

The effect of acidic residues and amphipathicity on the lytic activities of mastoparan peptides studied by fluorescence and CD spectroscopy

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Abstract Some mastoparan peptides extracted from social wasps display antimicrobial activity and some are hemolytic and cytotoxic. Although the cell specificity of these peptides is complex and poorly understood, it is believed that their net charges and their hydrophobicity contribute to modulate their biological activities. We report a study, using fluorescence and circular dichroism spectroscopies, evaluating the influence of these two parameters on the lytic activities of five mastoparans in zwitterionic and anionic phospholipid vesicles. Four of these peptides, extracted from the venom of the social wasp *Polybia paulista*, present both acidic and basic residues with net charges ranging from +1 to +3 which were compared to Mastoparan-X with three basic residues and net charge +4. Previous studies revealed that these peptides have moderate-to-strong antibacterial activity against Gram-positive and Gram-negative microorganisms and some of them are hemolytic. Their affinity and lytic activity in zwitterionic vesicles decrease with the net electrical charges and the dose response curves are more cooperative for the less charged peptides. Higher charged peptides display higher affinity and lytic activity in anionic vesicles. The present study shows that the acidic residues play an important role in modulating the peptides' lytic and biological activities

and influence differently when the peptide is hydrophobic or when the acidic residue is in a hydrophilic peptide.

Keywords Mastoparans · Antimicrobial peptides · Peptide net charge · Hydrophobicity · Circular dichroism · Fluorescence spectroscopy

Introduction

Mastoparans are low molecular weight peptides, generally tetradecapeptides, extracted from the venom sac of social wasps that act in the defense system of these insects. They are rich in hydrophobic and basic residues which are distributed in the chain in such way that, in adequate environment, they form amphipathic helical structures. Due to the distribution and amount of hydrophobic and basic residues these peptides present important biological activities such as antimicrobial, mast cell degranulation and hemolytic activities (Nakajima 1986).

Many peptides of this family present the sequence INW followed by a lysine or a leucine in the N-terminus and the last two residues in the C-terminus are hydrophobic, being the last one, mainly a leucine residue (Nakajima 1986). C-terminus is in general protected by amidation, which confers helical stability due to an extra hydrogen bonding (Sforça et al. 2004) and also helps to protect against proteolytic degradation (Andreu and Rivas 1999). Some members of this family, however, present an acidic residue, in general aspartic acid, in the N-terminus instead of asparagine with those peptides containing the regular asparagine residue, show that they differentiate in these activities. The presence of the acidic residue in the N-terminus could contribute with a stabilizing effect to the helical structure by increasing the helical macrodipole and

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providing an extra hydrogen bonding or salt bridge between lateral chains specially aspartic acid and lysine at positions i and $i + 4$ or $i + 3$ (Marqusee and Baldwin 1989).

Recently a novel family of peptides with acidic residues in the N-terminus extracted from the venom sac of the wasp *Polybia paulista* was described (Souza et al. 2005, 2009). Biological activity assays with Gram-positive and Gram-negative microorganisms revealed that their MIC ranges from 6 to 62 μM against *E. coli* and from 4 to 31 μM against *S. aureus*. Like other peptides extracted from the venom sac of wasps they present degranulating activity in rat mast cell. They showed low hemolysis in rat blood cell and chemotaxis. However comparisons of the biological activities of the peptides containing sequences with aspartic acid residue in the N-terminal region instead of asparagine, show that they differentiate in these activities. The peptides with IDWL in the N-terminal showed lower degranulating and hemolytic activities than their analogs with N to D substitution, and some of them present very high antimicrobial activity (Souza 2007). Dathe et al. (2002) showed that the peptide net charge modulates both, the peptide affinity to the bilayer and the lytic activity; however, the balance of the net charge due to the presence of acidic and basic residues has not been explored. Besides the presence of the acidic residue at the N-terminus, these peptides differentiate in their hydrophobicities and angle of the polar faces, which have also been shown to act synergistically in modulating the lytic and biological activities and consequently the peptide bilayer-specificity (Taheri-Araghi and Ha 2007).

The presence of acidic and basic groups allows not only for the helical stabilization by intra-molecular salt and hydrogen bonding, but also for the formation of bridges and bonds with the lipid polar head groups (Sakai and Matile 2003; Pantos et al. 2008). Studies by molecular dynamics of a cell penetrating peptide, Tat HIV, showed that the peptide translocation is strongly influenced by these types of hydrogen bonding (Herce and Garcia 2007). Recently it was proposed that the mechanism of action of mastoparans is similar to transportan, a cell penetrating peptide, which involves the same type of translocation (Almeida and Pokorny 2009; Yandek et al. 2009).

To understand the role played by the acidic residue at the N-terminus and hydrophobicity on the lytic activities of four peptides, derived from *Polybia paulista* wasp venom extract, we explore its influence on the structural parameters, such as net charge, helicity, hydrophobic moment and broadness of the polar face, in modulating the peptides' affinity to and their lytic activity in model phospholipid membranes and searched correlations with their biological activities.

Materials and methods

Chemicals

Lipids egg phosphatidylcholine and phosphatidylglycerol were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and carboxyfluorescein (CF) from Sigma Chemical Co (St. Louis, MI, USA). Unless otherwise indicated other chemicals were of high quality analytical grade. Buffers: Tris/ H_3BO_3 5 mM, 0.5 mM Na_2EDTA , pH 7.5 for CD and Tris/HCl 10 mM, 1 mM Na_2EDTA , pH 7.5, either containing 25 mM CF (vesicles formation) or 150 mM NaCl for the leakage experiments and fluorescence spectroscopy experiments.

Peptide synthesis and purification

Polybia MP-1, Polybia MP-2, Polybia MP-3, the synthetic analog Polybia N2-MP-1 and Mastoparan (MP-X) were synthesized, as described by Souza et al. (2005, 2009) by step-wise manual solid-phase synthesis using fluorenylmethoxycarbonyl (Fmoc) strategy. The crude peptides were dissolved in water and chromatographed under RP-HPLC using a semi-preparative column, under isocratic elution with different concentrations of the mobile phase for each peptide: 60% (v/v) acetonitrile in water [containing 0.1% (v/v) trifluoroacetic] for Polybia N2-MP-1 and 38% (v/v) acetonitrile in water [containing 0.1% (v/v) trifluoroacetic] for Polybia-MP-2 and Polybia-MP-3. The elution was monitored at 214 nm with a UV-DAD detector (SHIMADZU, mod. SPD-M10A), and each fraction eluted was manually collected into 1.5 mL glass vials. The homogeneity and correct sequence of the synthetic peptides were assessed using a gas-phase sequencer PPSQ-21A (SHIMADZU) based on automated Edman degradation chemistry and ESI-MS analysis.

Mass spectrometry analyses

The homogeneity of preparations was ascertained by using mass spectrometry; samples were analyzed on a triple quadrupole mass spectrometer, model QUATTRO II, equipped with a standard electrospray (ESI) probe (Micromass, Altrincham), adjusted to ca 250 $\mu\text{L}/\text{min}$. During all experiments the source temperature was maintained at 80°C and the needle voltage at 3.6 kV, applying a drying gas flow (nitrogen) of 200 L/h and a nebulizer gas flow of 20 L/h. The mass spectrometer was calibrated with intact horse heart myoglobin and its typical cone-voltage induced fragments. The molecular masses were determined by ESI-MSI, adjusting the mass spectrometer to give a peak width at half-height of 1 mass unit and the cone sample to skimmer lens voltage, controlling the ion transfer to mass

analyzer, was set to 38 V. About 50 pmol (10 μ L) of each sample was injected into electrospray transport solvent. The ESI spectra were obtained in the multi-channel acquisition mode; the mass spectrometer data acquisition and treatment system was equipped with MassLynx and Transform software for handling spectra.

Peptide solution

Peptides were dissolved in TRIS buffer (tris/HCl 10 mM, 1 mM Na₂EDTA, 150 mM NaCl, pH 7.5 for fluorescence experiments or tris/H₃BO₃ 5 mM, 1 mM Na₂EDTA, pH 7.5 for CD experiments), under gentle agitation. The peptide concentrations were determined spectrophotometrically in 280 nm using tryptophan molar absorptivity $\varepsilon = 5570 \text{ M}^{-1}\text{cm}^{-1}$.

Vesicle preparation

Liposomes composed respectively by L- α -phosphatidylcholine (PC) and 70% L- α -phosphatidylcholine and 30% L- α -phosphatidyl-DL-glycerol (PCPG 70:30) have been prepared according to general procedures with slight modifications. Shortly phospholipids dissolved in chloroform have been dried under N₂ flow on round bottom flasks. The lipid film was completely dried under vacuum for at least 3 h and afterwards hydrated with Tris buffer (Tris/H₃BO₃ 5 mM, 1 mM Na₂EDTA, pH 7.5 or with Tris/HCl 10 mM, 1 mM Na₂EDTA, pH 7.5, either containing 25 mM carboxyfluorescein (CF) for leakage experiments or 150 mM NaCl for fluorescence spectroscopy. In both preparations the final lipid concentration was around 10 mM. The suspension was sonicated under N₂ flow, in ice/water bath, for 50 min, or until clear, to produce small unilamellar vesicles (SUVs), necessary for the CD measurements. Titanium debris has been removed by centrifugation. Large unilamellar vesicles (LUVs) used in dye release experiments, potential zeta titrations and tryptophan fluorescence spectroscopy were obtained by two extrusion steps using an Avanti Mini-Extruder (Alabaster, AL, USA) and double-stacked polycarbonate membrane (Nuclepore Track-etch Membrane, Whatman): firstly 6 times through 0.4 μ m and then 11 times through 0.1 μ m membranes. For the dye-entrapped LUVs free dye was separated by gel filtration on a Sephadex G25 M column (Amersham Pharmacia, Uppsala, Sweden). Vesicles were used within 48 h of preparation. The lipid concentration was determined by phosphorus analysis (Rouser et al. 1970). Laser light scattering measurements with Zeta Sizer Nano NS-90 (Malvern Instruments, Worcestershire, UK) have revealed homodisperse LUV suspensions, with an average diameter of $100.0\text{--}110.0 \pm 0.2 \text{ nm}$ among several preparations.

Dye leakage

To complete a final volume of 1.2 mL, an aliquot of fresh LUV suspension was injected into a 1 cm quartz cell, containing magnetically stirred peptide solutions in Tris/HCl buffer at different concentrations, ranging from 0.25 to 9 μ M, according to the permeabilizing efficiency, to reach around 100% leakage within 20–30 min contact time. CF release from the vesicles was monitored fluorometrically by using a ISS PC1 spectrofluorometer (Champaign, IL, USA) at 520 nm, 0.5 nm slit width (excited at 490 nm, 1 nm slit width) by measuring the decrease in self-quenching at 25°C. Percentage of dye leakage was determined after regular time intervals, and calculated with the equation:

$$\text{percentage of leakage} = 100 \times (F - F_0)/(F_{100} - F_0) \quad (1)$$

where F is the observed fluorescence intensity, F_0 and F_{100} correspond, respectively, to the fluorescence intensities in the absence of peptides and to 100% leakage, as determined by the addition of 20 μ L of 10% Triton X-100 solution. F_{100} has been corrected for the corresponding dilution factor. The observed release of CF from vesicles is described by a single exponential function as proposed by Schwarz and Robert (1990). However, in the present study the leakage time course curves could only be fitted for all kinds of vesicles and peptide concentrations tested, with correlation factors above 0.97, most of them above 0.99, using the relationship:

$$\text{leaked fraction} = L_{\max} - L_i \exp(-kt) \quad (2)$$

where L_{\max} represents the maximum (steady state) leakage, or the fraction of CF released at the rate k , L_i is a constant that accounts for the leakage that occurs immediately at vesicle addition, i.e., when at $t \sim 0$, $L \neq 0$, t is the time elapsed after the addition of vesicle and k is a constant measuring the rate of leakage. The efflux function is given by:

$$E(t) = 1 - \text{leaked fraction} \quad (3)$$

and was employed to obtain the apparent average pore number (p), as:

$$p = -\ln E(t) \quad (4)$$

which is based on the assumption that vesicles are homogeneous in size (Schwarz and Robert 1990).

Circular dichroism measurements

CD spectra were obtained at 20 μ M peptide concentration in different environments: Tris/H₃BO₃ buffer or in the presence of zwitterionic PC and in anionic PCPG (7030)

SUVs at 100 μM concentration. Buffer, peptide and lipid concentration have been chosen to minimize noise-to-signal ratio and light scattering, CD spectra were recorded from 260 to 190 nm with a Jasco-710 spectropolarimeter (JASCO International Co. Ltd., Tokyo, Japan), which was routinely calibrated at 290.5 nm using d-10-camphorsulfonic acid solution. Spectra have been acquired at 25°C using 0.5 cm path length cell, averaged over six scans, at a scan speed of 20 nm/min, bandwidth of 1.0 nm, 0.5 s response and 0.1 nm resolution. Following baseline correction, the observed ellipticity, θ (mdeg) was converted to mean residue ellipticity $[\Theta]$ (deg cm^2/dmol), using the relationship $[\Theta] = 1000/(l \cdot c \cdot n)$, where “ l ” is the path length in centimeters, “ c ” is peptide millimolar concentration, and “ n ” the number of peptide residues. The α -helix fraction (f_H) was evaluated from the observed mean residue ellipticity at 222 nm ($\Theta_{222}^{\text{obs}}$) according to Deber and Li (1995):

$$f_H = \frac{(\Theta_{222}^{\text{obs}} - \Theta_{222}^0)}{(\Theta_{222}^{100} - \Theta_{222}^0)} \quad (5)$$

where Θ_{222}^0 is zero and Θ_{222}^{100} is given by:

$$\Theta_{222}^{100} = -39500 \left(1 - \frac{2.75}{n} \right) \quad (6)$$

where n is the peptide length, $n = 14$ in the present study.

Fluorescence spectroscopy

Tryptophan emission fluorescence spectra were collected using a quartz cell with a 1 cm path length, at 25°C with the ISS PC1 spectrofluorometer (Urbana Champaign, IL, USA). The spectra were recorded from 300 to 450 nm with excitation at 280 nm, and increment of 1 nm, averaging in five scans. Excitation and emission bandwidth were set at 1 nm and at 0.5 nm, respectively. The Trp emission spectra were recorded after 1 h of sample preparation. Correction for scattering was carried out by using cross-polarization, parallel at emission and perpendicular at excitation (Ladokhin et al. 2000), and/or subtracting spectra obtained for each vesicle composition from blank of peptide samples. Blue shifts ($\Delta\lambda_{\text{max}}$) were calculated as the differences in wavelength of the maxima in emission spectra of the peptide acquired in the presence or absence of vesicles. Standard deviation for the blue shift was 1 nm.

Acrylamide fluorescence quenching

Acrylamide fluorescence quenching experiments were performed by adding aliquots of a 2.77 M acrylamide aqueous solution in a, constantly stirred, 5.0 μM peptide solution in Tris/HCl 10 mM buffer in the absence and in

the presence of 500 μM lipid vesicles at 25°C. Tryptophan emission fluorescence spectra were collected from 310 to 450 nm, excited at 285 nm. The fluorescence quenching data were analyzed according to the Stern–Volmer equation for collisional quenching:

$$F_0/F = 1 + K_{SV}[Q] \quad (7)$$

where F_0 and F are fluorescence intensities, measured manually with the cursor at the maximum intensity peak, in the absence and in the presence of quencher respectively. K_{SV} is Stern–Volmer constant for collisional process and $[Q]$ is the quencher concentration. In the case of static quenching the equation used for the numerical fitting was:

$$F_0/F = 1 + K_{SV}[Q] \exp(V[Q]) \quad (8)$$

where V is a static quenching constant (Eftink and Ghiron 1976).

Results

Dye leakage

Vesicle permeabilization has been used as a model system to explore the lytic activity of bioactive peptides whose functions are centered at the bilayer lipidic phase such as antimicrobial and hemolytic peptides. The constituents of the cell membrane have been shown to work as a selective barrier for peptides action, since anionic lipids are preponderant in prokaryotic outer leaflet, whereas in mammalian membranes zwitterionic lipids are more abundant (Yeaman and Yount 2003; Zasloff 2002). We have used zwitterionic PC and anionic PCPG (70:30) as model systems to mimic mammal and bacterial membranes, respectively.

Fluorescent dye release induced by the five peptides studied was monitored by the fluorescence de-quenching. The dose response curves, the percentage of leakage as a function of the ratio of peptide to total lipid molar concentrations ($[P]/[L]$), showed sigmoid profiles for peptides in both zwitterionic and anionic vesicles as shown in the Fig 1a and b. The lytic activity is characterized by a threshold $[P]/[L]$ value, above which, cooperative dye release takes place. The threshold ratio, the leakage efficiency and cooperativity are found to be dependent on the vesicle surface charge density and on the peptide net charge. In zwitterionic vesicles it is observed that the ($[P]/[L]$) ratios increase with the peptide net charge. As the net charge increases the dose response curves become less cooperative. Comparing the percentage of leaked dye after the same contact time (10 min), also shows that the less charged peptides induce a higher leaked fraction. In anionic PCPG vesicles the behavior seems to be similar to that

observed for zwitterionic vesicles. The dose response curves for the less charged peptides ($Q = +1$ and $+2$) are characterized by low $[P]/[L]$ threshold ratios, slightly higher than those found in neutral PC vesicles; nevertheless, the leakage is more cooperative in anionic vesicles. For peptides with higher net charges ($Q = +3$ and $+4$) the $[P]/[L]$ threshold ratios are lower for anionic vesicles as compared with neutral ones with more cooperative dye release and achieving higher percentage of leaked dye.

The time course curves of the dye release, for which some typical examples were shown in inset of the Fig 1a and b were well fitted for zwitterionic and anionic vesicles using the Eq. 2. The release kinetics was fitted with correlation factors above 0.97, most of them above 0.99 in the range of peptide concentrations tested. The efflux ratio given by Eq. 3 at different peptide and lipid ratios was used to estimate the average pore number, p^* . Fig 2a and b shows dependence of this parameter with $[P]/[L]$ in zwitterionic and anionic vesicles, respectively. It can be observed that for Polybia MP-3 and -MP-2 in electrically neutral vesicles, the dependence of the apparent average pore number with $[P]/[L]$ shows that, above threshold $[P]/[L]$ values, p^* increases quickly suggesting a cooperative process of pore formation. This behavior is somewhat different from that observed previously for Polybia MP-1 for which the apparent average pore number increases linearly with $[P]/[L]$ suggesting a process of pore inactivation (dos Santos Cabrera et al. 2008). The leakage process for Polybia N2-MP-1 and MP-X was observed to be similar, but less cooperative as compared to Polybia MP-2

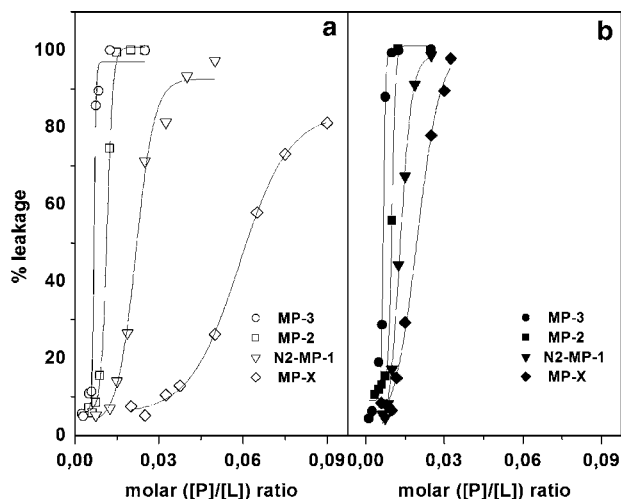


Fig. 1 Dose-response curves: % leakage after 10 min as a function of peptide to lipid molar ratio ($[P]/[L]$). **a** Zwitterionic vesicle at 100 μ M. Threshold $[P]/[L]$ values are 0.006 for MP-3, 0.0085 for MP-2, 0.0137 for N2-MP1 and 0.033 for MP-X. **b** Anionic vesicle at 100 μ M. Threshold P/L values are 0.0055 for MP-3, 0.0084 for MP-2, 0.079 for N2-MP1 and 0.009 for MP-X. Continuous line fitted with Boltzmann sigmoidal equation

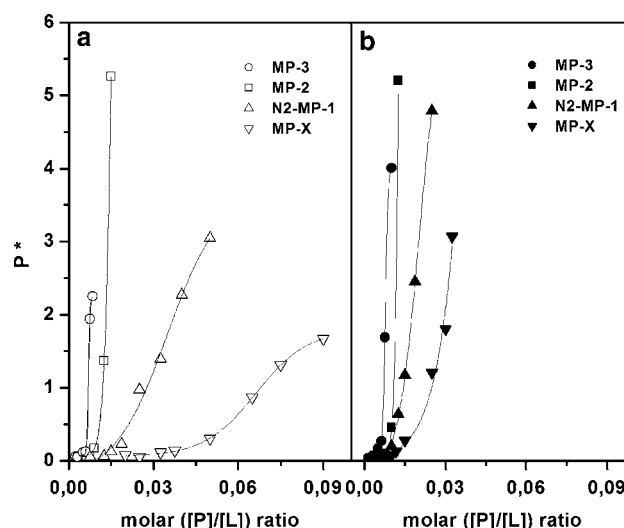


Fig. 2 Apparent average pore number p^* as a function of peptide to lipid ratio ($[P]/[L]$). **a** Zwitterionic vesicle addition at 100 μ M. **b** Anionic vesicle addition at 100 μ M. Continuous line fitted with Boltzmann sigmoidal equation

and -MP-3. In anionic vesicles it was also observed that the apparent average pore number increases with the $[P]/[L]$ ratio in a cooperative way. The values of the threshold $[P]/[L]$ and the respective average pore numbers obtained from these experiments are summarized in Table 1.

Circular dichroism

Circular dichroism spectra of the peptides in membrane (SUVs) present two negative dichroic bands at 222 and 208 nm characteristic of helical structure, while they were observed to be random coil in buffer, as shown in Fig 3. The Polybia MP-3 and MP-2 CD spectra in zwitterionic vesicles show a more intense dichroic band at 222 nm than at 208 nm suggesting a slight tendency to aggregation in these vesicles, but not in anionic vesicles. The helical content, summarized in Table 1, was observed to be dependent on the peptide net charge. In zwitterionic vesicles, the less charged peptides Polybia MP-3, -MP-2 and -MP-1 show higher helical contents and decrease for the most charged Polybia-N2-MP-1 and MP-X. In anionic vesicles it was observed higher helical content compared to electrically neutral vesicles, suggesting that the helix induction is modulated by electrostatic interaction between lipid head groups and charged residues in the peptides. The comparison of the helical fraction of Polybia MP-3 and -MP-2 indicates that the substitution of the acidic residue at position 2 by asparagine reduces the helical content by 11 and 36% in zwitterionic and in anionic vesicles, respectively. The same substitution in Polybia MP-1 leads to a decrease of 20% in electrically neutral vesicles, but in anionic ones the helical content increased significantly

Table 1 Structural and physico-chemical features, biological activity data compared to the quantitative parameters of the lytic activity and spectroscopy data of the interaction of Polybia mastoparans with zwitterionic and anionic vesicles

Polybia MP3	Ile- Asp -Trp-Leu-Lys-Leu-Gly-Lys-Met-Val-Met- Asp -Val-Leu-NH ₂									
Polybia MP2	Ile-Asn-Trp-Leu-Lys-Leu-Gly-Lys-Met-Val-Ile- Asp -Ala-Leu-NH ₂									
Polybia MP1	Ile- Asp -Trp-Lys-Lys-Leu-Leu- Asp -Ala-Ala-Lys-Gln-Ile-Leu-NH ₂									
Polybia N2-MP1	Ile-Asn-Trp-Lys-Lys-Leu-Leu- Asp -Ala-Ala-Lys-Gln-Ile-Leu-NH ₂									
MP-X	Ile-Asn-Trp-Lys-Gly-Ile-Ala-Ala-Met-Ala-Lys-Lys-Leu-Leu-NH ₂									
	Polybia MP-3		Polybia MP-2		Polybia MP-1		Polybia N2-MP-1		MP-X	
Q	+1		+2		+2		+3		+4	
<H>	0.06		0.08		−0.11		−0.10		0.01	
μ	0.26		0.27		0.24		0.25		0.2	
Φ	60		60		120		120		100	
	PC	PCPG	PC	PCPG	PC	PCPG	PC	PCPG	PC	PCPG
[P]/[L]	0.006	0.0055	0.0085	0.0084	0.15 ^b	0.25 ^b	0.0137	0.079	0.033	0.009
p*	0.084	0.17	0.13	0.13	0.23 ^b	0.15 ^b	0.13	0.075	0.1	0.093
Δλ (nm)	21.0	21.0	20.0	24.0	19.0	22.0	20.0	21.0	18.0	21.0
K _{SVL} /K _{SVB}	0.27	0.35	0.25	0.26	0.32	0.27	0.27	0.29	0.38	0.28
f _h	0.26	0.42	0.23	0.27	0.27 ^b	0.36 ^b	0.21	0.48	0.12	0.40
MIC (μM) <i>E. coli</i>	62 ^a		8 ^a		8 ^a		5.8		na	
MIC (μM) <i>S. aureus</i>	31 ^a		4 ^a		15 ^a		7.8		na	
EC ₅₀ (μM) WRRBC	50 ^a		50 ^a		nh		26		na	

Sequences and structural characteristics of Polybia-peptides in comparison with MP-X. Negatively charged Asp residues are shown in bold. Net electrostatic peptide charge, *Q*; average hydrophobicity per residue, <H> and mean hydrophobic moment μ, obtained from Eisenberg et al. (1984) consensus scale, averaged over the number of residues; angle subtended by the polar face (polar, charged residues), estimated from the helical wheel projection, Φ. Lytic activity on zwitterionic, PC, and anionic, PCPG, vesicles: threshold concentration ratio, [P]/[L], and the respective average number of pore, p*, in zwitterionic and anionic vesicles; Spectroscopy data on zwitterionic, PC, and anionic, PCPG, vesicles: blue shift, Δλ (nm); Stern–Volmer constants ratio in vesicles (K_{SVL}) and in buffer (K_{SVB}); α-helical content, f_h. Biological activity: minimum inhibitory concentration (μM), MIC, in Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria and hemolytic activity, EC₅₀ (μM), in washed rabbit red blood cells (WRRBC)

nh not hemolytic, na not available

^a Souza et al. 2009

^b dos Santos Cabrera et al. 2008

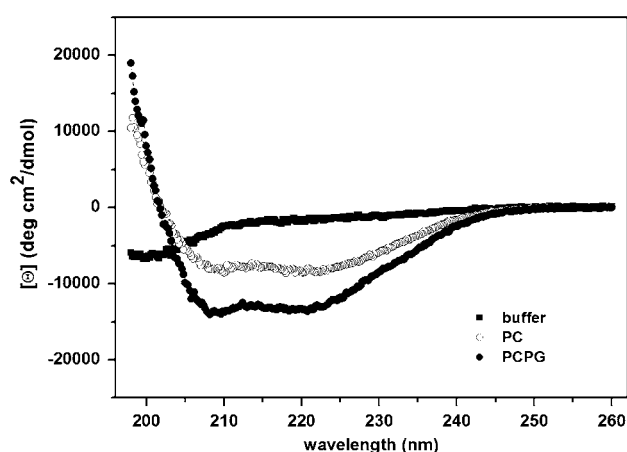


Fig. 3 Circular dichroism spectra of MP-3 at 20 μM. Spectra were recorded in the absence and the presence of SUVs at 100 μM in Tris H₃BO₃ 5 mM, 150 mM NaF, 0.5 mM Na₂EDTA, pH 7.5, at 25°C

suggesting that the N-terminus substitution N2D and consequent increase in net positive charge might have different effects on the helical content when peptides are hydrophobic, as Polybia MP-2, or hydrophilic, as Polybia-N2-MP-1. The electrostatic contribution, however, seems not to be the mandatory factor modulating the helical content, when peptides are hydrophobic: the less charged peptide Polybia MP-3 presents higher helical content in anionic vesicle than the two peptides with net charge +2.

Fluorescence spectroscopy

Tryptophan fluorescence emission spectra for the five peptides, in the presence of vesicles, showed significant spectral blue shift compared with the respective spectra in buffer, indicating that upon interaction with bilayers the tryptophan residue moves to a more hydrophobic

environment. In Fig 4a and b the differences between the positions of the tryptophan spectral peaks in the presence and in the absence of lipids were displayed as a function of the total molar lipid concentration. In zwitterionic vesicles the largest spectral shift was observed for the less charged peptides, suggesting that the peptide affinity to neutral vesicles decreases with the net charge, in very good agreement with the results obtained by circular dichroism and dye leakage. It is intriguing that for Polybia MP-3 and -MP-2 the blue shift increases quickly when a minimum amount of vesicle was added and could be indicating that besides peptide adsorption another process, possibly peptide aggregation induced by the lipid bilayer, takes place, nevertheless it was not observed tryptophan self-quenching which should be expected in the peptide aggregated state. The less charged peptides Polybia MP-3 and -MP-2 showed also greater blue shifts in anionic vesicles followed by the other peptides in good agreement with the leakage data.

The significant blue shift observed for tryptophan suggests that this residue penetrates in the bilayer. To quantify

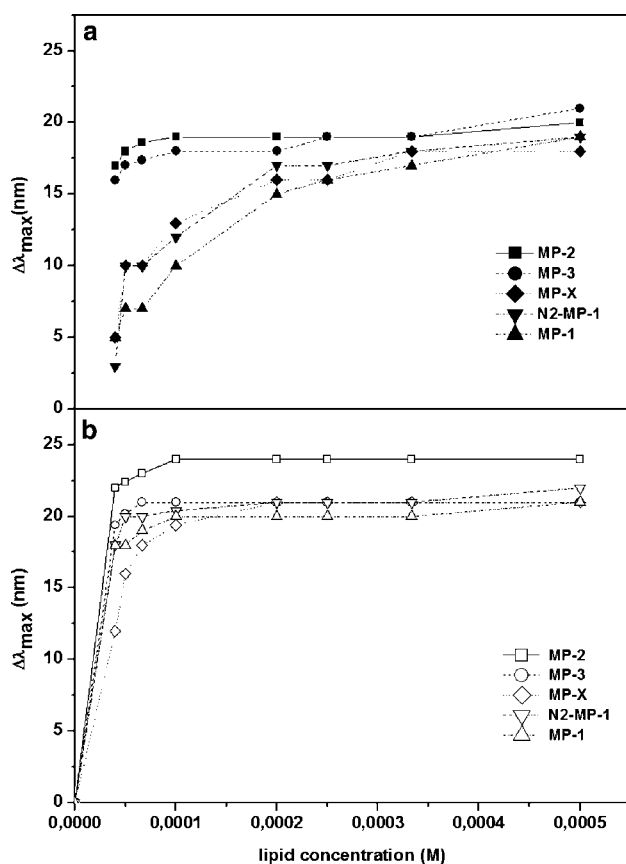


Fig. 4 Tryptophan blue shift with increasing lipid concentration of the five peptides at 5 μ M incubated with different lipid concentration up to 500 μ M in 10 mM Tris/HCl, 1 mM EDTA, 0.15 NaCl, pH 7.5 at 25°C during 1 h for equilibration. **a** Zwitterionic lipid. **b** Anionic lipid

the extent of this penetration in the hydrophobic phase of the bilayer, fluorescence quenching experiments were performed. Water soluble acrylamide was used as a quencher for tryptophan and the loss of fluorescence intensity, due to the addition of this quencher, was analyzed with Stern–Volmer plots, showed in Fig 5. Considering the acrylamide concentration range used, the Stern–Volmer plots for peptides in buffer were not linear suggesting that besides the collisional quenching process there is a static one, possibly due to the presence of acrylamide adjacent to the tryptophan, when it is excited (Eftink and Ghiron 1976). In the presence of vesicles the static quenching was reduced and the relative change in the fluorescent intensities increases linearly with the quencher concentration. The values of the Stern–Volmer constants were obtained from the slopes of the linear plots according to Eq. 7 and from the numerical fitting of non linear plots using Eq. 8. The Stern–Volmer constants in the presence of vesicles have shown that the bilayer protects the fluorophore from the quencher; however this screening effect was not complete. To compare the quenching data, the Stern–Volmer constants for each peptide (K_{SVL}) in vesicles were normalized by the respective value in buffer (K_{SVB}) and the results are shown in Table 1. Since acrylamide is accessible to all tryptophan species in solution, with exception to those buried in the lipidic phase of the bilayer (Mishra et al. 1994), these normalized constants represent the fraction of fluorescence intensity remained after the incorporation of the fluorophore in the bilayer and qualitatively estimates the peptide-bilayer affinity. Notwithstanding the similarity of the constants for Polybia

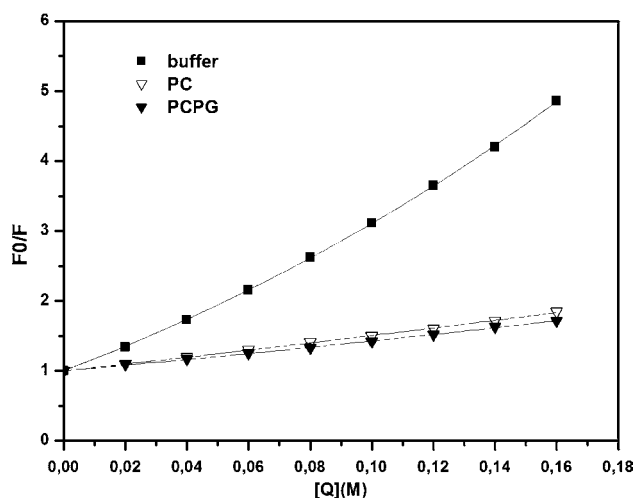


Fig. 5 Stern–Volmer plots of N2-MP1 tryptophan fluorescence quenched by acrylamide in buffer and in the presence of LUVs. Liposomes were composed of PC and PCPG. The concentration of lipids was 500 μ M in a volume of 1300 μ L of 10 mM Tris/HCl, 1 mM EDTA, 0.15 NaCl, pH 7.5. Continuous lines are the best fit obtained with Eq. 7 for the linear plot and Eq. 8 for the non-linear

mastoparans it is possible to observe that tryptophan residues in these peptides were differently shielded in zwitterionic and anionic vesicles.

Discussion

Besides the difference in the net charge, four of the five peptides used in the present study show the presence of acidic residues, two of them (Polybia MP-3 and -MP-1) with an aspartic acid residue in the N-terminus (D2). This is not a ubiquitous aspect among linear peptides that present antibacterial activity and to which the cationic charge is accepted to play an important role in the specificity to the bacterial anionic outer leaflet membrane. Previous results in biological activity have shown that the peptides with smaller net charges present multi-functional activity, including reasonable antibacterial activity against *E. coli* and *S. aureus*, relatively low hemolytic effect compared with mellitin and mast cell degranulation activity (Souza et al. 2005; Souza et al. 2009).

The lytic activities of these peptides were evaluated in model zwitterionic and anionic membranes in assays of fluorescent dye released from vesicles. Two parameters have been used: the threshold peptide to total lipid molar concentration ratios, to start the cooperative dye leakage, and the average pore number at these critical ratios. This evaluation showed that the less charged peptide Polybia MP-3 presents very low threshold ratios with approximately the same threshold $[P]/[L]$ for neutral or anionic vesicles. The average pore numbers, however, showed that this peptide displays preference for electrically neutral vesicles which is in good agreement with its low antibacterial and mild hemolytic activities. For Polybia MP-2 with the substitutions: N2D, I11 M and A13 V, it was observed that the critical $[P]/[L]$ and the average pore number were the same for zwitterionic and anionic vesicles. The biological activity showed that this peptide presented lower values of minimum inhibitory concentration for Gram-positive and Gram-negative bacteria and similar hemolytic efficacy compared with Polybia MP-3 (Table 1). Estimative of the helical propensities for Polybia MP-1 and MP-2 based on Chou-Fasman calculations (Chou and Fasman 1974), reveals that excepting the N2D substitution in Polybia MP-2, the other two, I11 M and A13 V, maintain the same helical propensity ($P_\alpha = 120$) for the segment K8 to L14. Asparagine is less helicogenic than aspartic acid according to the Chou-Fasman scale of helix propensity, otherwise the increase in the helical macrodipole due to the acidic residue in the N-terminus and possible hydrogen or saline bond between this residue and a lysine in an neighbor position, helps to stabilize the helix in Polybia MP-1 both in zwitterionic and anionic vesicles. N2D

substitution in the N-terminus. Beyond its relatively high helical content, influenced by the presence of the acidic residue at the N-terminal, Polybia MP-2 is slightly more hydrophobic with mean hydrophobicity $\langle H \rangle = 0.08$, according to the Eisenberg consensus scale (Eisenberg et al. 1984) compared to Polybia MP-3 with $\langle H \rangle = 0.06$, while their mean hydrophobic moments are approximately the same $\langle \mu \rangle = 0.26$ and $\langle \mu \rangle = 0.27$ for Polybia MP-3 and -MP-2, respectively. They are characterized by broad hydrophobic face and Φ the angle subtended by the positive charges is estimated in 60° , according to the helical wheel projection shown in Fig. 6. These characteristics, relatively high helical content in zwitterionic vesicles, hydrophobicity and broad hydrophobic face, were responsible for the higher lytic activity in zwitterionic vesicles as well as the hemolytic activity. Studying peptides designed to vary one of these parameters, Kiyota et al. (1996) and Dathe et al. (2002) showed that association of hydrophobicity, low polar angle and high helicity results in more hemolytic activity. The smaller net charge in Polybia MP-3 contributed to the lower affinity in anionic vesicles, as indicated by its K_{SVL}/K_{SVB} constant ratio (Table 1), despite its higher helical content, hydrophobicity and the broad hydrophobic face. Electrostatic net charge and consequently the binding affinity have been shown to be more important in modulating the activity in anionic vesicles (Dathe and Wieprecht 1999; Sitaram and Nagaraj 1999; Seelig 2004). The spectral shifts and the normalized Stern-Volmer constants support the lower affinity of Polybia MP-3 for anionic vesicles and similar affinities for neutral and anionic vesicles for Polybia MP-2. The greater net charge and higher hydrophobicity of Polybia MP-2 acted in concert to modulate its affinity for neutral and anionic vesicle.

Compared to Polybia MP-2, the two extra charged groups in the N-terminal, one acidic (D2) and one basic (K5) in Polybia MP-1, increased the width of the polar face ($\Phi = 120^\circ$) and the differences in the sequence resulted in a hydrophilic peptide with a mean hydrophobicity $\langle H \rangle = -0.11$. As a result, the threshold $[P]/[L]$ and the average pore number for Polybia MP-1, points out for a more efficient lytic activity in anionic vesicles judging from its average pore numbers and from the less cooperative dose response curve for zwitterionic vesicles (dos Santos Cabrera et al. 2008). Notwithstanding the higher threshold $[P]/[L]$ ratio in anionic vesicles, the $[P]/[L]$ at 50% leakage normalized by the threshold $[P]/[L]$ was 1.08 in anionic vesicles and 1.7 in the zwitterionic showing that the lytic process is more cooperative and efficient in anionic vesicle. In Polybia MP-1 hydrophilicity and a broader hydrophilic face contributed to a decrease in the lytic activity in zwitterionic vesicles. The same characteristics modulated positively this activity in anionic vesicles and also the peptide affinity to these vesicles, as supported

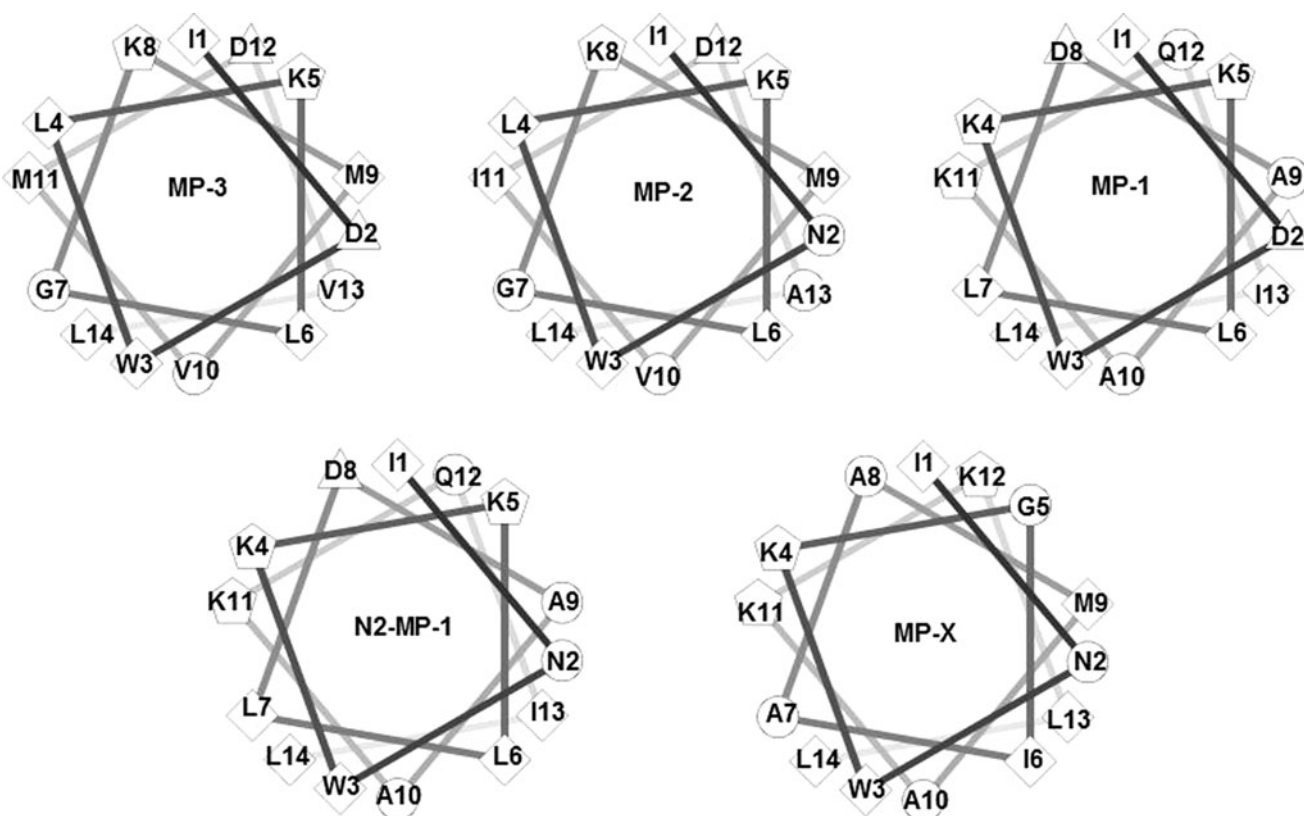


Fig. 6 Helical wheel projections of the peptides. The representations of the residues are the following: *circles* hydrophilic, *diamonds* hydrophobic, *triangles* the potentially negative and *pentagon*

potentially positive. Helical wheels were prepared by the software Helical Wheel projections; wheel.pl, version 1.3 (Zidovevetski et al. 2003)

by the blue shifts and Stern–Volmer constants. The substitution N2D in Polybia N2-MP1 resulted in a slightly less hydrophilic peptide with higher net charge and with the same width of the estimated polar face. The Chou–Fasman calculations show a segment of ten residues from K5 to L14 with high helical propensity with $P_{\alpha} = 120$. Although the N2D substitution in Polybia N2-MP-1 is outside this helical segment, the loss of the acidic residue decreased the helical content decreased in electrically neutral vesicles and probably due to the extra charge and hydrophilicity the helical fraction increased in PCPG vesicles, when compared to its parent peptide. As a result of these characteristics Polybia N2-MP-1 presented higher lytic activity in anionic vesicles, with smaller pore number. The Stern–Volmer constants and the spectral shifts, however, are quite similar in neutral and anionic vesicles. It is worth noting that this substitution resulted in a relatively higher antimicrobial and hemolytic activities compared with Polybia MP-2 and -MP-1. The reversion of the bilayer specificity due to the increase in the electrostatic net charge beyond a critical value in the same sequence leading to a simultaneous increase in the antimicrobial and hemolytic activity was also observed in magainin (Dathe et al. 2002).

MP-X the higher charged of the studied peptides, with three basic groups, is not hydrophilic, $\langle H \rangle = 0.01$, with slightly narrower polar face (100°) than Polybia-MP1. Its mean hydrophobic moment is the smaller between the studied peptides $\langle \mu \rangle = 0.2$. This peptide has the segment from A7 to L14 with high propensity and the Chou–Fasman calculation shows a $P_{\alpha} = 128$. The helical content in zwitterionic vesicles is the smallest between the peptides studied, probably due to its net charge and its smaller hydrophobic moment, which, in consequence, modulates its lower affinity to the electrically neutral vesicles and consequently the higher $[P]/[L]$ ratio. The greater net charge favored the interaction with anionic vesicles resulting in higher helical content and lower critical $[P]/[L]$ ratio. The higher affinity to anionic vesicles was also observed in the spectral shift and Stern–Volmer constants.

The results reported above have emphasized that the presence of an acidic residue in the N-terminus stabilized the peptide helical conformation by increasing the helical macrodipole (Schoemaker et al. 1985). The conformation stabilizing effect could be improved if this residue could be salt bridged with a basic residue as the third or fourth nearest neighbor. The present study shows that the acidic

residue in a hydrophobic peptide plays an important role in modulating their affinity and lytic toward neutral vesicles which are different from the acidic residue in a hydrophilic peptide with higher affinity and lytic activity in anionic vesicles. The data showed that the interplay between the acidic residue in the N-terminus and peptide hydrophobic/hydrophilic balance drives the peptide bilayer specificity and consequently its lytic and biological activities.

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Conflict of interest statement None.

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