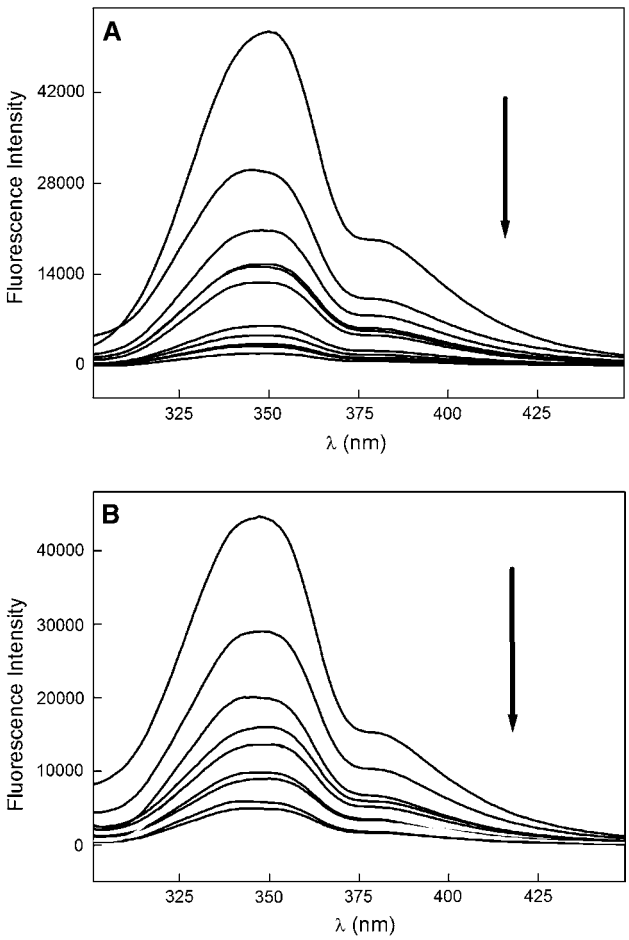

Fig. 3 Peptides fluorescence emission spectra of different concentrations (2–73 μM): **a** L_0 and **b** L_1 in the presence of DPPC liposome, according to "Materials and methods". The *arrow* indicates an increase of peptide concentration in solution

at approximately 190 nm and a maximum at 210 nm, the characteristic features of a *β*-structure (Yang et al. 1994). An increase in magnitude of the ellipticity according to *P/L* ratio increase also showed, possibly due to the saturation of the peptides on the lipids layer.

The CD spectrum of the peptide L_1 in water, pH 7.0, has a minimum at approximately 197 nm and maximum at 224 nm, characteristic of a random coil structure with interactions among molecules of tryptophan (Yang et al. 1994; Andersson et al. 2001). Addition of DPPC liposome to the solution of L_1, in different peptide/lipids molar ratios (*P/L*), resulted in change in the peptide CD spectrum to a minimum at approximately 195 nm and the disappearance of the maximum at 224 nm (Fig. 2b). This is probably due to the interaction of the peptide with the lipid layer, which leads to the separation of the tryptophans (Sforça et al. 2005; Woody 1996; Kelly and Price 2000; Andersson et al. 2001).

A similar result was obtained with the A*β* (1–40) peptide, where a fast change in CD spectrum was observed, in the presence of the ganglioside lipid; it changed from random coil structure to *β*-structure (Choo-Smith and Surewicz 1997). Another study with a 21-residue peptide, derived from a human prion protein, showed a conformational change from *α*-helix to *β*-structure after interaction with SDS micelles (Kelly and Price 2000).

The tryptophan amino acid was used as a probe to analyze the local environment and the influence of the N-terminus modifications (Ladokhin et al. 2000). The presence of a tryptophan residue allows the study of the conformational changes that can occur in these molecules. The greater or smaller proximity of the side chains and terminus ionizable groups of the peptide with this residue can affect the fluorescence intensity (Van der Wel et al. 2007) by the quenching process. This amino acid also can be used as a probe to analyze the local environment; it

allows the evaluation of the migration from one environment to the other. These studies are possible due the variation of fluorescence emission spectroscopy and fluorescence quenching (Park et al. 1995; Ladokhin et al. 2000).

DPPC–liposomes interaction was studied with different peptide concentrations. As can be seen in Fig. 3a, the increase of L_0 and L_1 concentration results in the decrease of fluorescence intensity. This reduction in fluorescence intensity can be attributed to the conformational changes suffered by the peptide. The modification in structure can promote a greater approximation of the side chains of some amino acids with the indol group of tryptophan, acting as suppressors and affecting this parameter (Chen and Barkley 1998; Ladokhin et al. 2000). It is important to note that only one maximum was found in the spectra, indicating that both tryptophans have the same behavior in this membrane mimetic, probably due to their proximity and/or strong interaction between them.

It should be noted that the fluorescence spectra have also revealed a blue shift of λ_{max} emission for the L_0 peptide as much as for the L_1 (Fig. 4). Initially, both peptides presented a discrete reduction in λ_{max} (L_0 reduced 5 nm and L_1 only 3 nm) for the mol ratio *P*/*L* of around 0.005. This value remains constant with the increase of the *P*/*L* ratio to 0.009 and 0.011, for L_0 and L_1, respectively. After this baseline, a new reduction in λ_{max} was observed, reaching a new level at 339 and 344 nm for L_0 and L_1, respectively. This behavior of λ_{max}, apparently in two stages, followed by the reduction in fluorescence intensity with the increase of *P*/*L* ratio, can be correlated with distinct phenomena that occur during the interaction of the peptide with the lipid present in liposome. Therefore, in small *P*/*L* relationships, as the first step, the peptide probably undergoes an aggregation stage in contact with the lipid membrane, which results in its adsorption in the interface (when they are present in the interface at a ratio of approximately one peptide for every 14 lipids). This interpretation is associated with the low value of fluorescence reduction and λ_{max} that is attributed to the tryptophan migration to a more apolar environment (Christiaens et al. 2002; Lakowicz 2006). In the second step, due to the increase in the peptide adsorption in the lipid interface, possibly, a redistribution of the peptides in the more internal layer is occurring. Hence, the peptide that is probably more immersed in the liposome is L_0 because it has the lowest λ_{max} emission (L_0 339 nm and L_1 344 nm).

It should be highlighted that the susceptibility of the bilayer depends on the peptide concentration on the membrane surface, which is, when a limit ratio between the peptide and lipid concentration is reached; it leads to the immersion on the lipid bilayer, depending on its composition (Huang 2000). Besides that, the phospholipid composition of the membrane, and its size and charge can influence in the orientation, insertion and depth of the peptide in relation to the membrane surface (Deber and Li 1995).

The fluorescence quenching can be numerically measured from the obtention of the Stern–Volmer (K_{sv}) constant for each peptide, in a lipid microenvironment as in an aqueous environment. K_{sv} is a parameter obtained from the quenching tangent curve, given by the fluorescence

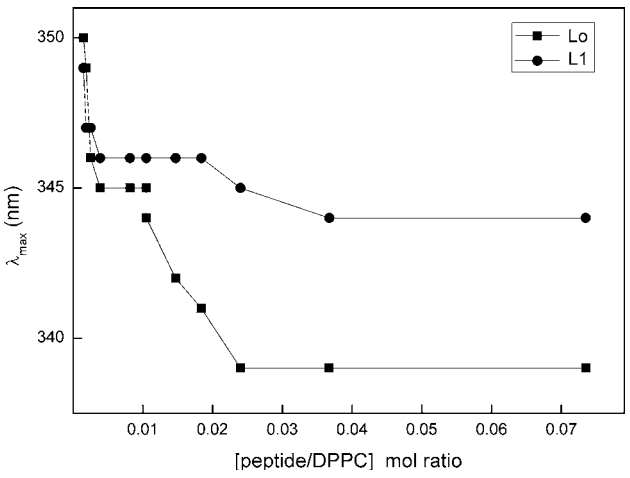

Fig. 4 Peptides λ_{max} emission variation of L_0 and L_1 studied in aqueous media alone, or in the presence of DPPC liposomes, according to "Materials and methods"

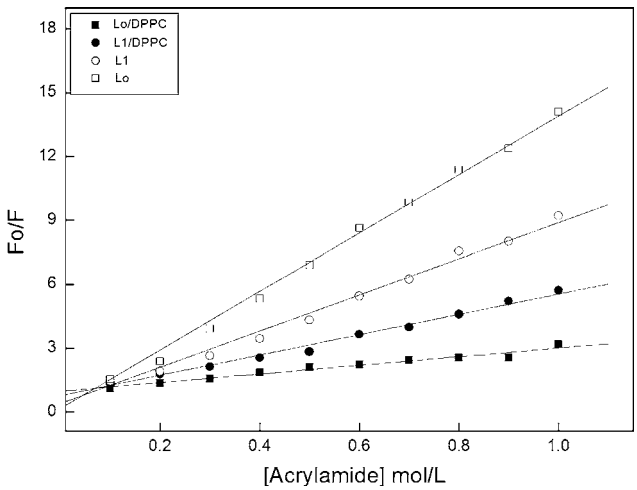

Fig. 5 Fluorescence quenching (F_0/F) of the Tryptophans present in L_0 (*squares*) and L_1 (*circles*) in the absence (*open symbols*) and in the presence (*full symbols*) of DPPC liposomes, pH 7.0, with acrylamide quencher. These experiments were made by serial addition of small aliquots (5 μL) of concentrated acrylamide solution stock (6 M) in a cuvette containing a fixed concentration (50 μM) of peptide solution alone or in the presence of 14 lipid/peptide ratios, up to a final concentration of 1 M acrylamide, according to "Materials and methods"

Table 1 λ_{max} and K_{sv} values of L_0 and L_1 peptide in the presence (+) or absence (−) of DPPC liposomes

Peptide	DPPC liposomes	λ_{max}	K_{sv} (M^{-1})
L_0	(+)	339	2.02
	(−)	350	13.7
L_1	(+)	345	4.8
	(−)	349	8.5

intensity initial value in relation to the punctual values obtained (F_0/F) in the presence of increasing concentrations of acrylamide (Deva and Behere 1999; Eftink and Ghrion 1976; Christiaens et al. 2002; Kelkar and Chattopadhyay 2006; Lakowicz 2006; Sahu and Behera 2008).

As can be seen in Fig. 5, both peptides show tryptophan fluorescence quenching by the acrylamide—fluorescence intensity decreases. This reduction of fluorescence can be due to a higher exposition of tryptophan in the apolar media. The linear curve obtained in this study probably indicates that both tryptophans have the same quenching by acrylamide and the same insertion in the mimetic membrane.

In another experiment, the fluorescence quenching was also done with the L_0 and L_1 peptides, separately, in aqueous solution, yielding information about the tryptophan exposition level in the absence of liposome (Fig. 5). All the K_{sv} constants determined in the presence and absence of liposomes are summarized in Table 1. We can observe that peptides have shown a greater quenching in aqueous medium (higher values of K_{sv}) than in the presence of DPPC liposomes (lower values of K_{sv}) due to a greater exposition of tryptophan when in solution. Thus, we can conclude that the L_0 peptide interacts more with the lipids present in liposomes than L_1 peptide because it has a lower K_{sv}.

It should be noted that each of the two peptides studied (L_0 and L_1) have two tryptophan residues in their structure; however, through this quenching experiment it was not possible to differentiate them because we have obtained only one line in the regression value of F_0/F. This can be explained by the fact that both tryptophans have similar behavior or yet, due to a limitation of the technique that does not allow us to differentiate them.

In the DSC study, the disturbances undergone by the lipids present in DPPC liposomes in the presence of L_0 and L_1 peptides were observed. Surface adsorption, membrane insertion, and peptide-specific bonding are frequently followed by system energy changes that can be conveniently measured by the DSC technique. For this study, a membrane model DPPC was used, and in Fig. 6, the thermograms in the absence or presence of L_0 and L_1 peptides are respectively shown.

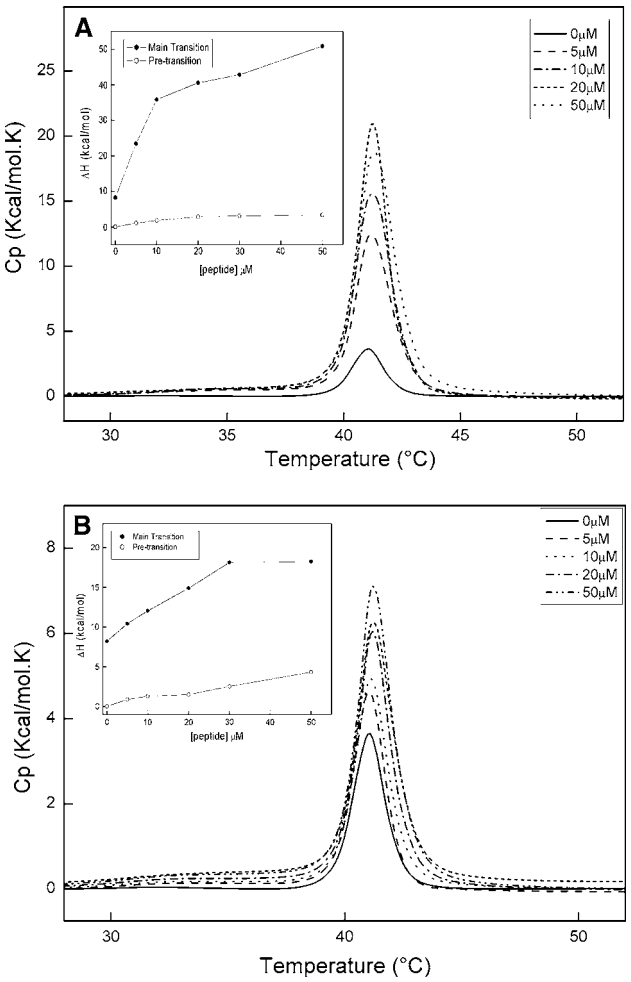

Fig. 6 DSC thermograms of DPPC bilayers in the presence of increasing concentrations of **a** L_0 and **b** L_1 determined using a Nano-DSC II (CSC), according to "Materials and methods". *Inset* enthalpy variation $-\Delta H$ (kcal/mol) of the pre-transition and main transition of DPPC against L_0 and L_1 concentration, respectively

The DPPC typically presents two transition temperatures: the pre-transition at T_c 31.75°C and the main transition to the liquid-crystalline phase at T_c 41.03 (Pedersen et al. 2002; Sospedra et al. 2002; Dave et al. 2005; Kun et al. 2008).

In DPPC liposomes, both L_0 and L_1 had minimal effect on the main transition temperature T_c (for DPPC the temperature of the transition between lamellar gel, P_β, and liquid crystalline phase, L_α) at around 41°C. A slight increase was observed in the temperature of the pre-transition (lamellar tilted gel, L_β, to the ripple phase, P_β), 31.7–33.9°C (L_0) and 31.7–33.1°C (L_1), indicating a slight stabilization of the L_β over the P_β phase, while L_1 decreased cooperativity more than L_0 (indicated by the increase in half-width) of the pre-transition (Fig. 6).

Moreover, for both peptides, ΔH increased with increasing peptide concentrations, which is indicative of

lipids associating with peptides or greater lipid packing and/or lipid sequestering, resulting in the inability of the lipids to participate in the main transition (Inset of Fig. 6). This variation was bigger for L_0 peptide, increased to 50.8 kcal/mol, while L_1 increased to 18.2 kcal/mol (Inset of Fig. 6).

In a study done with DPPC and alamethicin, an antimicrobial peptide, through DSC, a great increase of the peak after the peptide–lipid interaction was observed and conferred to the peptide–lipid interaction (Dave et al. 2005). In another study, done with cyclic antimicrobial peptide rhesus theta defensin (RTD-1) and open chain analog (oRTD-1), the peptides were not able to notably alter the phase transition properties of DPPC. They decreased the cooperativity of the pre-transition, resulting in a very flat wide peak (Abuja et al. 2004), similar to what L_0 and L_1 peptides had shown.

Antibacterial and hemolytic effects of peptides

After the advent of high-throughput experiments and post-genomic technologies, more than 80% of drug substances were natural products or inspired by a natural compound. This fact illustrates the importance of natural products for human health (Calderon et al. 2009). The modern nanobiotechnology considers the design and engineer modifications of bioactive peptides for new antibiotic formulations (Thennarasu and Nagaraj 1996). Thus, the cytotoxic potential, in vitro, of L_0 and L_1 peptides over the bacterial lineages *Streptococcus mutans* (Gram-positive) and *Aggregatibacter actinomycetemcomitans* (Gram-negative) was evaluated, according to "Materials and methods". The results show that only the L_0 peptide had viability reduction

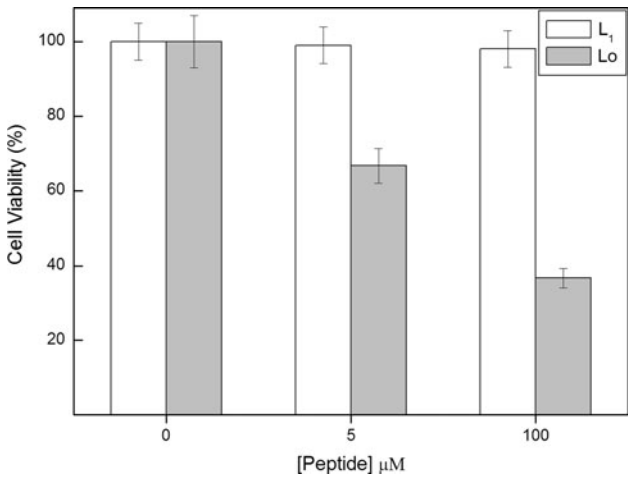

Fig. 7 Peptide toxicity of L_1 (*white bars*) and L_0 (*gray bars*) in Gram-positive bacteria culture (*Streptococcus mutans*) for 30 min. Bacteria survival percentages were obtained according to "Materials and methods"

in a concentration-dependent manner; however, for *S. mutans* there was a 25% reduction in the presence of 5 μM of the peptide and 56% with 100 μM (Fig. 7). These results indicate that L_0 kills Gram-positive bacteria membrane, and probably acts on the inner membrane.

Similar results were obtained with the linear SPFK peptide, 13 residues, in the presence of the *Escherichia coli* bacteria (Sitaram et al. 1992). Peptides derived from toxiri pardaxin (PX), 18 amino acids, exhibited antimicrobial activity against *Escherichia coli* and not against Gram-positive microorganisms (Thennarasu and Nagaraj 1996). Studies directed toward structure–function correlation with short bioactive peptides such as L_0 and L_1, which are composed of only ten residues and thus are easily obtainable by chemical synthesis, would be appropriate for possible therapeutic uses (Sitaram et al. 1992).

For the Gram-negative bacteria, no cytotoxicity was observed, neither for L_0, nor for L_1, in the concentration range up to 100 μM.

L_0 and L_1 peptides have not shown hemolytic activity up to concentrations of 100 μM. It should be clear that for a peptide to be toxic it does not have to forcibly be hemolytic. In many cases, toxicity of the peptide is associated with the membrane rearrangement-generated processes, specific interaction, enzyme/protein membrane inactivation, phase state changes forming membrane microdomains, etc., even if it does not break its integrity. This reason can explain the fact that the peptide is toxic only to Gram-positive and not to Gram-negative bacteria.

Similar results have been described for peptides from pardaxin toxin and SPF fragment of the seminalplasmin peptide (SPFK). On the latter study, besides having used the same methodology, the SPF peptide is highly hydrophobic and has tryptophan in its composition, such as labaditin (Thennarasu and Nagaraj 1996; Shin et al. 2001).

Conclusion

The Fmco/tBu method was adequate for the L_1 synthesis and cyclization for the obtention of Labaditin (L_0). The HPLC purification was efficient (over 95%), and Mass spectrometry has confirmed the planned peptides. Both L_0 and L_1 interact with liposome. That was proved through the λ_{max} shift through fluorescence, the conformational change observed by CD and DSC, with a significant increase of enthalpy. Independent of the technique used, the L_0 peptide always showed greater interaction with DPPC liposomal systems than its linear analogous L_1.

By using this technique it is also possible to observe the conformational restriction influence of the cyclic peptide (L_0) when compared to the linear analogous (L_1). Hence, cyclic peptides are considered good models for the study of

the structure–interaction relationship, as already described by other authors (Rezai et al. 2006a, b; Kwon and Kodadek 2007). This is probably due to the fact that cyclization can increase permeability in the membrane eliminating terminal charges and internally favoring conformations by hydrogen bonds. Based on that, according to what was described by Seelig (2004), the adsorption and insertion into the membrane can be followed by conformational transitions, favored by, in part, a hydrogen bond system. There are two very well-known conformational transitions induced by membrane, α-helix to β-structure (Kelly and Price 2000) done by amphypatic peptides (Seelig 2004) and of random coil structure to β-structure (Choo-Smith and Surewicz 1997) done by hydrophobic peptides. A probable interaction mechanism of the L_0 peptide can be based, initially, on the hydrophobic peptide–membrane interaction. Next, the adsorption of the peptide on the lipid surface occurs. The exact location of this adsorption layer is difficult to define, depends on the forces involved. On this layer, peptides are directly in contact with the lipids, all accessible. On the third step, the bond process occurs with consequent conformational change of the peptide. This mechanism is based on the λ_{max} emission variations observed (Fig. 4), where initially, a discrete λ_{max} emission reduction occurs followed by a constant plateau, according to the increase of the *P*/*L* ratios. In this condition, the membrane adsorption should be happening. After this plateau, a new reduction in the λ_{max} emission values is observed, reaching a new plateau at 339 nm, which can be related to the insertion moment. A similar example that was well documented is the example of the Cyclosporine A hydrophobic peptide. When a cyclic decapeptide interacts with the membrane, it alters its conformation from random coil to β-structure (Seelig 2004). Labaditin shows similar characteristics, including immunosuppressant activity (Kosasi et al. 1989).

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