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# Solid Phase Chemical Synthesis and Structure - Activity Study of Brevinin - 2R and Analogues as Antimicrobial Peptides

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**ARTICLE INFO**

***Article type:***
Original Article

***Article history:***
Received: 23 Dec 2012
Revised: 27 Jan 2013
Accepted: 20 Feb 2013

***Keywords:***
Brevinin-2, Rana
Protein Structure,
Secondary
Circular Dishroism

**ABSTRACT**

**Background:** Brevinin-2R, as 25 amino acids peptide of the skin of *Rana ridibunda* frog, possesses potent antimicrobial and low hemolytic activity. It has an N-terminal hydrophilic region and a C-terminal loop that is delineated by an intra-disulfide bridge. In our study, Brevinin-2R and its diastereomer as well as its cyclic analogue were synthesized and characterized in order to investigate its structural features and biological implications.

**Methods**: MIC determination is based on the recommended classical method of national comittee for labratory safety standard (NCLSS) and standard by Hancock With some change on cationic peptides. In this study All bacterial strains were obtained from Industrial-Scientific Research center.

**Results**: Both analogues showed lower antimicrobial activities compared to Brevinin-2R. In spite of Brevinin-2R peptide which shows low hemolytic activity, these analogues failed to show any hemolytic activity even at higher concentrations (up to 400 µg/ml). Based on proteolytic stability measurements, diastereomer and cyclic analogues displayed 90% and 60% residual antimicrobial activity, respectively, while antimicrobial activity of Brevinin-2R was 20%. The CD analysis revealed that amphipathic $\alpha$-helical conformation of the synthesized peptides is involved in antimicrobial effects.

**Conclusion**: CD studies and HPLC based measurement of retention time using a reverse phase column indicated that the Brevinin-2R can form an amphipathic loop resulting in an enhanced hydrophobicity. The hemolytic activity of Brevinin-2R and its analogues appeared to correlate with the retention time as well as the α-helicity. Accordingly, it seems that the combination of incorporating of D-amino acids into lytic peptides and their cyclization may result in developing new antimicrobial peptides with improved properties for treating infectious diseases.

- ***Please cite this paper as:*** Yaghoubi H, Asef A, Banki A. Solid phase chemical Synthesis and Structure-Activity Study of Brevinin -2R and Analogues as Antimicrobial Peptides. *J Med Bacteriol.* 2013; **2** (1, 2): pp. 41-53.

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## Introduction

Granular glands in the skin of certain anurans (e.g., frogs and toads) are capable of synthesizing and storing polypeptides which belong to the super family of defensins with a broad range of antimicrobial activity. These defensins are a vital component of the innate immunity system that protects anurans from colonization and invasion with pathogenic microorganisms (1, 2). These peptides have attracted attention as potential therapeutic agents, for use against multi-resistant bacteria (3). The genus *Rana* seem to be a successful group of frogs with at least 250 species distributed worldwide (4). They are valuable sources of antimicrobial defensins with unique peptide sequences among different *Rana* species (5). Although there are no common amino acids sequences and that of and biological activities, these peptides display invariable cationic and relatively hydrophobic characteristics. They also possess the propensity to form an amphipathic helix in a membrane-mimetic environment (6). These kinds of defensive antimicrobial peptides have attracted researchers' attention because of their low toxicity against mammalian cells and their unique biological potential of perturbing the membrane of the microorganism (7-9).

It is believed that these peptides act on the lipid membranes of microorganisms even though their precise mechanisms are yet to be fully understood (10-14). Basically, the positively charged peptides first bind to the negatively charged lipid membranes of the pathogen, which result in adapting α-helical or β-sheet structures. Such process can increase the permeability of the lipid membranes either by channel formation (barrel- stave model) (15) or by the perturbation of the structure of the bilayer (carpet model) (15), culminating in death of the target cells.

Several structure-activity relationship studies about linear antimicrobial peptides indicate that the net positive charge, hydrophobicity and α-helical structure are dominant factors for the activity and specificity (16-23). Furthermore, the high α-helicity and / or high hydrophobicity appear to be the major factors which are critically related to the mammalian cell toxicity, rather than antibacterial activity (14, 24-26). There are also reports about the unique contribution of net positive charge and hydrophobicity and their relationship to antimicrobial activity and cytotoxicity in mammalian cell.

In the present study, we designed and synthesized Brevinin-2R (BR) and its diastereomer (BR-D) as well as its cyclic analogue (BR-C) in order to investigate its structural features and biological implications. Peptides with similar net positive charge and investigated the effect of their α-helical structure and hydrophobicity on the Gram-positive and Gram-negative bacteria. To pursue such aim, the Brevinin-2R was selected due to following reasons:

1. This peptide has an amphipathic α-helical structure and high net positive charge (27)
2. It shows very potent activity against cancer cells (27)
3. It is a novel peptide with antimicrobial properties.

## Materials and Methods

*Materials*

Fmoc-amino acids, 2-cltritylcl resin, and 2-(1H-benzotriazol-1-yl)- 1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) were obtained from Bachem (Switzerland). diisopropylethylamine (DIPEA), piperidine and other solvents such as dichloromethane (DCM), dimethyl formamide (DMF), triisopropylsilane (TIS), dimethylsulfoxide (DMSO), triflouroacetic (TFA) and acetonitrile (ACN) were from Merck (Germany). Cell concentrations were estimated by measuring the optical density of colony- forming units (CFU) / ml at 620 nm. MIC determination based on the recommended classical method of national comittee for labratory safety standard (NCLSS) and standard by Hancock With some change on cationic peptides. (51)

In this study all bacterial strains were obtained from Industrial-Scientific Research center. (*Methicillin resistant Staphylococcus aureus*) MRSA . (*Bacillus cereus*) 1015. (*Staphylococcus aureus*) 1112. (*Staphylococcus epidermidis*)1114.

*Peptide Synthesis and Purification Procedures*

Two different analogues were designed, synthesized and characterized and named as follows:

BR-D

(K**L**KNFAKGVAQS**LL**NKASCK**L**SGQC) containing D-amino acids (Leu)

BR-C

(CKLKNFAKGVAQSLLNKASKLSGQC) which is cyclized by a N-terminal to C-terminal disulfide bond. For synthesis of Brevinin-2R and its analogues, reaction was manually accomplished in a reaction vessel using 2-cltritylcl resin, following standard Fmoc strategy. Fmoc-Cys (Trt)-OH was attached to the 2-cltritylcl resin with DIPEA in anhydrous DCM: DMF (1:1) at room temperature for 2 h. 20% (v/v) piperidine in DMF was used for deprotection step. Coupling was performed using a coupling reagent (i.e., TBTU) in the presence of DIPEA as a base. A solution of Fmoc-AA-OH, TBTU, and DIPEA in DMF was added to the resin-bond free amine, shook for 3h at room temperature and then checked with Kaiser Test for the peptide bond formation. These steps were repeated until the last amino acid was added to the peptide-resin. Cleavage of the peptide from the resin was fulfilled by treatment with a mixture of trifluoroacetic acid (TFA) / thioanisole / ethandithiol / $H_2O$ in the ratio of 90: 5: 2.5: 2.5 (v/v) at room temperature for 4 h. After filtration and washing of resin with TFA, a gentle stream of nitrogen was applied to remove excess TFA. Crude peptide was triturated with diethyl ether chilled at -20˚C and then centrifuged at 3000 rpm for 10 min. Synthesized peptides were purified on Vydac C18 semi-prep column ( 1 cm diameter, 25 cm length) using ÄKTA purifier10 HPLC system (Amersham Bioscience, USA). Separation was achieved with a linear gradient of 0-60% B in 60 min in the eluent A (0.1% TFA in deionized water) and the eluent B (0.1% TFA in CAN). Purified peptide was confirmed by mass spectrometer (LTQ orbitrap, Thermo Electra, San Jose, CA).

*Synthesis of the Intra-disulfide Bridge*

The linear form of Brevinin-2R and analogues were diluted below 20 mM in 0.01 M phosphate buffer at pH 7 in the presence of 10% (v/v) DMSO (28). After stirring at 400 rpm and room temperature for 24 h and

completion of the oxidation, excess solvent was removed using a rotary evaporator and the peptide was purified by RP-HPLC. For characterization of oxidized peptide, mass spectrometer (LTQ orbitrap, Thermo Electra, San Jose, CA) was employed.

*Antibacterial Activity*

Brevinin-2R and analogues were tested for antibacterial activity against Gram-positive and Gram-negative bacteria. The antibacterial activity of peptides was carried out in sterile 96-well microplate (Nunc F96 microtiter plate, Denmark) in a final volume of 100 µl. Aliquots (90 µl) of a suspension containing bacteria at a concentration of $10^6$ colony-forming units (CFU)/ml in culture medium (MHB) were added to 10 µl of water containing the peptide in 2-fold dilutions in water. The plates were incubated at 37°C for 24 h and the absorbance at 620 nm was measured by ELISA reader (Spectra, Anthos 2020, Austria) to assess cell growth. The minimal inhibitory concentration (MIC) was defined as the dose at which 50% inhibition of growth is observed. Each MIC was determined from three independent experiments performed in duplicate.

*Hemolytic Activity*

Hemolytic activity was assayed as previously described with minor modifications (29). Freshly prepared human erythrocytes (3 ml) were washed (×3) with PBS at pH 7.4. They were then diluted to a final volume of 20 ml with the same buffer. Peptides stock solutions (20 µl), serially diluted in PBS, and were added to 180 µl of the cell suspension. Following a gentle mixing, tubes were incubated at 37°C for 60 min prior to centrifugation at 4000 g for 5 min. Supernatant (100 µl) was diluted to 1 ml with PBS and the absorbance was determined at 567 nm. The hemolysis affected by 0.2% Triton X-100 was considered 100% hemolysis.

*Proteolytic Stability*

In order to evaluate the proteolytic stability, Brevinin-2R and analogues were dissolved in PBS buffer at pH 7.4, and aliquoted into Eppendorf tubes. Trypsin was mixed with peptides at 1: 250 ratios (enzyme / peptide, w/w). The enzymatic degradation of peptides by trypsin was carried out at 37°C for 4 h, and stopped by adding a trypsin inhibitor type II-S, soybean (Sigma) at different time points. The samples were tested for their residual antimicrobial activity and analyzed using RP-HPLC (30, 31).

*Circular Dichroism (CD)*

The CD spectra were measured in either 20 mM sodium phosphate buffer (NaPB), or 50% (v/v) trifluoroethanol (TFE) in 20 mM NaPB, using solutions with peptide concentrations of about 0.2 mg/ml by means of a circular dichroism, JASCO J-715 spectopolarimeter (Japan). The CD results were expressed as molar ellipticity [θ] (deg. $cm^2d.\ mol^{-1}$) based on a mean amino acid residue weight (MRW) assuming its average weight 110 (32). The molar ellipticity was determined as [θ] = (θ. 100 MRW) / (cl), where c is protein concentration (mg/mL), l is the light path length in cm, and θ is the measured ellipticity in degree at wavelength λ. Noise of data was smoothed through the fast Fourier-transform noise reduction routine which allows enhancement of most

noisy spectra without distorting their peak shape (32). The percentage of α-helix was calculated from the mean residue ellipticity at 222 nm in CD spectra by the method of Chen et al. (33).

## Results

*Design and Synthesis of Brevinin-2R and its Analogues*

The sequences and characteristics of brevinin-2R and its analogues exploited in the present investigation are described in Table 1. Two analogues, with the same net charge as Brevinin-2R, but with different structure were synthesized to investigate the function of α-helicity and hydrophobicity. In was previously reported that Brevinin-2R have potent effects on cancer cells (27). Thus, to advance our previous study, we synthesized BR-D and BR-C to look at the possible relationship between the structural features of Brevinin-2R of and their biological activities. All peptides were synthesized in solid phase and characterized by mass spectrometer. The purity of each synthetic peptide was above 95% as measured by analytical HPLC. The hydrophobic moment of the peptides was calculated using the Eisenberg method (34) and were compared with the α-helicity measured in the lipid membrane condition by CD spectroscopy. As shown in Table 1, Brevinin-2R and its analogues had different retention times on the C18 reverse phase HPLC column containing $CH_3CN$-$H_2O$ as eluent. The retention time, which reflects the hydrophobic interactions between the peptides and the C18 stationary phase, appeared to be parallel with the hydrophobic moment of Brevinin-2R and its analogues. Comparison of the retention times revealed that Brevinin-2R had a late retention time and high hydrophobic moment than that of BR-D and BR-C analogues.

*Antimicrobial Activity*

The activities of the peptides against Gram-positive and Gram-negative are shown in Table 2. Although all peptides had antimicrobial activity, based upon the MIC results, Brevinin-2R imposed greater impacts than that of analogues on Gram-positive and Gram-negative bacteria. These data showed that BR-C activity on Gram-negative bacteria is more effective than BR-D, whereas both of them had relatively similar effect on Gram-positive bacteria. These results indicated that hydrophobicity plays an important role in antimicrobial activity. Since the antimicrobial activity was affected by a delicate balance between net positive charge, hydrophobicity and α-helical structure; increasing the hydrophobicity resulted in improving the antimicrobial activity (35).

*Hemolytic Activity and Proteolytic Stability*

The results of hemolytic activity measurements of Brevinin-2R, BR-C and BR-D are shown in Figure 1. It is clear that, in contrast to Brevinin-2R which showed very low hemolytic activity up to 400 µg/ml (1%), tested peptides of BR-C and BR-D had no hemolytic activity in similar condition, perhaps due to decreased hydrophobicity and α- helicity in BR-C and BR-D. This literally means that the hemolytic activity of the majority of antimicrobial peptides increases with increasing their hydrophobicity and decreasing their net positive charge (36-38).

The proteolytic stability of the peptides to trypsin is shown in Figure 2. The activity of the trypsin-treated, BR-D and BR-C peptides was 90 and 60% of the control after 4 h, respectively, whereas antimicrobial activity of Brevinin-2R decreased to 20% of the control after 4 h. Degradation was observed by analyzing the trypsin-treated peptides using RP-HPLC. These results showed that the presence of D-amino acids and cyclization have important consequences in the proteolytic stability of the peptides.

**Table 1.** Sequences and characteristics of Brevinin-2R and its analogues

| Peptide | Sequence [a] | μH [b] | $t_R$ (min)[c] | α-helicity (%)[d] |
|---|---|---|---|---|
| **Brevinin-2R** | KLKNFAKGVAQSLLNKASCKLSGQC | 0.2 | 28 | 53 |
| **BR-C** | CKLKNFAKGVAQSLLNKASKLSGQC | 0.14 | 24 | 14 |
| **BR-D** | K**L**KNFAKGVAQS**LL**NKASC**K**K**L**SGQC0.7 | 0.1 | 19 | 12 |

[a] Underlined bold amino acids are D-amino acids.
[b] Hydrophobic moment (μH) were calculated using Einsenberg method (34).
[c] Retention times were determined on a C18 reverse column
[d] α-Helicity was evaluated by the method of Chen et al. (33).
The underlined letters represent cysteine residues linked together with disulphide bonds

**Table 2.** Antimicrobial activity of Brevinin-2R and its analogues

| Bacteria | Minimal inhibition concentration (MIC*, μg/ml) | | |
|---|---|---|---|
| | **Brevinin-2R** | **BR-D** | **BR-C** |
| (***Methicillin resistant Staphylococcus aureus***) **MRSA** | 6.25 | 25 | 25 |
| ***Bacillus cereus* 1015** | 0.78 | 3.12 | 3.12 |
| ***Staphylococcus aureus* 1112** | 1.56 | 6.25 | 6.25 |
| ***Staphylococcus epidermidis* 1114** | 0.39 | 0.78 | 1.56 |
| ***Pseudomonas aeruginosa* ATCC27853** | 25 | 100 | 25 |
| ***Klebsiella pneumoniae* ATCC13883** | 12.5 | 25 | 25 |
| ***Escherichia coli* ATCC2592** | 0.39 | 12.5 | 6.25 |

*Each MIC was determined from three independent experiments performed in duplicate

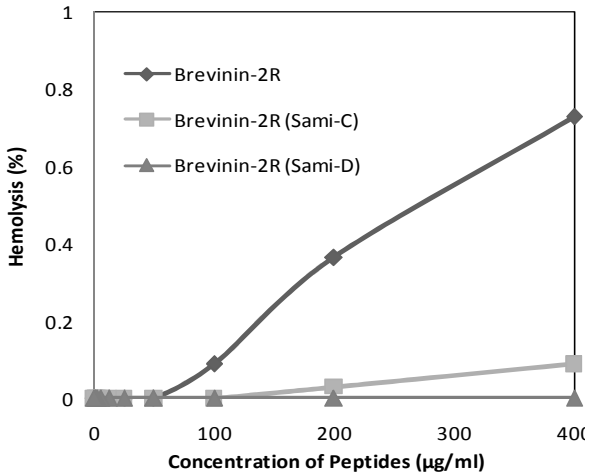


**Figure 1.** Low hemolytic activity of Brevinin-2R and its analogoues toward hRBC. hRBC hemolysed with 0.2% Triton-X100 served as a positive control and hRBC treated with PBS served as a negative control. Hemolysis was determined from triplicate measurements of three independent experiments

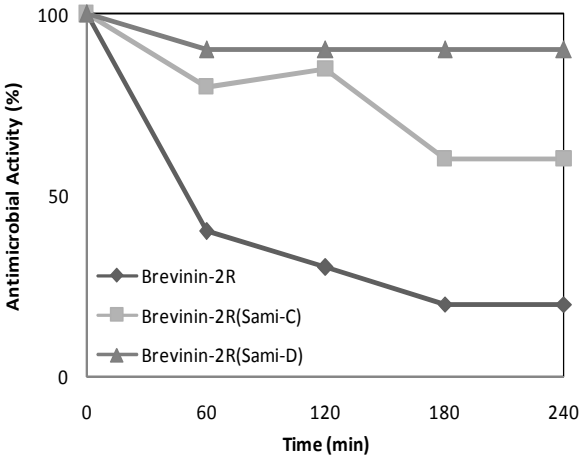


**Figure 2.** Antimicrobial activity of Brevinin-2R and its analogoues by trypsin treatment. Trypsin was mixed with peptides at 1:250 ratios (enzyme/peptide, w/w). Each enzymatic degradation was determined from two independent experiments performed in duplicate

*CD spectroscopy of Brevinin -2R and its Analogues*

Structure-activity studies on antimicrobial peptides indicated that the secondary structure, induced in membrane-mimetic environments, rather than that in phosphate buffer, these results are well agreed with those of activity, measured at corresponding conditions (36-38). To investigate the effect of D-amino acids substitution and cyclization on the secondary structure of the Brevinin-2R, CD spectra were measured. As shown in Figure 3A, peptides had random structure in phosphate buffer. The mean residue ellipticity at 222 nm ([θ] 222) and the corresponding fractional helicity of Brevinin-2R and its analogues are summarized in Table 1. It should be noticed that CD spectra with negative ellipticity at 208 and 222 nm revealed that Brevinin-2R had a predominantly α-helical structure in the presence of 50% TFE (pH 7.4) as shown in Figure 3B. The α-helical content of BR-D and BR-C was decreased and both of these peptides had similar secondary structure in 50% TFE, indicating that the incorporation of D-Leu in BR-D and BR-C destabilizes α-helical structure.

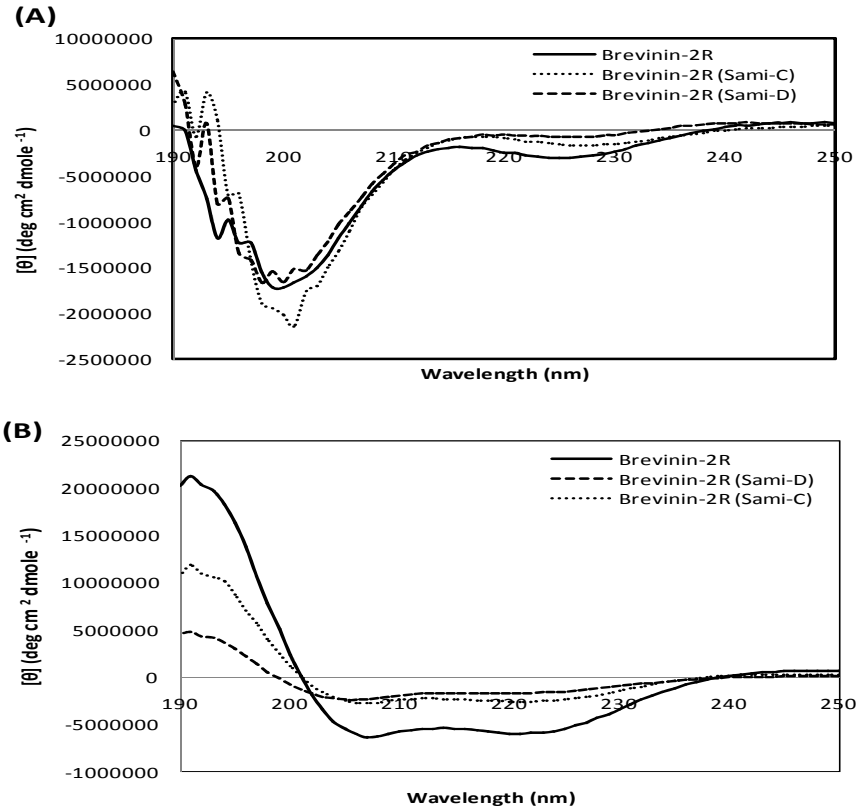


**Figure 3.** CD spectra of Brevinin-2R and its analogoues in 20 mM phosphate buffer, pH7.4 (A) and in 20 mM phosphate buffer (pH7.4) with 50% TFE (v/v) (B). TFE: Trifluoroethanol

## Discussion

So far, it has been reported that structural parameters such as net positive charge, hydrophobicity, peptide helicity, hydrophobic moment, and the size (angle) of hydrophobic / hydrophilic domain influence the activity and selectivity of membrane-active peptides (16-23). Among these parameters, net positive charge, helicity, and hydrophobicity appear to be the most fundamental factors for activity and selectivity (17, 20). Besides, it is generally believed that the change of net positive charge and hydrophobicity, which result in the change of α-helical structure, is an important factor for the specificity toward neutrally charged membrane (23-26). The Brevinin-2R is a novel peptide that unlike other Brevinins showed no significant hemolytic activity. In this study, to investigate the biological implications of Brevinin-2R and its structure-activity relationship, we synthesized the analogues of Brevinin-2R (BR-D and BR-C) with net positive charge and different α-helical structure and hydrophobicity similar to that of Brevinin-2R. Based on our previous investigation showing the Brevinin-2R impacts on different cancer cells (Patent No.: WO / 2006 / 128289), we decided to study the effect of α-helical structure and hydrophobicity on the specificity against bacteria. Accordingly, we synthesized BR-D and BR-C analogues. Based upon our findings, increase of α-helical structure and hydrophobicity enhanced antimicrobial activity on Gram-positive and Gram-negative bacteria also on cancer cells (data not shown) than BR-D and BR-C analogues.

Considering the general biological mechanism of membrane-active peptides, it is expected that they bind to lipid membrane of microorganisms to impose their biological action. In fact, interactions between the positively charged peptides and the negatively charged lipid membranes are the most critical force for such binding. However, if sufficient charge-charge interactions have already existed, the decrease of the α-helical structure and hydrophobicity may affect binding. Therefore, since the α-helical structure and hydrophobicity of the peptides are the fundamental requirements for their activity (17, 18, 20, 21), it seems that there is a threshold of hydrophobicity and α-helical structure which is essential for insertion of peptides into lipid membranes. We speculate that the hydrophobicity and α-helical structure of the peptides employed in our study were beyond of such threshold.

Based upon our findings, modifications of Brevinin-2R did not change net positive charge, which made it possible to elucidate the effect of the secondary structure and hydrophobicity on the activity. Interestingly, we observed a good linear correlation between the antibacterial activity and α-helical content of Brevinin-2R and its analogues (Tables 1, 2).

The relationship between biological activity and retention time of peptides has been so far well documented (39-43). According to our results, we speculate that the retention time on C18 column was related to the hydrophobicity of the peptide in the stationary phase. Note that Brevinin-2R has an intra-disulfide bridge located in the positively charged C-terminal region which can form an amphiphilic loop. This may

result in an increase in retention time and accordingly hydrophobicity. Nevertheless, such structure was not observed in BR-D, which may be ascribed to disruption of α-helical structure and hydrophobicity by D-Leu. In fact the hydrophobicity of BR-C is higher than BR-D, hence it displayed greater antibacterial activity against Gram-negative bacteria compare to BR-D. Such relationship between hydrophobicity and biological activity has been already reported (44). From cell membrane and lipid bilayer viewpoint, it can be also concluded the greater the hydrophobicity, the higher the fusion into lipid bilayer and cell membrane.

The observed change of hydrophobicity is well interpreted parallel with the change of hemolytic activity. As shown in Fig. 1, there exists a relationship between hemolytic activity and hydrophobicity. This relationship is in consistent with several studies, in which it has been shown, that hydrophobicity of the peptides effectively modulate hemolytic activity (45). That is because the phospholipids composing the inner membrane of Gram-negative and the single membrane of Gram-positive bacteria are negatively charged. However, the outer leaflet of normal mammalian cells is composed predominantly of zwitterionic phosphatydil choline and sphingomyelin phospholipids. Thus, Brevinin-2R possessed a high hydrophobicity than its analogues and subsequently it imposed hemolytic activity higher than analogues on mammalian cells.

We have also investigated peptide stability by determining the stability in tyrpsin. A facile method for increasing the stability of peptides is to replace natural amino acids in the cleavage site by D-amino acids. Using such method, we found that the stability of BR-D was increased while BR-C showed just 60% remaining activity. Perhaps, this is because N-terminal and C-terminal had linked together in BR-C and trypsin could not degrade the peptide easily (46–49). Brevinin-2R also showed 20% remaining activity. In contrast to its analogues, Brevinin-2R displayed very potent site to degradation with trypsin. To improve the stability of antimicrobial peptides without affecting their activity, therefore, the effect of the replacement of D-amino acid and cyclization on the secondary structure and hydrophobicity must be considered (46–50). Given all these results, we propose that there must be a threshold of hydrophobicity and α-helicity, if sufficient charge-charge interactions have already existed.

## Conclusion

The modulation between the hydrophobicity and α-helical content without changing net positive charge, improves antimicrobial activity while increases hemolytic activity. It is demonstrated that the hemolytic activity of the majority of antimicrobial peptides increase with increasing their hydrophobicity. Moreover, incorporation of D- amino acids and cyclization could also improve proteolytic stability.

## Acknowledgment

None declared.

## Conflict of Interest

None declared conflicts of interest.

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