Correlation between the activities of α-helical antimicrobial peptides and hydrophobicities represented as RP HPLC retention times

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Abstract

PTP7 is a 13-amino acid residue peptide designed from gaegurin 6, an antimicrobial peptide isolated from skin secretions of Rana rugosa. In order to examine the effect of hydrophobicity on antimicrobial activity, a series of PTP7 derivatives were constructed and analyzed the activity against bacteria and artificial membrane. We found that the mean hydrophobicity by simple summation of hydrophobicity of each constituent amino acid did not necessarily describe the hydrophobic property of antimicrobial peptides. The mean hydrophobicity did not show close correlation with the observed hydrophobicity by measuring reverse phase high performance liquid chromatography (RP HPLC) retention time. The observed hydrophobicity represented as RP HPLC retention time correlated well with the activity against artificial membrane and Gram positive bacterial species, such as Staphylococcus aureus, Staphylococcus epidermidis, and Micrococcus luteus, rather than mean hydrophobicity. However, antimicrobial activity against Gram negative bacteria, such as Escherichia coli, did not show correlation with RP HPLC retention time. These data indicate that the RP HPLC retention time should be exploited rather than the mean hydrophobicity in the analysis of the relationship between hydrophobicity and antimicrobial activity.

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Keywords: RP HPLC retention time; Hydrophobicity; Antimicrobial peptide; PTP peptide; Gaegurin

1. Introduction

Many animals have innate immunity mediated by antibiotic peptides [7,9]. In many cases, they are released from secretory glands into internal body fluids or onto mucosal epithelia. They constitute the first line of host defense, and combat invading pathogens or control the natural flora. Many different families of antimicrobial peptides have been isolated from insects to mammals [3,9,17] and peptides from different structural classes frequently show synergy with each other [25]. Since magainins had been isolated from the skin of Xenopus laevis, a number of cationic peptides from the skin of various amphibians have been isolated and found to have a broad-spectrum of antimicrobial activity [1].

In order to improve antimicrobial activity, considerable efforts have been done on obtaining short synthetic peptides with increased potency than the native forms [10,15] and elucidating the relationship between the physicochemical parameters and activity [5,13,14,19,22]. Net positive charge, helicity, hydrophobic moment, angle subtended by charged residues, and hydrophobicity have been suggested as major parameters influencing the biological activity of α-helical antimicrobial peptides. Among these, the hydrophobic property of antimicrobial peptides has been recognized as important factor to promote hydrophobic interaction with hydrophobic core of the lipid bilayer [18]. With this mechanism, the increase of hydrophobicity of peptide enhances the antimicrobial activity [23,24]. However, some studies indicated that
no strong correlation exists between antibacterial activity and hydrophobicity suggesting the methods employed to measure hydrophobicity of peptides may not be appropriate to expect antimicrobial activity [8,21].

Previously, we made antimicrobial peptide derivatives based on gaegurin 6 (FLPLLAGAANFLPTICISYKC) by deletions and substitutions of amino acid [10]. PTP7 was the most potent derivative with near half the size of gaegurin 6. In this report, we designed a series of antimicrobial peptides based on PTP7, analyzed and found that the observed hydrophobicity by reverse phase high performance liquid chromatography (RP HPLC) retention time rather than the mean hydrophobicity by simple summation of the hydrophobicity of each constituent amino acid, correlates well with the activity of peptide against artificial membrane and Gram positive bacterial species.

2. Materials and methods

2.1. Materials

Chemicals for peptide synthesis were obtained from Applied Biosystems (Foster City, CA). Preparative 10 μm C18 RP HPLC column (22 mm i.d. × 250 mm) and analytical 5 μm C18 RP HPLC column (4.6 mm i.d. × 250 mm) were obtained from Vydac (Hesperia, CA). Egg phosphatidylcholine (PC), egg phosphatidylglycerol (PG), and calcine were purchased from Sigma. Sephadex G50 was obtained from Pharmacia. The bacterial species used were Staphylococcus aureus (ATCC6538), Micrococcus luteus (ATCC12228), Escherichia coli (ATCC4698) and Micrococcus luteus (ATCC12228) and were exported from Vydac (Hesperia, CA).

2.2. Synthesis of peptides

Peptides were synthesized on the basis of standard Fmoc (9-fluorenyl-methoxycarbonyl) chemistry with an Applied Biosystems 431A automatic peptide synthesizer [16]. Chlorotriyl resin was used as the support to obtain a carboxyated C-terminal peptide. The peptides were cleaved from the resin by incubating with a mixture of trifluoroacetic acid (TFA), 1,2-ethanedithiol, and thianisole (90:5:5, v/v) for 2 h at room temperature. After removing the resin by filtration through glass wool, the peptides were precipitated and washed in the presence of tert-butyl methyl ether (MTBE). The peptide pellet was dissolved in water and purified by preparative RP HPLC on a 10 μm C18 Vydac analytical column. The purity of peptides was checked by analytical RP HPLC on a 5 μm C18 Vydac analytical column. Theoretical molecular weights were calculated using DNA/S (Toshiba, Japan) and they were confirmed by mass spectrometry on a matrix-assisted laser desorption ionization (MALDI-II) using Voyager Biospectrometry Workstation (PerSeptive Biosystem, Framingham, USA). The purity and the concentration of peptides were also assessed on a 20% acetic–urea gel by comparing the band intensity of peptide with those of the serially diluted melittin at known concentration [20].

2.3. Sequence analysis

The mean hydrophobicity was calculated as mean per residue hydrophobicity. The amphipathicity of peptides was calculated by determining the mean hydrophobic moments (mH) of an idealized α-helix, in which side-chain protrude perpendicular to the helix axis at regular 10° intervals, using the Eisenberg equation [6]. In all calculations, the numerical values we used are in the consensus scale of Eisenberg’s.

2.4. RP HPLC analysis of peptides

Each peptide was subjected to a RP HPLC using an analytical C18 column (4.6 mm × 250 mm). The peptides were eluted using a linear gradient of 0–100% acetonitrile in 0.1% trifluoroacetic acid at flow rate of 2 ml/min for 30 min. The absorbance was measured at 224 nm. The RP HPLC retention time of each peptide was determined when the peak was at its maximum height.

2.5. CD spectroscopy

CD spectra were collected using a Jasco J-715 CD spectropolarimeter. The spectra were scanned at 20 °C in a quartz optical cell with a 0.2 mm path length. Spectral scans were performed from 250 to 190 nm with a step resolution of 0.5 nm, a speed of 50 nm/min. Samples were measured at peptide concentrations between 20 and 30 μM in 10 mM SDS micelles. The helicity was represented as θ222/θ208 which had been suggested as offering a concentration independent measure of helix content [13].

2.6. Antimicrobial assay

Stock cultures of bacteria were grown in Luria-Bertani (LB) media at 37 °C for 18 h. Minimal inhibitory concentrations (MICs) of antimicrobial agents were measured by the standard microdilution method as previously described [10]. In brief, bacterial cells (1 × 10⁶ CFU/ml) were incubated in LB broth containing peptides dissolved in deionized water. The MIC was defined as the lowest concentration of peptide or other antimicrobial agent that visibly inhibited bacterial growth after incubation at 37 °C for 16 h. Each determination was performed three times.

2.7. Preparation of lipid vesicle

Small unilamella vesicles (SUVs) were prepared for dye leakage as previously reported [26] with minor modifications. PC/PG mixture (7.5 mg; 2:1, w/w) was evaporated under a stream of nitrogen gas to form a thin lipid film on
the wall of a glass test tube. Dried lipid was resuspended with 2 ml of 10 mM Tris–HCl buffer (pH 7.4) containing 154 mM NaCl, 0.1 mM EDTA, and 70 mM calcein by vortexing for 10 min. The resulting lipid dispersions were then sonicated for 15 min until clear. The untrapped dye was removed from the SUVs by gel filtration chromatography on a Sephadex G-50 column using 10 mM Tris–HCl buffer. Lipid concentration was determined by phosphorus analysis [2].

2.8. Dye leakage

Calcine leakage from vesicles was determined by measuring the decrease in self-quenching. The fluorescence intensities of calcine released from SUV suspensions were monitored at 535 nm (excitation at 490 nm) on a Wallac 1420 Victor 2 (Perkin-Elmer, Finland) after 10 min incubation with peptide. The fluorescence intensity corresponding to 100% leakage was determined by addition of Triton X-100 to the sample (finally 0.1%, v/v). Effective concentration causing 50% dye release (EC50) was estimated from dose-response curves at 116/M lipids.

2.9. Hemolytic activity

Hemolytic activity of the peptides was determined as previously reported [20]. Hemolysis induced by the peptides were measured by incubating 10% (v/v) suspension of human RBCs in phosphate-buffered saline with 100/g/ml of peptides at 37 °C for 10 min. After centrifugation at 10,000 rpm for 10 min, optical density at 350 nm of the supernatant was measured. Zero percent and 100% hemolysis were determined in phosphate-buffered saline and 0.1% Triton-X100, respectively.

![Helical wheel projection of PTP7. Black circles refer to charged amino acids.](image)

3. Results

3.1. Design and chemical synthesis of peptides

In order to examine the factors influencing the activity of antimicrobial peptides, we designed a series of antimicrobial peptides based on PTP7, the 13-residued antimicrobial peptide based on gaegurin 6 with most potent activity in our previous report [10]. As shown in the helical presentation of PTP7 (Fig. 1), the hydrophobic and charged residues of PTP7 are clustered. Subsequent PTP7 derivatives were designed in a way that the amphipathic characters of peptides was preserved. In order to make peptides with main-

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
<th>Charge</th>
<th>Angle</th>
<th>Φ</th>
<th>Ψ</th>
<th>Helicitya</th>
<th>t (min)</th>
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</thead>
<tbody>
<tr>
<td>PTP7</td>
<td>FLGALKALSKLL</td>
<td>+2</td>
<td>60</td>
<td>0.338</td>
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<td>20.408</td>
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<td>0.618</td>
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<tr>
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<td>0.204</td>
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<td>0.132</td>
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</tr>
<tr>
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<td>0.116</td>
<td>0.537</td>
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<td>0.161</td>
<td>0.587</td>
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<td>100</td>
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<td>0.106</td>
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<tr>
<td>PTP7v3</td>
<td>FLGALKALSKLL</td>
<td>+4</td>
<td>120</td>
<td>0.447</td>
<td>−0.002</td>
<td>0.669</td>
<td>19.650</td>
</tr>
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</table>

Bold letters designate the changed amino acid residues from PTP7.
a Φ, mean hydrophobicity per residue.
b Ψ, hydrophobic moment.
c RP-HPLC retention time.
d Helicity was represented as θ222 /θ208.
tained mean hydrophobicity, leucine and/or phenylalanine residues were substituted to valine. Substitutions of alanine to valine were done to increase mean hydrophobicity of peptides and substitutions of leucine to alanine were done to decrease mean hydrophobicity. The amino acid sequences used in this study are summarized in Table 1. The purity of peptides was checked by analytical reverse phase HPLC (>95%) and their corresponding molecular weights were confirmed by MALDI-TOF mass spectrometry.

3.2. Physicochemical parameters of peptides

The physicochemical parameters of the peptides were determined experimentally or theoretically (Table 1). The mean hydrophobicities per residue and the hydrophobic moments were calculated using Eisenberg consensus scale of hydrophobicity and the observed hydrophobicities were determined by measuring the RP HPLC retention times of the peptides. According to Eisenberg's consensus scale, the hydrophobicities of leucine and valine are very similar, 0.53 and 0.54, respectively [6]. Valine, however, has lower helix propensity than leucine [4]. With this propensity of leucine and valine, the insertions of a valine residue at each leucine position made less helical peptides while maintaining similar mean hydrophobicity (Table 1). Plots of RP HPLC retention times against mean hydrophobicity showed that mean hydrophobicity did not necessarily describe the hydrophobic property of peptides (Fig. 2). Peptides with reduced mean hydrophobicity, such as PTP7a6, PTP7a2a5, PTP7k3, and PTP7k3k10, were eluted earlier than PTP7. PTP7v4v8 with increased mean hydrophobicity, was eluted later than PTP7. Some peptides, however, did not show correlation between the mean hydrophobicity and the RP HPLC retention time. The RP HPLC retention times of such peptides were decreased by the insertion of valine in spite of the similar mean hydrophobicity. Furthermore, PTP7v9 was eluted earlier than PTP7 in spite of the increased mean hydrophobicity.

3.3. Biological activity of the peptides

In order to examine which hydrophobicity measure, either mean hydrophobicity or RP HPLC retention time, correlated better with biological activity of peptide, peptides were tested for their antibacterial activity (Table 2). The resultant MICs against Gram positive bacterial species, such as S. aureus, S. epidermidis, and M. luteus, are shown in Table 2. PTP7v4v8, the peptide eluted latest from a C-18 column, showed the most potent antimicrobial activity, and PTP7a2a5, the peptide eluted first, showed the lowest antimicrobial activity. Plots of RP HPLC retention time against antimicrobial activity indicated a significant correlation. Plots of RP HPLC retention time against antimicrobial activity for specific peptides, such as PTP7v4v8 and PTP7v8a9v11, in particular, showed almost 3 min of difference in RP HPLC retention time with respect to PTP7 in spite of similar mean hydrophobicity.

Table 2

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Antimicrobial activity, MIC (µg/ml)</th>
<th>EC50 (µg/ml)</th>
<th>%Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. aureus</td>
<td>S. epidermidis</td>
<td>M. luteus</td>
</tr>
<tr>
<td>PTP7</td>
<td>5</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>PTP7v2</td>
<td>12</td>
<td>22</td>
<td>6</td>
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<tr>
<td>PTP7v5</td>
<td>11</td>
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<td>6</td>
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<td>PTP7v9</td>
<td>12</td>
<td>22</td>
<td>7</td>
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<td>PTP7v12</td>
<td>13</td>
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<td>8</td>
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<td>PTP7v13</td>
<td>11</td>
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<td>6</td>
</tr>
<tr>
<td>PTP7v6</td>
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<td>5</td>
</tr>
<tr>
<td>PTP7v5v6</td>
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<td>3</td>
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<td>28</td>
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<td>16</td>
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<tr>
<td>PTP7k3k10</td>
<td>9</td>
<td>10</td>
<td>3</td>
</tr>
</tbody>
</table>

Fig. 2. Relationship between mean hydrophobicity and retention time.
Fig. 3. Correlation between the retention time on RP HPLC and the antibacterial activity against *S. aureus* (a) and *E. coli* (b).

Cated that the increase of RP HPLC retention time enhanced the antibacterial effect (Fig. 3a). The antimicrobial activity against *E. coli*, however, did not show correlation with the RP HPLC retention times (Table 2). Plots of retention time against antimicrobial activity showed that the relationship between the antimicrobial activity and the RP HPLC retention time of *E. coli* was quite different from that of Gram positive bacteria (Fig. 3b). In addition to the relationship between RP HPLC retention time and activity, the peptides with increased net charge, PTP7k3 (+3) and PTP7k3k10 (+4), showed increased antimicrobial activity (Table 2). The peptides were also tested for cytolytic activity on human red blood cells. As shown in Table 2, hemolytic activity of the peptides with +2 charges was increased as RP HPLC retention times increased. The increase of RP HPLC retention time, however, did not show significant effect on hemolytic activity. PTP7v4v8, which was eluted latest from RP HPLC column, showed only 7.67% hemolysis. It should be noted that the increased net charge to +3 and +4 led to two- and three-fold increase of hemolytic effect, respectively.

3.4. Peptide-induced membrane permeation

The peptides were tested for their membrane permeability activity by measuring the efflux of fluorescence dye, calcein, from lipid vesicles of PG:PC (1:2) composition. EC_{50} were determined at lipid concentrations of 116 μM. All peptides showed a permeabilizing activity against lipid vesicles (Table 2). The activity pattern on the lipid vesicles showed similar tendency to that on Gram positive bacteria (Fig. 4). PTP7v4v8, the most potent peptide against Gram positive bacteria, showed the highest activity on artificial membrane whereas PTP7a2a5, the least potent peptide against Gram positive bacteria, showed the lowest activity. PTP7v8a9v11, a peptide with similar mean hydrophobicity but with 3 min difference of RP HPLC retention time compared to PTP7, showed almost 10-fold weaker activity than PTP7 on lipid vesicles. These results showed that RP HPLC retention time correlated well with membrane permeabilizing activity of peptide rather than mean hydrophobicity.

4. Discussion

In developing antimicrobial agent originated from antimicrobial peptide, it is very important to elucidate the relationship between activity and physicochemical features, such as hydrophobicity, net positive charge, helicity, hydrophobic
moment, and angle subtended by charged residues. It was suggested that hydrophobicity is important in interaction between antimicrobial peptide and the hydrophobic core of lipid bilayer [18,23,24]. Several reports, however, indicated that no strong correlation exists between antimicrobial effect and hydrophobicity [8,21]. This disagreement may be caused from the difference between actual hydrophobicity in liquid environment and theoretical mean hydrophobicity. To confirm this notion, we examined the effect of hydrophobicity represented as RP HPLC retention times and mean hydrophobicity on activity of peptides.

It was suggested that the access of water to CO groups in peptide backbone is important in hydrophobicity of a helical peptide [12]. The CO groups in frayed region may be more favorable for water to access than those in helix region and cause reduction of the hydrophobic property of peptides with similar mean hydrophobicity. Therefore, experimented hydrophobicity of similar mean hydrophobicity may be dependent on helicity. Our results showed that the experimented hydrophobicity obtained by measuring retention time on HPLC and the mean hydrophobicity and did not show close correlation (Fig. 2) and that peptides with similar mean hydrophobicity but with reduced helicity inserting valine residues were eluted earlier from RP HPLC column than those with higher helicity (Table 2). The activity against Gram positive bacteria and artificial membrane showed close correlation with RP HPLC retention time (Figs. 3a and 4). These results showed that RP HPLC retention time represented hydrophobic property of antimicrobial peptide better than mean hydrophobicity.

Alanine has been reported as an amino acid with high helix-making propensity [4]. The substitution of leucine or phenylalanine to alanine, however, dropped the helicity of PTP7 derivatives (Table 1). This conflict is caused from the difference of environmental condition. It was reported that helix propensities depend on temperature and hydrophobicity of environment [11,12]. Leucine showed more helical propensity than alanine in more hydrophobic environments, such as 10 mM SDS or 30% TFE buffer at room temperature whereas alanine showed more helical propensity than leucine in phosphate buffer at 0 and 5 °C [12]. Therefore, the peptides with alanine were less helical than PTP7 in our experimental conditions.

PTP7 and PTP7v4v8 showed strong antimicrobial activity against Gram positive bacterial species (Table 2). They, however, showed low activity against E. coli. This discrepancy between the activity against Gram positive and negative bacteria may be caused from the difference in the cell wall composition. Because Gram negative bacteria have both the inner membrane and the outer membrane with lippopolysaccharide (LPS), the peptide should move across the outer bilayer to reach the inner membrane. Therefore, the antimicrobial activity against Gram negative bacteria depends also on its ability to diffuse through the cell outer membrane as well as the ability to interact and permeate the bacterial inner membrane. Therefore, other peptide characteristics which play a key role to reach bacterial inner membrane are required for a peptide to be potent against Gram negative bacteria.

Hemolytic activity of peptides with +2 charges was increased as the RP HPLC retention time was increased (Table 2). The effect of increase in charge on hemolysis, however, was more drastic than the effect of hydrophobicity increase. Previously, it was reported that charge increase led to a striking increase of hemolytic effect of magainin derivatives and the low hemolytic activity was restored by decrease of hydrophobicity of hydrophobic helix surface [5]. An increase in net positive charge enhances accumulation of peptide lipid head groups of erythrocyte membrane whereas an increase in hydrophobicity may increase the ability of peptide to interact with membrane. As shown in Fig. 1, hydrophobic property of hydrophobic helix surface of PTP7 is high enough to interact with membrane of erythrocyte. Therefore, net positive charge may be limiting factor of hemolytic activity of PTP peptides. These results suggested that the net positive charge is a very important factor in modulating hemolytic activity of peptides with high hydrophobic property.

In conclusion, actual hydrophobic property of peptide is dependent not only on hydrophobicity of constituent amino acid but also on helical propensity of peptide. Furthermore, experimented hydrophobicity represented as RP HPLC retention time showed close correlation with activity against Gram positive bacteria and PC/PG membrane. Therefore, RP HPLC retention time, rather than mean hydrophobicity, should be considered as hydrophobicity in the analysis of the relationship between physicochemical parameters and activity. These results may provide insight into the factors that should be considered in the design of antimicrobial peptides.

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