FEBS Letters 554 (2003) 100–104 FEBS 27747

Individual substitution analogs of Mel(12–26), melittin's C-terminal 15-residue peptide: their antimicrobial and hemolytic actions

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Received 18 August 2003; revised 15 September 2003; accepted 24 September 2003

First published online 7 October 2003

Edited by Hans Eklund

Abstract Residues 1–9 of M(12–26) (GLPALISWIKRKRQ-Q-NH_2), the C-terminal 15-residue segment of melittin, were substituted individually to change the hydropathicities in these positions. Antimicrobial and hemolytic activities of these peptides were determined. The results showed increased antimicrobial activities with increased hydrophobicities at almost all the positions studied. The effects at positions 2, 5, 8 and 9 were significant while the effects at the other positions were small. These two groups of residues were located on the opposite faces of the α-helix. In other words, the hydrophobicities of the two faces were favorable, but one face (the more favorable face) contributed more to the antimicrobial activities than the other (the less favorable face). The hydrophobicity, not the amphipathicity, seems to be crucial for antimicrobial activity. In contrast, the hydrophobicity of one face was favorable but the other was unfavorable for the hemolytic activity, indicating that the amphipathicity may be important for hemolysis. Interestingly, the more favorable face for antimicrobial activity was located opposite to the favorable face for hemolytic activity, indicating the direction of the hydrophobic face for the antimicrobial activity and direction of the amphipathicity for the hemolytic activity were also important.

Key words: Melittin; Antimicrobial peptide; Hemolysis; Hydrophobic; Amphipathic

1. Introduction

The widespread use of antibiotics has caused serious antibiotic resistance by pathogens. Therefore, in recent years, much attention has been paid to search new classes of antibiotics that can avoid the resistance of the bacteria. The endogenous antimicrobial peptides and their derivatives are attractive candidates (for reviews, see [1–6]). Among the antimicrobial peptides, short linear peptides (<40 amino acids) such as cecropin, magainin, melittin, etc., have attracted much attention. This class of peptides shares some common structural features, i.e. net positive charge, around 50% hydrophobic amino acid residues and a potential to form an amphipathic α-helical structure [7–10]. These structural features are also believed to be the cause of hemolytic activities of some antimicrobial peptides such as melittin and pardaxin [11,12], and are similar to the structural features of calmodulin-binding peptides [13,14] as well. Some antimicrobial peptides such as melittin, crabrolin, mastaparans, seminalplasmin and substance P bind tightly to calmodulin. In our previous papers [14,15], we proposed a model for the prediction of the calmodulin-binding domain of the calmodulin-binding peptides and an empirical formula to predict the binding affinities. It was predicted that the calmodulin-binding domain of melittin spans the residues 12–26. The tested affinities of synthetic deletion peptides of melittin, M(15–26), M(14–26), M(13–26), M(12–26) and M(11–26), for calmodulin were in good agreement with the predicted values [14,16]. We also found that the last three peptides retained antibacterial activity and all the synthetic peptides had a much-reduced hemolytic activity to human red blood cells [17]. Subbalakshmi et al. also found that the C-terminal 15-residue fragment of melittin possessed a slightly lower antimicrobial activity and much lower hemolytic activity than melittin [18]. The structure of these peptides can be separated into two parts: N-terminal hydrophobic segment (residues 1–9, for example, for M(12–26)) and C-terminal basic residue cluster. In this paper, using M(12–26) as the model, residues in the hydrophobic segment (residues 1–9) were substituted individually to change the hydropathicities of the positions. The purpose was to study the effect of the hydropathicities of individual positions on the antimicrobial and hemolytic activities, and the difference between the two effects as well.

2. Materials and methods

2.1. Melittin purification and peptide synthesis

Melittin was purified from bee venom [19]. Melittin analogs were synthesized by the solid-phase peptide synthesis technique using *t*-butyloxycarbonyl (Boc) chemistry on a *p*-methylbenzhydrylamine resin (Tianjin Nankai Hecheng Co., PR China). The Boc amino acids were obtained from Advanced ChemTech (USA). All protected amino acids were added with coupling mediated by dicyclohexylcarbodiimide and 1-hydroxybenzo-triazole (ACROS). The peptides bound on the resin were cleaved using liquid HF. The synthesized peptides were first passed through a Sephadex G-25 (Pharmacia) column and then fractionated by the use of reversed-phase high-performance liquid chromatography (RP-HPLC) on a C_{18} μ-Bondpak semi-preparative column (300×19 mm, Waters Co.). The purified peptides, which were shown to be homogeneous (>95%) by analytical HPLC, were subjected to amino acid analysis and mass spectroscopy to confirm their compositions.

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0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)01113-X

2.2. RP-HPLC analyses of peptides

Peptides were analyzed by RP-HPLC on a Nova-Pak C_{18} column (3.9 mm×150 mm, Waters Co.) at a flow rate of 1 ml/min. The sample concentration was 1 mg/ml peptide in mobile phase, which comprised 28% acetonitrile/72% water (v/v) containing 0.1% trifluoroacetic acid. The retention times (t_R) of the peptides were determined at 25°C.

2.3. Antimicrobial activities

The antimicrobial activities of the peptides were assayed in nutrient broth (5 g of bactonutrient broth and 5 g of NaCl per liter medium) under aerobic conditions. Different concentrations of peptides were added to 1 ml medium containing the inocula of the test organism ($\sim 10^6$ CFU) in mid-logarithmic phase of growth. Growth inhibition was determined by measuring the OD_{600}, following incubation for 24 h at 37°C. The antibacterial activity is expressed as minimal inhibitory concentration (MIC). The microorganisms used were Gram-positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis*, and Gram-negative bacterium *Escherichia coli*.

2.4. Hemolytic activity

The hemolytic activities of the peptides were tested against human erythrocytes. The erythrocytes were suspended in phosphate-buffered saline (PBS). Peptides dissolved in PBS were then added to 500 μl of a solution of the stock erythrocytes in PBS to reach a final volume of 1000 μl (final erythrocyte concentration, 2.5% v/v). The resulting suspension was incubated for 60 min at 37°C with gentle mixing. The samples were then centrifuged and the absorbance of the supernatants was measured at 414 nm. Controls for zero hemolysis (blank) and 100% hemolysis consisted of erythrocyte suspended in PBS and 1% Triton, respectively. The hemolytic activity is expressed as concentrations required for 100% (HC_{100}), 50% (HC_{50}), or 10% (HC_{10}) lysis of human erythrocytes, respectively.

2.5. Circular dichroism (CD) spectroscopy

The CD spectra were measured with a JASCO-J-715 spectra polarimeter under nitrogen flush in 1-cm path length cells at 25°C. The spectra were recorded between 250 and 200 nm and the average of three recordings was taken. The calibration was carried out with *d*-camphorsulfonic acid. The peptides were scanned at concentrations of 1.5×10^{-5}–2.0×10^{-5} M. The percentage of α-helical structure was calculated as follows: α-helix (%) = $([\theta]_{222}-[\theta]^0_{222})/[\theta]^{100}_{222}$, where $[\theta]_{222}$ is the experimentally observed absolute mean residue ellipticity at 222 nm. Values for $[\theta]^0_{222}$ and $[\theta]^{100}_{222}$, corresponding to 0 and 100% helix content at 222 nm, were estimated to be −2000 and −28 400 degrees cm^2/dmol, respectively [20].

3. Results

3.1. Design of peptides

In our previous papers it was shown that the C-terminal segment, M(12–26), of melittin retained antibacterial activity but had a much reduced hemolytic activity to human red blood cells [17]. M(12–26) consists of an N-terminal hydrophobic segment (residues 1–9) and a cluster of basic residues (Lys^{10}-Arg^{11}-Lys^{12}-Arg^{13}) in the C-terminus. In order to study the effect of the hydrophobicity of individual residues of the hydrophobic segment on the antimicrobial and hemolytic activities, a series of analogs with modified hydrophobicities were designed, as shown in Table 1. The hydrophobic residues Leu^2, Leu^5, Ile^6 and Ile^9 in M(12–26) were individually replaced by a hydrophilic amino acid, Ser, in four parallel syntheses. Similarly, the hydrophilic residues Gly^1, Ala^4 and Ser^7 were individually replaced by a hydrophobic amino acid, Leu, in three other parallel syntheses. The effect of the hydropathicity at each of these positions was studied by comparing each of the substituted peptides with the model peptide, M(12–26). Trp at position 8 is a sole aromatic amino acid residue in the model peptide. It was reported that Trp might play a role for the antimicrobial and hemolytic activities in antimicrobial peptides [21,22]. In order to eliminate the influence of aromatic residue on the antimicrobial and hemolytic activities in the comparison, two peptides were synthesized. In one of the peptides Trp^8 was replaced by a hydrophobic amino acid, Leu, and in the other by a hydrophilic one, Ser. The influence of the hydropathicity at this position was compared using the two substituted peptides. Pro is the only amino acid with the α-amino group in a ring structure (secondary amine group) in the 20 natural amino acids, which is an N-terminal helical boundary residue but a strong helical breaker [23]. Okada et al. [24] reported that when melittin was bound to phosphatidylcholine vesicles the C-terminal Leu^{13}-Lys^{21} (corresponding to Leu^2-Lys^{10} of the M(12–26)) segment had an α-helical conformation. Pro^{14} (corresponding to Pro^3 of M(12–26)) was thus located in the interior of the α-helical conformation. When Pro occurs in the interior of an α-helix it breaks at least two adjacent H bonds and its ring pushes the proceeding turn of backbone away by an ångstrom [25]. To eliminate this effect, similar to the substitutions at position 8, Pro^3 was replaced with a hydrophobic amino acid, Leu, and by a hydrophilic one, Ser, respectively, and the influence of the hydropathicity at this position was compared using the two substituted peptides.

3.2. Antimicrobial and hemolytic activities of the peptides

The MICs of melittin and its analogs against Gram-positive (*S. aureus* and *B. subtilis*) and Gram-negative species (*E. coli*) were determined by serial dilution microbroth, as shown in Table 2. Melittin was used as the control. All the peptides had much lower MICs against the Gram-positive bacteria than

Table 1
Sequences, calculated hydrophobicities, hydrophobic moments, and retention times on RP-HPLC of melittin and its analogs

Peptide	Sequence	H_m[a]	μ_H[a]	t_R (min)
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2	−0.086	0.22	>80
Mel(12–26)	GLPALISWIKRKRQQ-NH_2	−0.28	0.20	20.32
Mel(12–26, L^1)	**L**LPALISWIKRKRQQ-NH_2	−0.26	0.21	20.72
Mel(12–26, S^2)	G**S**PALISWIKRKRQQ-NH_2	−0.33	0.16	4.6
Mel(12–26, L^3)	GL**L**ALISWIKRKRQQ-NH_2	−0.24	0.17	45
Mel(12–26, S^3)	GL**S**ALISWIKRKRQQ-NH_2	−0.29	0.21	–
Mel(12–26, L^4)	GLP**L**LISWIKRKRQQ-NH_2	−0.30	0.19	28.59
Mel(12–26, S^5)	GLPA**S**ISWIKRKRQQ-NH_2	−0.33	0.15	3.61
Mel(12–26, S^6)	GLPAL**S**SWIKRKRQQ-NH_2	−0.35	0.20	5.53
Mel(12–26, L^7)	GLPALI**L**WIKRKRQQ-NH_2	−0.23	0.15	18.49
Mel(12–26, L^8)	GLPALIS**L**IKRKRQQ-NH_2	−0.27	0.20	13.1
Mel(12–26, S^8)	GLPALIS**S**IKRKRQQ-NH_2	−0.32	0.20	5.10
Mel(12–26, S^9)	GLPALISW**S**KRKRQQ-NH_2	−0.35	0.14	5.17

[a]Mean hydrophobicities (H_m) and hydrophobic moments (μ_H) were calculated using consensus values of hydrophobicity scale [26].

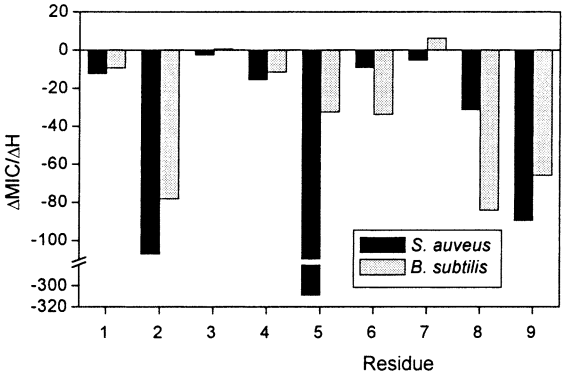

Fig. 1. The effect of hydrophobicity of each individual position on the antimicrobial activity.

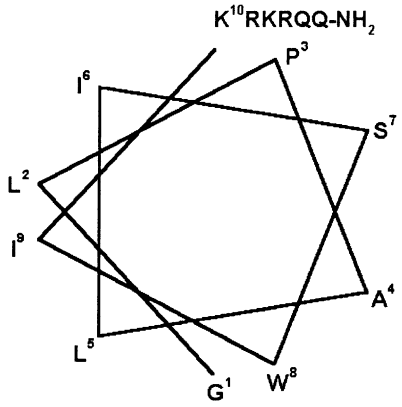

Fig. 2. Helical wheel projection of M(12–26).

against the Gram-negative one. Some of the MICs against *E. coli* exceeded the concentration ranges tested. The antimicrobial activity analysis was thus limited to the antimicrobial activity against the Gram-positive species below. It was found from Table 2 that the antimicrobial activity increased with increasing hydrophobicity at any individual position studied, with the exception of positions 3 and 7 against *B. subtilis*. Hemolytic activities of the peptides to human erythrocytes were determined and listed in Table 2. Most of the synthetic peptides had very low hemolytic activities. The concentrations required for 100% lysis (HC_{100}) or 50% lysis (HC_{50}) exceeded the concentration ranges tested for most of the peptides. In order to quantitatively compare the hemolytic activities of the peptides, the concentrations required for 10% lysis (HC_{10}) were determined.

3.3. Effect of the hydropathicities of the individual residues on the antimicrobial and hemolytic activities

The Eisenberg consensus scale [26] was used as the measure of hydropathicity of single amino acid residues. The effect of the hydropathicity of each individual residue on the antimicrobial activity was measured by the antimicrobial activity change (ΔMIC) divided by the Eisenberg consensus hydropathicity change (ΔH) between the peptides with hydrophobic and hydrophilic residues at the position. Similarly, the effect of the hydropathicity of each individual residue on the hemolytic activity was measured by the hemolytic activity change (ΔHC_{10}) divided by the hydropathicity change (ΔH). Fig. 1 shows the plot of $\Delta MIC/\Delta H$ against the residue number. It is clear that the antimicrobial activity increased with increasing hydrophobicity at any of the positions, except for positions 3 and 7 against *B. subtilis*. However, the dependences of the antimicrobial activities on the hydrophobicities at different positions were different. The effects at positions 2, 5, 8 and 9 were significant while the effects at positions 1, 3, 4, 6 and 7 were small. Compared with the helical-wheel projection (Fig. 2), these two groups of residues were located on opposite sides. Fig. 2 was made based on the report of Okada et al. [24] that five C-terminal residues (Arg^{22}-Gln^{26}, corresponding to Arg^{11}-Gln^{15} of M(12–26)) did not show a unique conformation when melittin was bound to phosphatidylcholine vesicles.

Fig. 3 is the plot of $\Delta HC_{10}/\Delta H$ as a function of residue number. In contrast to the antimicrobial activities, the hemolytic activities increased with increasing hydrophobicities at some positions, such as residues 1, 4, 7, 8 and 9, and decreased at other positions, such as residues 2, 5 and 6. The former group of residues (with the exception of residue 9) and the latter group are located on opposite sides of the α-helix. The unusual behavior of residue 9 probably correlated with its direct link to the basic residue cluster. Little effect was observed at position 3. Interestingly, the face (left-hand side in Fig. 2) whose hydrophobicity was more favorable for the antimicrobial activity and the face (right-hand side in Fig. 2) whose hydrophobicity was favorable for the hemolytic activity were located on opposite sides.

Table 2
Antimicrobial and hemolytic activities of melittin and its analogs

Peptide	MIC (μg/ml)			Hemolytic activity (μg/ml)		
	S. aureus	*B. subtilis*	*E. coli*	HC_{100}	HC_{50}	HC_{10}
Melittin	0.72	0.18	11.4	10	5	1.78
Mel(12–26)	5.6	5.6	88.9	–	–	4 500
Mel(12–26, L^1)	1.1	2.2	70.8	–	1 450	192
Mel(12–26, S^2)	90	67.5	>270	–	–	1 588
Mel(12–26, L^3)	1.2	2.2	70.8	–	120	35.2
Mel(12–26, S^3)	3.1	1.6	6.25	–	403	44.0
Mel(12–26, L^4)	1.3	2.4	76.7	–	1 300	203
Mel(12–26, S^5)	250	31.2	>250	–	–	3 840
Mel(12–26, S^6)	14.6	39	311	–	–	650
Mel(12–26, L^7)	1.4	0.64	81.7	–	620	15.4
Mel(12–26, L^8)	13.5	10.0	161	–	–	9 350[a]
Mel(12–26, S^8)	38.2	76.4	>305	–	–	13 800[a]
Mel(12–26, S^9)	94.2	70.8	>94.4	–	–	6140

[a]Data were obtained by extrapolating to higher concentrations than the testing range.

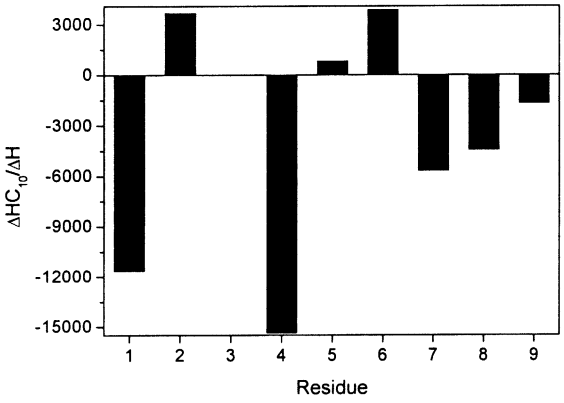

Fig. 3. The effect of hydrophobicity of each individual position on the hemolytic activity.

3.4. α-Helical structures determined by CD

The CD results showed that melittin and its analogs adopted random coil structures (data not shown) in 10 mM Tris–HCl buffer. The α-helical content of melittin increased from 10% in salt-free buffer to 47% in 2 M NaCl, which was in accordance with the results reported by Goto and Hagihara and was believed to be caused by melittin aggregation [27], while no remarkable increase in the content of α-helical structure for all the synthetic peptides was observed (data not shown), indicating the synthetic peptides could not form aggregates in this condition. The α-helical content of the synthetic peptides and melittin increased markedly in 30 mM SDS and 10% HFIP (27–82%), respectively, which mimic the membrane environment, indicating the peptides have an inherent helicity. No obvious correlations between the bioactivities and α-helical structures were observed.

3.5. Correlations of antimicrobial activities with hydrophobicities of the peptides

The calculated hydrophobicities, hydrophobic moments and retention times of melittin and its analogs are listed in Table 1. Plots of H_m against t_R and μ_H against t_R (not shown), respectively, indicated that the calculated hydrophobicities and the retention times were linearly correlated, while no correlation between the hydrophobic moments and the retention times was found. Analyzing the data we found that both the calculated hydrophobicities and the retention times correlated well with the antimicrobial activities, and the retention times

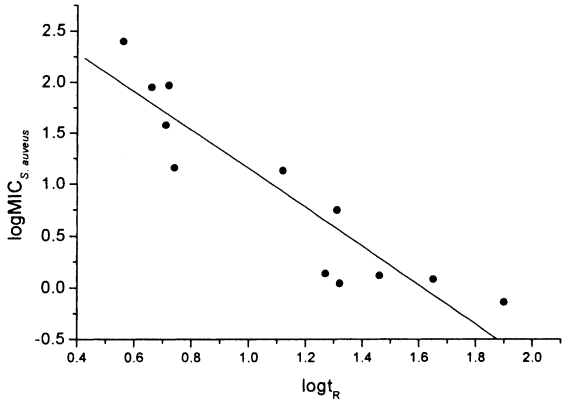

Fig. 4. Correlation between the antimicrobial activity against *S. aureus* and the retention time on RP-HPLC.

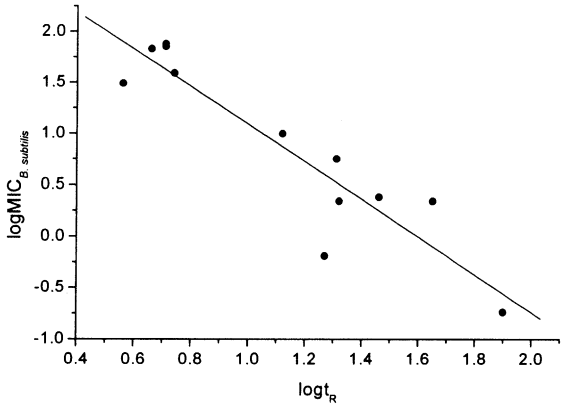

Fig. 5. Correlation between the antimicrobial activity against *B. subtilis* and the retention time on RP-HPLC.

had a better correlation than the calculated hydrophobicities. Thus the retention times are good measurements of the hydrophobicities of the peptides. Figs. 4 and 5 show the correlations between the retention times and the antimicrobial activities. The data reveal no correlation of the hydrophobic moments to the antimicrobial activities, and no correlation of the calculated hydrophobicities, hydrophobic moments and retention times, respectively, to the hemolytic activities as well.

4. Discussion

One of the main aims of the current study on the antimicrobial peptides is to obtain the maximum possible antimicrobial activity accompanied by the minimum toxicity toward the host. To attain this purpose, investigation of the structure–activity relationships or the mechanisms of antimicrobial activity and the toxicity toward the host (usually hemolysis) may be most important. The selectivity was believed to be caused by the different compositions of the membranes of bacteria and mammalian cells. Our results showed that the hydrophobicity at all directions of the α-helix of this series of peptides contributed to the antimicrobial activity, but the hydrophobicity on one face was more favorable than that on the other (see Figs. 1 and 2). These results indicated that the peptides might adopt α-helical structures upon binding to the membranes of bacteria. The direction of the faces probably depended on the electrostatic interactions. The hydrophobicity and the direction of the hydrophobic face, not the amphipathicity, seem to be crucial for antimicrobial activity. In contrast, amphipathicity was important for hemolysis (see Figs. 2 and 3). Interestingly, we found that the face of the α-helix whose hydrophobicity was favorable for hemolysis was opposite to the face whose hydrophobicity was more favorable for antimicrobial activity, indicating that the direction of the amphipathicity was also important for hemolysis. The first step of the action of peptides to the membranes seems to be electrostatic interactions between the positively charged peptides and negatively charged phospholipid membranes. Then the orientation of the left face (see Fig. 2) probably exactly faces the hydrophobic fatty acyl chains in the membrane of bacteria. The right face might interact with a less hydrophobic environment, e.g. membrane proteins. In the binding of the peptides to the membrane of erythrocytes, the orientation of the right face probably exactly faces the hydrophobic fatty acyl chains.

It has been proposed that electrostatic interactions between the positively charged peptides and the negatively charged phospholipid membranes seem to play an important role in initial interactions, but biological activity appears to be driven by the hydrophobic interactions between the non-polar amino acids and the hydrophobic core of the lipid bilayer [1]. As both faces of the α-helix contributed to the hydrophobic interaction in the binding of the peptide to the membranes of bacteria, while only one face contributed to the binding of the peptide to the membranes of erythrocytes, the former binding was probably stronger than the latter. Therefore membranes of bacteria might be a much stronger promoter of α-helical structures than those of erythrocytes. This is supported by the results of Shai and coworkers [28–32], who designed a series of diastereomers of natural antimicrobial or model amphipathic peptides. The diastereomers were composed of both L- and D-amino acids which probably disrupt the formation of a helical structure. The CD spectra indicated that the diastereomers tested had little or no specific secondary structure in trifluoroethanol, a solvent strongly promoting α-helical structure [28–30]. However, ATR-FTIR data showed that the diastereomers adopted distorted or 3_{10}-helices when they were bound to phosphatidyletyanolamine (PE)/ phosphatidylglycerol (PG) (7/3, w/w) phospholipid, which mimics *E. coli* membranes [31,32]. These diastereomers retained antimicrobial activities and had much-reduced hemolytic activities.

It has been reported that retention times of peptides on RP-HPLC correlated with their hydrophobicities for some groups of peptides [33–35] or with their amphipathicities, which have often been quantitatively measured by hydrophobic moments, for others [36,37]. Our results belong to the former case, i.e. the antimicrobial activity of the peptides correlated well with their hydrophobicities. However, no correlation between the hemolytic activities and the hydrophobicities or the hydrophobic moments existed. These results are not surprising, as the hydrophobicities of all the directions of the α-helical structure were favorable for the antimicrobial activities, although one face was more favorable than the other, while not only the amphipathicities but their directions influenced the hemolytic activities.

5. Conclusion

By comparing the effect of the hydrophobicity at each position of residues 1–9 of M(12–26) on the antimicrobial and hemolytic activities, three main conclusions can be drawn: (1) The hydrophobicities in all the directions of the α-helical wheel projection of this series of peptides was favorable for the antimicrobial activity, but one face of the α-helix had a greater effect than the other. Thus the peptides might adopt α-helical structures upon binding to the membranes of bacteria. (2) The hydrophobicity on one face was favorable for the hemolytic activity, but unfavorable on the other. Thus amphipathicity might be important for hemolysis. (3) The direction of the face whose hydrophobicity was more favorable for the antimicrobial activity was opposite to that of the face whose hydrophobicity was favorable for hemolytic activity. The directions might depend on the electrostatic interactions.

Acknowledgements: This work was financially supported by the National Key Technology R&D Program from Ministry of Science and Technology of China (Grant No. 9690105154) and the Key Foundation Program from Tianjin Municipal Science and Technology Commission (Grant No. 033801811).

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