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Original Article

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D-amino acid substitution enhances the stability of antimicrobial peptide polybia-CP

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Abstract

With the increasing emergence of resistant microbes toward conventional antimicrobial agents, there is an urgent need for the development of antimicrobial agents with novel action mode. Antimicrobial peptides (AMPs) are believed to be one kind of ideal alternatives. However, AMPs can be easily degraded by protease, which limited their therapeutic use. In the present study, D-amino acid substitution strategy was employed to enhance the stability of polybia-CP. We investigated the stability of peptides against the degradation of trypsin and chymotrypsin by determining the antimicrobial activity or determining the HPLC profile of peptides after incubation with proteases. Our results showed that both the all D-amino acid derivative (D-CP) and partial D-lysine substitution derivative (D-lys-CP) have an improved stability against trypsin and chymotrypsin. Although D-CP takes left-hand α-helical conformation and D-lys-CP loses some α-helical content, both of the D-amino acid-substituted derivatives maintain their parental peptides' membrane active action mode. In addition, D-lys-CP showed a slight weaker antimicrobial activity than polybia-CP, but the hemolytic activity decreased greatly. These results suggest that D-CP and D-lys-CP can offer strategy to improve the property of AMPs and may be leading compounds for the development of novel antimicrobial agents.

Key words: antimicrobial peptide, polybia-CP, stability, D-amino acid substitution

Introduction

The frequency of emergence of multidrug resistant (MDR) bacteria has increased during the past two decades, such as methicillin resistant *Staphylococcus aureus* (MRSA) [1], as a consequence of the extensive use of antibiotics in medicine, agriculture, and food industry. MDR bacteria are defined as non-susceptible to one or more antimicrobials on three or more antimicrobial classes [2]. The emergence of MDR bacteria limited the use of conventional antibiotics to defend the infection of microbes and imposed great risk to human life and health. However, since 1962 only a few novel structural classes have been introduced to the antibacterial armamentarium [3]. Thus, there is an urgent need to develop novel antimicrobial agents. Nowadays, antimicrobial peptides (AMPs) are believed to be potential alternatives of conventional antibiotics and attract great interest of researchers.

AMPs were found among almost all kind of life, such as bacteria, protozoan, insects, invertebrates, amphibians, plants, vertebrates, and human [4,5]. They have been demonstrated to possess antibacterial activity, antifungal activity, anticancer activity, antivirus activity, and so on [6]. AMPs were proposed to always target the cell membrane, not the protein or enzyme [7]. This is different from the conventional antibiotics. Although the resistance toward

AMPs has been reported and the possible mechanisms have been proposed [8–10], it is still difficult for bacteria to develop resistance against AMPs by the change of membrane composition [11]. However, the obvious disadvantage of natural AMPs is their potential lability to proteases generated by human and microbes. For example, trypsin-like proteases can degrade proteins and peptides with basic residues. LL-37 can be degraded by V8 proteases from *S. aureus* and lose its antimicrobial activity [12]. Several strategies have been proposed to address this issue, including the use of unnatural or D-amino acids, the use of non-peptidic backbones, and chemical modification to create protease-resistant prodrug molecules [13–15]. Unfortunately, although the modification can improve the stability of AMPs against the degradation of protease, such strategies always have unfavorable effects on the conformation and antibacterial activity of AMP.

Polybia-CP is a typical amphiphilic antimicrobial peptide whose primary amino acid sequence is ILGTILGLLKSL-NH_2 (1239.73 Da). It was originally isolated from the venom of the social wasp *Polybia paulista* [16]. It has been shown to have antitumor activity, antibacterial activity, and antifungal activity with membrane active action mode [17–19]. However, polybia-CP is susceptible to proteases and has potentially unfavorable pharmacokinetics.

In the present study, in order to improve the stability of polybia-CP, D-amino acid substitution strategy was employed. Our results showed that the D-counterpart of polybia-CP (D-CP) is resistant to the degradation of trypsin and chymotrypsin, while the D-lysine substituted derivative (D-lys-CP) is resistant to trypsin only. Notably, both the D-enantiomer of polybia-CP and the D-lysine substituted derivatives of polybia-CP have comparable antibacterial activity with polybia-CP.

Materials and Methods

Synthesis and purification of peptides

Polybia-CP, D-lys-CP, and D-CP were synthesized by the solid-phase method using N-9-fluorenylmethoxycarbonyl (F-moc) chemistry [20]. These peptides were purified by reverse-phase HPLC (Waters Corporation, Milford, USA) using a μBondapak C_{18} 19 mm by 300 mm column eluted with 20%–80% CH_3CN/H_2O in 0.1% trifluoroacetic acid (TFA). The molecular masses of the purified peptides were determined by an electrospray ionization-mass spectrometry. All the peptide fragments had a purity of at least 95% and were dissolved in double-distilled water and stored at −20°C before use [19].

Table 1. Amino acid sequence of polybia-CP and its derivatives

Peptides	Sequence	M_{Cal}[a]	M_{Obs}[b]
Polybia-CP	ILGTILGLLKSL-NH_2	1240.59	1239.8393
D-lys-CP	ILGTILGLLkSL-NH_2	1240.59	1239.8474
D-CP	ilgtilgllksl-NH_2	1240.59	1239.8350

[a]Mcal, calculated monoisotopic mass.
[b]Mobs, observed monoisotopic mass, which were deduced from the protonated molecule ([M+H^+]).

Strains and broth

Bacterial strains used in this study were purchased from the American Type Culture Collection (ATCC, Manassas, USA): *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 23857), *S. epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 25922), *E. coli ML-35* (ATCC 43837), *Klebsiella influenza* (ATCC 700603), *Sakazakii* (ATCC 29544). All strains were grown in MH-broth (Hopebio, Qingdao, China) and all tests were performed in the same culture using bacteria in exponential growth phase.

Antibacterial activity analysis

The determination of MIC

The minimum inhibition concentration (MIC) values of polybia-CP, D-lys-CP, and D-CP were determined using a standard serial dilution method with minor modification [10]. The antibacterial activity was tested in Mueller-Hinton Broth (MH-broth) against *S. aureus* (ATCC 29213), *B. subtilis* (ATCC 23857), *S. epidermidis* (ATCC 12228), *E. coli* (ATCC 25922), *E. coli ML-35* (ATCC 43837), *K. influenza* (ATCC 700603), and *Sakazakii* (ATCC 29544). The final concentrations of these peptides ranged from 1 to 256 μM. The inoculum sizes contained ~1 × 10^5 CFU/ml. The MIC values were determined after 18 h of incubation. The MIC values were defined as the lowest concentration of the antibacterial agents at which no visible turbidity was observed comparing with the drug-free control group.

The determination of MBC

In brief, 100 μl samples were taken from the wells of 96-well microtiter plates which were used to determine minimum bactericidal concentrations (MBCs) of peptides, and then were added on MH-broth agar plate for CFU counting. After incubation at 37°C for 24 h, MBCs were determined by counting the number of colonies in the plate. The MBCs were defined as the lowest concentration of the antibacterial agents at which 99.9% of the bacteria were killed comparing with the negative control group.

Time-killing kinetics of polybia-CP and its analogs against *S. aureus* (ATCC 29213)

The bacteria (1 × 10^5 CFU/ml) after suitable dilutions into sterile MH-broth medium were incubated with the peptides at different concentrations at 37°C. The final concentrations of polybia-CP, D-lys-CP, and D-CP ranged from 16 to 64 μM, 64 to 256 μM, and 8 to 32 μM. After incubation for different time periods, the mixtures were taken from the wells of 96-well microtiter plates with appropriate dilutions. Then they were added on MH-broth agar for CFU counting. Time-killing kinetics of peptides was obtained by plotting mean colony count (log_{10} CFU/ml) versus time.

Table 2. MIC/MBC[a] values of polybia-CP, D-lys-CP, and D-CP against the tested bacteria cells

Peptide	*S. aureus* ATCC 29213	*B. subtilis* ATCC 23857	*S. epidermidis* ATCC 12228	*E. coli* ATCC 25922	*E. coli* ATCC 43827	*K. influenza* ATCC 700603	*Sakazakii* ATCC 29544
Polybia-CP	8/8	8/128	8/16	32/64	32/128	128/128	64/128
D-lys-CP	64/64	16/128	16/32	64/128	64/256	128/256	128/256
D-CP	8/16	4/16	64/256	32/64	64/128	128/128	64/128

[a]The left of diagonal mark (/) is the MIC value in μM, while the right is the MBC value in μM.

Hemolysis of human RBCs

Human red blood cells (RBCs) were obtained in the Hospital of Lanzhou University. After centrifugation at 800 *g* for 10 min, fresh RBCs with heparin sodium were washed three times with 10 mM phosphate buffer saline (PBS). RBCs were diluted to the final erythrocyte concentration of 8%. The RBC suspension (100 µl) was added to a 96-well microtiter plate. The peptides dissolved in water were added to the wells of a 96-well plate by serial 2-fold dilution (100 µl/well). The final concentrations of these peptides ranged from 2 to 128 µM. PBS and 2% Triton X-100 were used as negative and

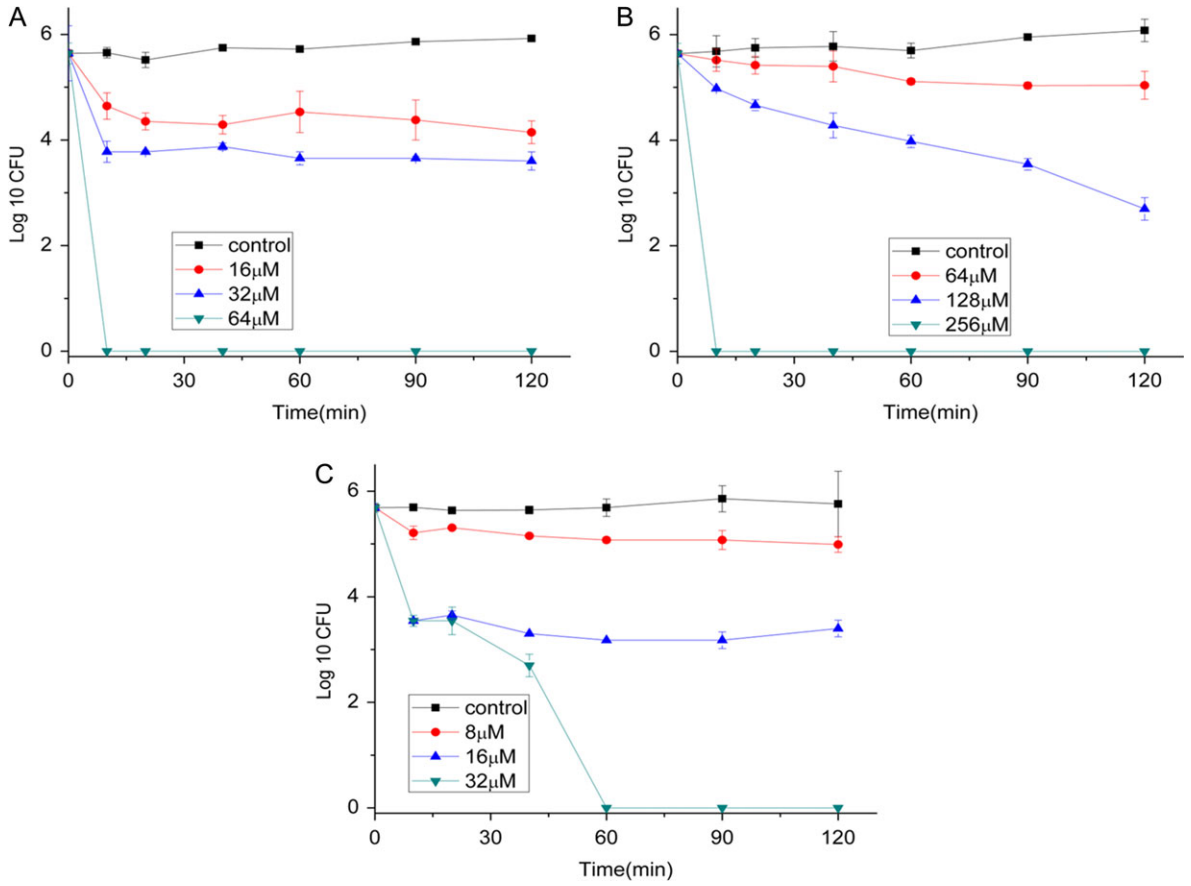

Figure 1. Time-kill kinetics of polybia-CP (A), D-Lys-CP (B), and D-CP (C) against *S. aureus* (ATCC 29213) The final concentrations of polybia-CP,D-lys-CP and D-CP ranged from 16 to 64 µM, 64 to 256 µM, and 8 to 32 µM. Different concentrations of peptides were incubated with *S. aureus* for indicated time, and 100 µl of cultures were taken out and appropriately diluted in MH-broth medium. Then, they were plated on MH-broth agar plates. After incubation at 37°C for 18 h, the number of colonies was counted. Control was sample treated with broth only.

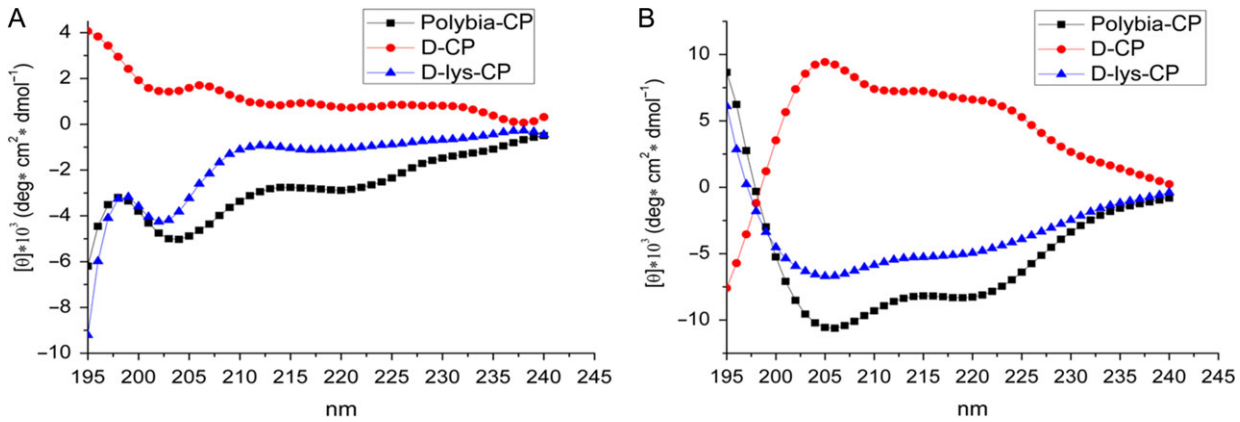

Figure 2. Peptide folding analyzed by CD spectroscopy (A) CD spectra of polybia-CP, D-CP, and D-lys-CP in the presence of PBS (10 mM, pH 7.4). (B) CD spectra of the three peptides in 50% trifluoroethanol (TFE) (v/v) as membrane-mimicking environment. The spectra were recorded between 195 and 240 nm. (■) polybia-CP, (●) D-CP, and (▲) D-lys-CP.

positive control, respectively. The mixtures were incubated in carbon dioxide incubator for 60 min at 37°C, and then centrifuged at 1200 *g* for 15 min. The OD values of the supernatants of the samples were measured with a microplate reader (Bio-Rad, Hercules, USA) at 490 nm. Hemolysis rate was calculated as follows:

Hemolysis rate = $[OD_{490(peptides)} - OD_{490(PBS)}]/[OD_{490(2\%Triton-100)} - OD_{490(PBS)}]$,

where $OD_{490(peptides)}$ is OD value of the blood samples after incubation with peptides, $OD_{490(2\%Triton-100)}$ and $OD_{490(PBS)}$ were the OD value of the blood sample treated by 2% Triton X-100 and PBS, respectively.

Circular dichroism analysis

The circular dichroism (CD) spectra of these peptides were determined by using Olis DSM 1000 CD spectrometer (Olis, Bogart, USA) at room temperature. Measurements of these peptides (50 μM) were performed in 10 mM PBS and in 50% (v/v) TFE using a quartz cuvette [21]. The spectra were recorded between 195 and 240 nm, and the average of four scans was taken. The percentage of a-helical structure was calculated as follows:

$$\alpha\text{-Helical content (\%)} = \left(\frac{[\theta]_{222} - [\theta]^0_{222}}{[\theta]^{100}_{222} - [\theta]^0_{222}}\right) \times 100,$$

where $[\theta]_{222}$ is the experimentally observed mean residue ellipticity at 222 nm, and values for $[\theta]^0_{222}$ and $[\theta]^{100}_{222}$ which correspond to 0% and 100% α-helix content at 222 nm, are estimated to be −2000 and −32,000, respectively, [22].

Determination of the integrity of membrane of bacteria by PI uptake assay

The propensity of peptides to disrupt the integrity of membrane was measured by propidium iodide (PI) uptake assay. Briefly, *Candida glabrata* (ATCC 2001) with inoculum size of 10^6–10^7 CFU/ml was incubated in the presence of polybia-CP, D-lys-CP, and D-CP at the concentration of 32 , 128, and 128 μM, respectively. The mixtures were incubated for 3 h at 35°C, then PI (Solarbio, Beijing, China) at the concentration of 50 μg/ml was added and incubated for 5 min at room temperature in the dark. Microscopic analysis was performed with laser confocal scanning microscope (LSM 710 META; Zeiss, Oberkochen, Germany).

Proteolysis of AMPs by trypsin and chymotrypsin

Growth inhibition assay

In brief, trypsin (Sigma, Shanghai, China) and chymotrypsin (Sigma) were dissolved in PBS (10 mM) by serial 10-fold dilution. The final concentrations of these two enzymes ranged from 2×10^{-6} mg/ml to 2 mg/ml. Polybia-CP, D-lys-CP, and D-CP (at the concentration of 128, 256, and 128 μM, respectively) were mixed with different concentrations of trypsin or chymotrypsin. The samples were incubated for 1 h or 6 h at 37°C. Then they were heated for 15 min at 60°C to terminate the enzyme reaction. Then, the trypsin-treated peptides were incubated with *E. coli* (ATCC 25922) in a 96-well microtiter plate overnight at 37°C. The growth inhibitory effect was determined at 600 nm by measuring the absorbance. The survival rate was calculated as follows:

Survival rate = $OD_{600(peptides)}/OD_{600(PBS)}$,

where $OD_{600(peptides)}$ is OD value of *E. coli* cells after incubation with peptides + protease, $OD_{600(PBS)}$ was that of *E. coli* cells treated by PBS + protease.

Spread plate assay

Briefly, 40 μl of polybia-CP, D-lys-CP, and D-CP (128, 256, and 128 μM, respectively) were mixed with 10 mM PBS buffer (40 μl) containing 1 mg/ml trypsin or chymotrypsin. After incubation at 37°C for 4 h, the solutions were heated at 60°C for 15 min to inactivate the enzyme. The bacteria cells were diluted as described previously in MH-broth medium, added to each sample, and incubated for 3 h. The sample mixtures were then taken out and plated onto MH-broth agar plate. After incubation on the MH-broth agar plate for another 18 h, the growth of bacteria was observed.

Radial diffusion assay

In short, the bacteria cells were added to 200 ml MH-broth agar (at 50°C). After the bacteria solution was quickly mixed, the media agar was poured into the plate to form an ~5 mm deep layer and then punched to make evenly spaced wells. Then 20 μl of the mixtures of peptides (128, 256, and 128 μM, respectively) and the enzymes (trypsin and chymotrypsin) were added to each well. And 20 μl of sterile double-distilled water without enzymes was taken as the control group. Then the plates were incubated at 37°C for 24 h and the growth of bacteria around the wells was observed.

Reverse-phase HPLC

Peptides at a concentration of 10 mM were pretreated with trypsin or chymotrypsin (0.2 mg/ml) at 37°C [23,24]. After preincucation, 40 μl of reaction solution was taken and mixed with 80 μl of acetonitrile with 1% TFA at 4°C for 15 min to inactivate the enzyme. Then, the mixture was centrifuged at 13,000 *g* for 10 min to precipitate protein. The supernatant (50 μl) was loaded onto the C18 reverse-phase column for HPLC analysis. Samples were eluted by a

Table 3. Mean residual ellipticity at 222 nm $[\theta]_{222}$ and percent of α-helical contents of the peptides in 50% TFE (v/v)

Peptides	$[\theta]_{222}$	α-Helix (%)
Polybia-CP	−7808.7	26.02
D-lys-CP	−3752.3	12.50

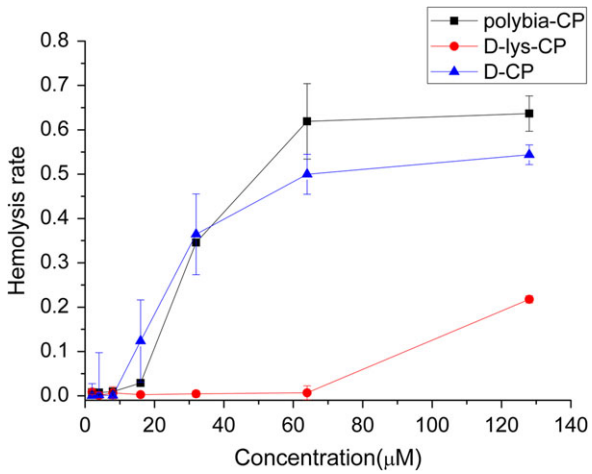

Figure 3. Hemolytic activity of polybia-CP, D-Lys-CP, and D-CP Erythrocytes were incubated with peptides (ranged from 2 to 128 μM) at 37°C for 1 h. Then, the hemolytic activities of these three peptides were determined by monitoring the release of hemoglobin from human RBCs at 490 nm. Hemolysis rate was expressed as the percentage of 2% Triton X-100 induced hemolysis. 2% Triton X-100 induced hemolysis was presented as 100% hemolysis.

linear gradient of 20%–80% CH_3CN/H_2O in 0.1% TFA at a flow rate of 1 ml/min in 30 min. The UV absorbance of the eluted peptides was detected at 220 nm with a UV monitor.

Statistical analysis

Origin 8.0 (OriginLab, Northampton, USA) was used for statistical analysis. Data were expressed as the mean ± standard error of the mean.

Results

Antibacterial activity of polybia-CP and its analogs

In the present study, polybia-CP, D-lys-CP, and D-CP were chemically synthesized. The sequences and molecular weights of the peptides were shown in **Table 1**. The antibacterial activity and bactericidal activity analyses were performed on eight standard laboratory bacterial strains with standard antimicrobial activity assay. As shown in **Table 2**, D-lys-CP showed a slightly lower antimicrobial activity than polybia-CP, while D-CP showed almost the same antimicrobial activity as polybia-CP. In addition, D-lys-CP and D-CP also showed bactericidal activity at the concentration ranging from 1 to 4 times of their MICs. The time-kill kinetics showed that both D-lys-CP and D-CP exert their bactericidal activity in a time- and concentration-dependent manner as their parent peptide polybia-CP (**Fig. 1**). These data indicated that polybia-CP, D-lys-CP, and D-CP could reduce inoculum by 100% within 120 min at the concentration of 64, 256, and 32 μM, respectively.

α-Helical formation of the peptides

To explore the conformation of the substituents of polybia-CP, the CD studies of D-CP and D-lys-CP were performed both in 10 mM

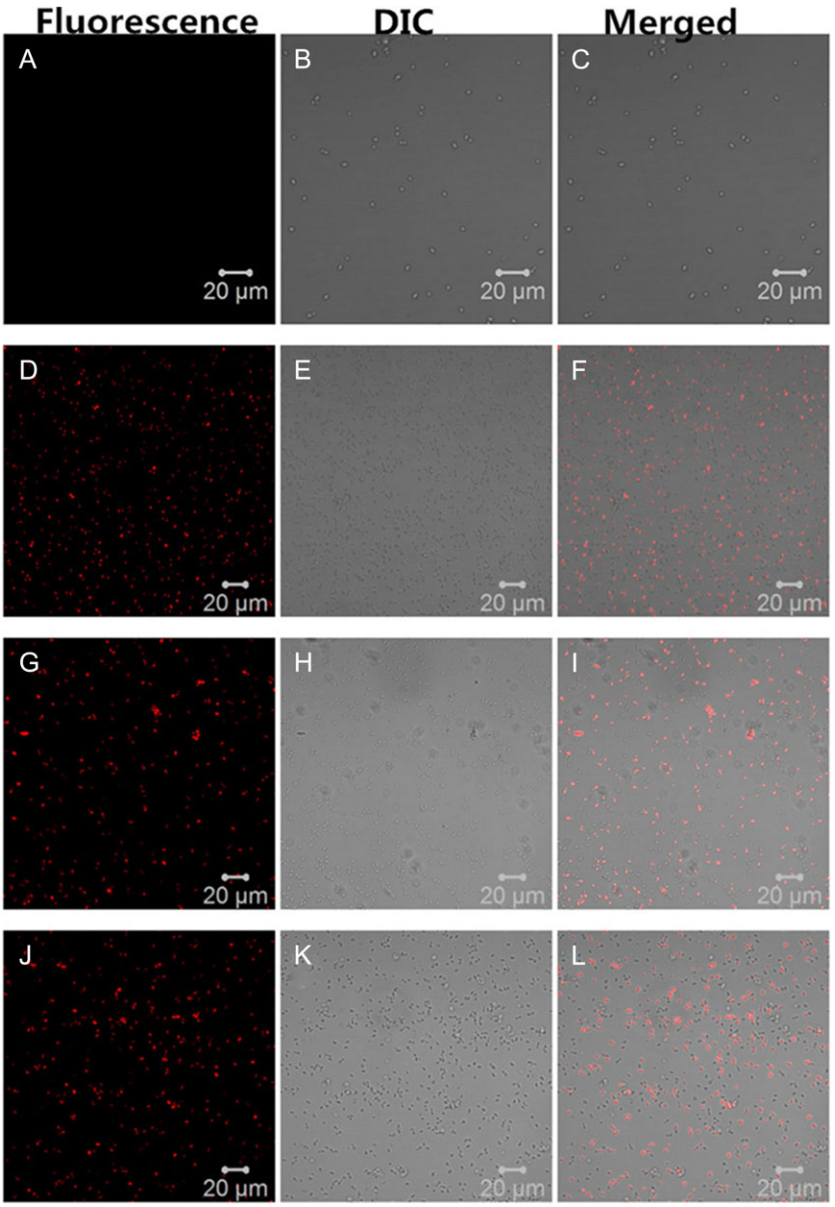

Figure 4. Effect of polybia-CP, D-lys-CP, and D-CP on the integrity of *C. glabrata* (ATCC 2001) cell membrane *C. glabrata* cells were incubated with PBS (A–C), polybia-CP (D–F), D-lys-CP (G–I), and D-CP (J–L) at the concentration of 32, 128, and 128 μM, respectively, for 3 h at 35°C. Then the fungal cells were stained with PI (50 μg/ml) and analyzed by confocal laser-scanning microscopy to assess the integrity of bacteria membrane. Results are the representative of three separate experiments. Scale bar, 20 μm.

PBS (pH 7.4) which mimics an aqueous environment and in 50% (v/v) TFE which mimics a membrane environment. The spectra of the D-lys-CP and D-CP were compared with that of polybia-CP (**Fig. 2**). Our results showed that D-lys-CP showed typical α-helical spectrum with two minimum peaks at 208 and 222 nm in 50% TFE solution, while D-CP showed a symmetric spectrum with polybia-CP, suggesting that D-CP takes a left-hand α-helical spectrum. Polybia-CP, D-lys-CP exhibited less α-helicity in TFE solution (**Table 3**). These data indicated that D-lys-CP still shows α-helical comformation with some decrease of α-helical content, while D-CP takes left-hand α-helical conformation.

The hemolytic activity

The cytotoxicity of D-lys-CP and D-CP against human RBCs was assessed by a hemolytic assay. As shown in **Fig. 3**, D-CP showed a comparable hemolytic activity to polybia-CP, while D-lys-CP showed significantly lower hemolytic activity. This suggested that although the partial D-amino acids-substituted peptides make a slight loss of antibacterial activity, the cytotoxicity toward normal host cells was decreased greatly.

Action mechanism of the peptides

As mentioned above, D-lys-CP had a slight effect on the secondary conformation of polybia-CP, while D-CP turned the right hand α-helical conformation of polybia-CP to left-hand α-helical conformation. In our previous study, we found that polybia-CP was a typical α-helical antimicrobial peptide with membrane lytic activity [18]. We proposed that the D-amino acids-substituted peptides might exert their antimicrobial activity by disrupting the integrity of the cytoplasmic membrane. To confirm this possibility, the mechanism of actions of D-CP and D-lys-CP was investigated by PI uptake assay. Our result showed that after incubation of peptides with fungi cells, both D-CP and D-lys-CP could induce the cells to uptake the fluorescent PI which binds with DNA, showing fluorescence under the view of laser confocal scanning microscopy. This result was identical to the effect of polybia-CP on the fungi cells (**Fig. 4**),

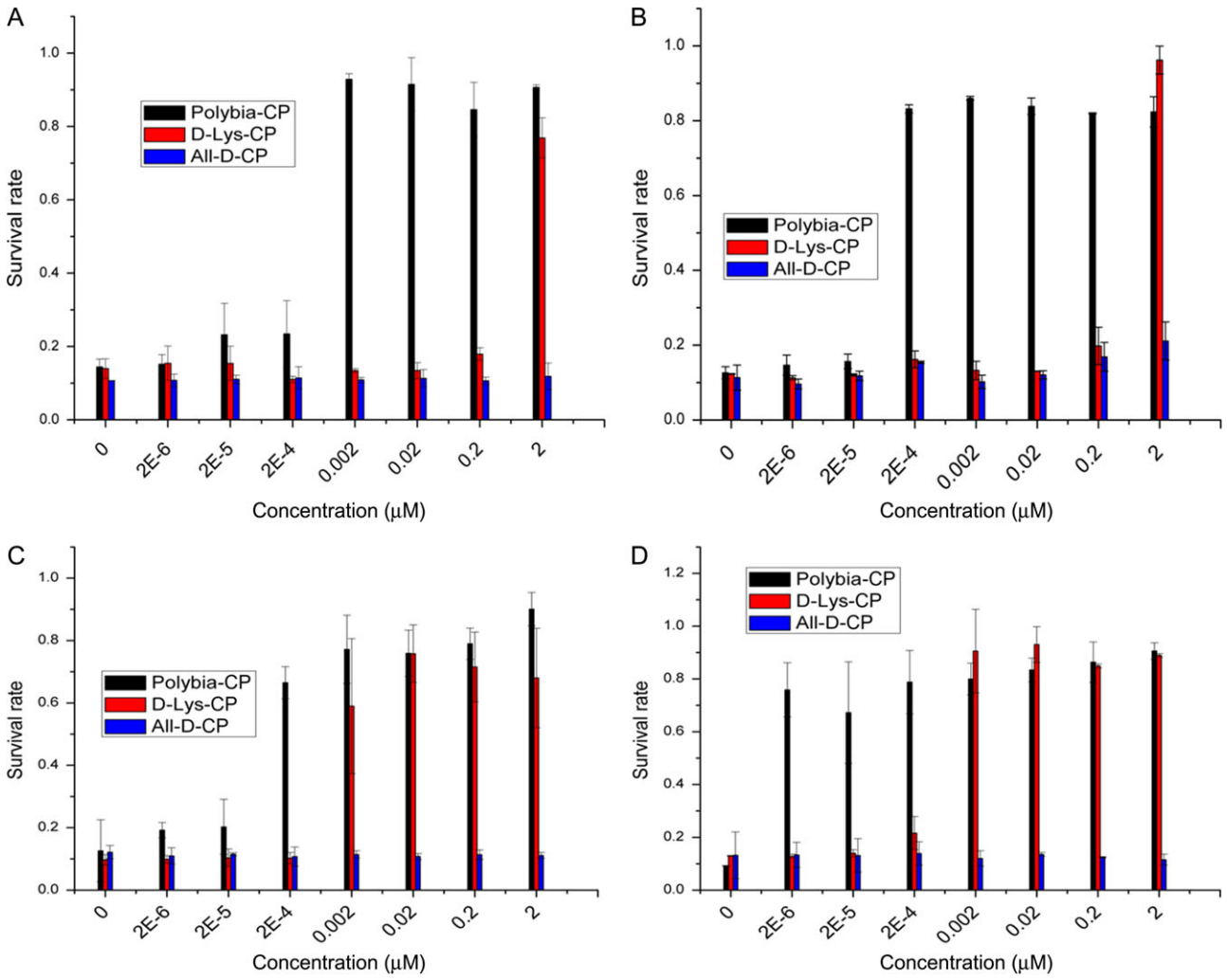

Figure 5. Effect of trypsin and chymotrypsin on the antibacterial activity of polybia-CP, D-lys-CP, and D-CP Peptides (128, 256, and 128 μM, respectively) were incubated with different concentrations of trypsin for 1 h (A) or 6 h (B), and chymotrypsin for 1 h (C) or 6 h (D) respectively at 37°C. After the enzymatic reaction was terminated, peptides were incubated with *E. coli* ATCC 25,922 (10^5–10^6 CFU/ml) in a 96-well microtiter plate overnight at 37°C. Then the absorbance at 600 nm was measured. Data are the average of three separate experiments.

suggesting that both D-lys-CP and D-CP exert their antimicrobial activity by disrupting the integrity of the membrane.

Protease resistance of D-lys-CP and D-CP

To test the protease resistance of these D-form amino acid-substituted derivatives of polybia-CP against trypsin and chymotrypsin, they were incubated with protease for indicated time periods. The effects of protease digestion on the antimicrobial activity and bactericidal activity of D-lys-CP and D-CP were determined by growth inhibition assay, spread plate method and radical diffusion assay. Our results showed that polybia-CP lost its antimicrobial activity after incubation with 2×10^{-3} mg/ml trypsin or 2×10^{-4} mg/ml chymotrypsin for 1 h, while it lost its antimicrobial activity after incubation with 2×10^{-4} mg/ml trypsin or 2×10^{-6} mg/ml chymotrypsin for 6 h. However, as shown in **Fig. 5**, D-lys-CP maintained its antimicrobial activity after incubation with 2×10^{-1} mg/ml trypsin and 2×10^{-4} chymotrypsin for 1 and 6 h, while D-CP still maintained its antimicrobial activity after incubation with tested maximum concentration of 2 mg/ml trypsin for 6 h. As shown in **Fig. 6**, in polybia-CP-, D-lys-CP-, and D-CP-treated plates, there is no colony formation. However, many clonies formed in the polybia-CP+trypsin/chymotrypsin group, while there are no or few colonies in the D-CP+trypsin/chymotrypsin group and D-lys-CP+trypsin/chymotrypsin group.

Radical diffusion assay further confirmed the effect of trypsin and chymotrypsin on the bactericidal activity of the D-form amino acid-substituted derivatives of polybia-CP. As shown in **Fig. 7**, after incubation with trypsin and chymotrypsin, D-CP induces significant clearance zones as the control group with peptides only, while there is no clearance zone in the polybia-CP+typsin/chymotrypsin group.

Furthermore, HPLC analysis was employed to determine the degradation of peptides by trypsin and chymotrypsin. Polybia-CP was susceptible to the treatment of trypsin and chymotrypsin, which resulted in a changed HPLC profile. D-CP was resistant to the treatment of trypsin and chymotrypsin and showed a similar HPLC profile of samples without enzyme treatment. D-lys-CP was resistant to trypsin after 6 h treatment, while after treatment with chymotrypsin, some changes occurred in the abundance of main peak in the HPLC profile, indicating partial degradation (**Fig. 8**). These data indicated that the stability of polybia-CP against the degradation of tested proteases was enhanced by D-amino acid substitution.

Discussion

In recent years, with the extensive use of antibiotics, the emergence of MDR bacteria increased dramatically. However, the lag of development of antibiotics with novel structure and action mode made this situation worse. Human life may be endangered in a non-antibiotics era again. So nowadays there is an urgent need to address this concern. AMPs, which are present in virtually every life form, attract much attention of researchers for their membrane action mode. Although there are some promises for AMPs to be developed into novel antimicrobial agents, there are also some challenges for them to be clinically used. That is why after nearly two decades of efforts in the research of AMPs, there is still limited success in clinic. Among all the challenges for AMPs, the susceptibility

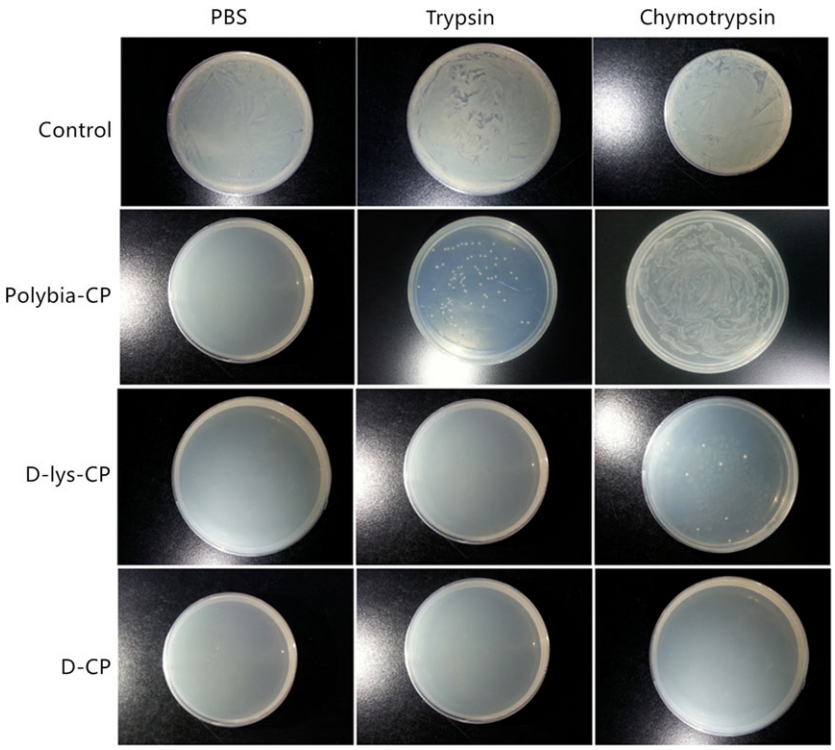

Figure 6. Viability of bacteria after incubation with peptides in the presence or absence of enzyme Polybia-CP, D-lys-CP, and D-CP at the concentration of 128, 256, and 128 μM, respectively, were incubated with 0.2 mg/ml trypsin, chymotrypsin or PBS buffer respectively at 37°C for 4 h. After incubation, trypsin was inactivated by heating at 60°C for 20 min. Subsequently, each peptide samples were collected directly into tubes, which contained an equal volume of *E. coli* (ATCC 25,922) cultures with an inoculum size of 10^6 CFU/ml, and incubate at 37°C for 3 h. Then bacteria samples (100 μl) were taken out and plated onto MH-broth plates. After overnight incubation, the colony formation in the plates was photographed respectively.

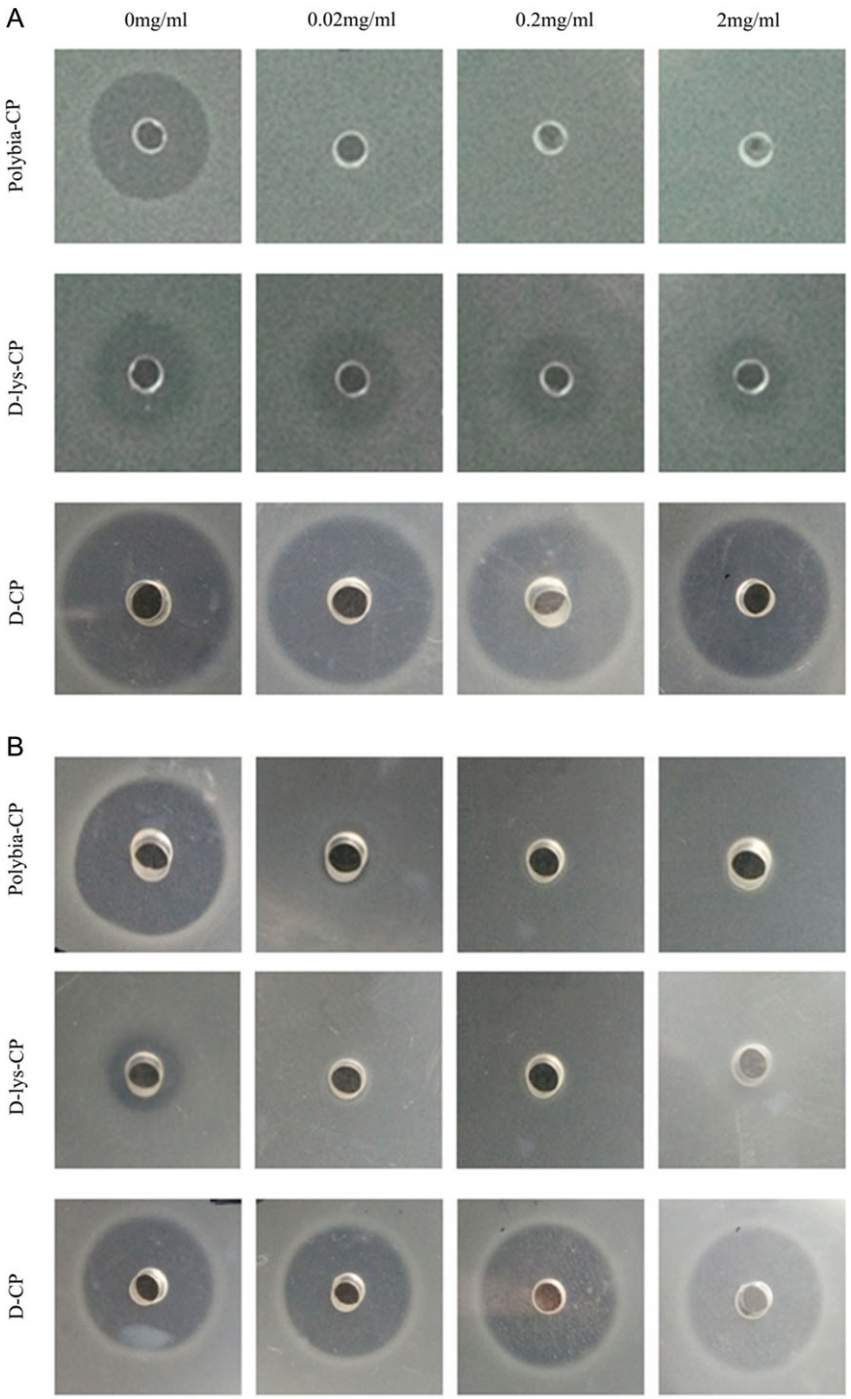

Figure 7. Effect of protease on the bactericidal activity of polybia-CP, D-lys-CP, and D-CP determined by radial diffusion assay Peptides (128, 256, and 128 μM, respectively) were incubated with trypsin (A) and chymotrypsin (B) at the indicated concentration for 4 h at 37°C. Then 25 μl of the enzyme-treated peptides were added into wells in the *S. aureus* ATCC 29213 (10^5–10^6 CFU/ml) mixed MH-broth agar plate and incubated overnight at 37°C.

to protease is one big issue. Almost all of the AMPs exist in natural life have basic amino acids in their sequences, which is an obligate feature of AMPs. Trypsin and chymotrypsin which are abundant in the digestive system of mammals always attack AMPs at cationic residues, like lysine, arginine, and histamine [25].

Chemical modification is a common strategy to improve the stability of natural peptides. Among them, introduction of non-natural amino acids, especially D-form amino acids into the sequence of peptides was an effective strategy to prevent peptides from proteolytic degradation [26]. However, chemical modification may change the conformation of natural peptides and have some unfavorable effects on their bio-activity. For AMPs, all D-amino acid replacement could increase the stability of AMPs without loss of antimicrobial activity [27]. In the present study, D-CP takes a left-hand α-helical conformation, which was a reverse conformation to its parental peptide polybia-CP. The α-helical conformation is associated with its

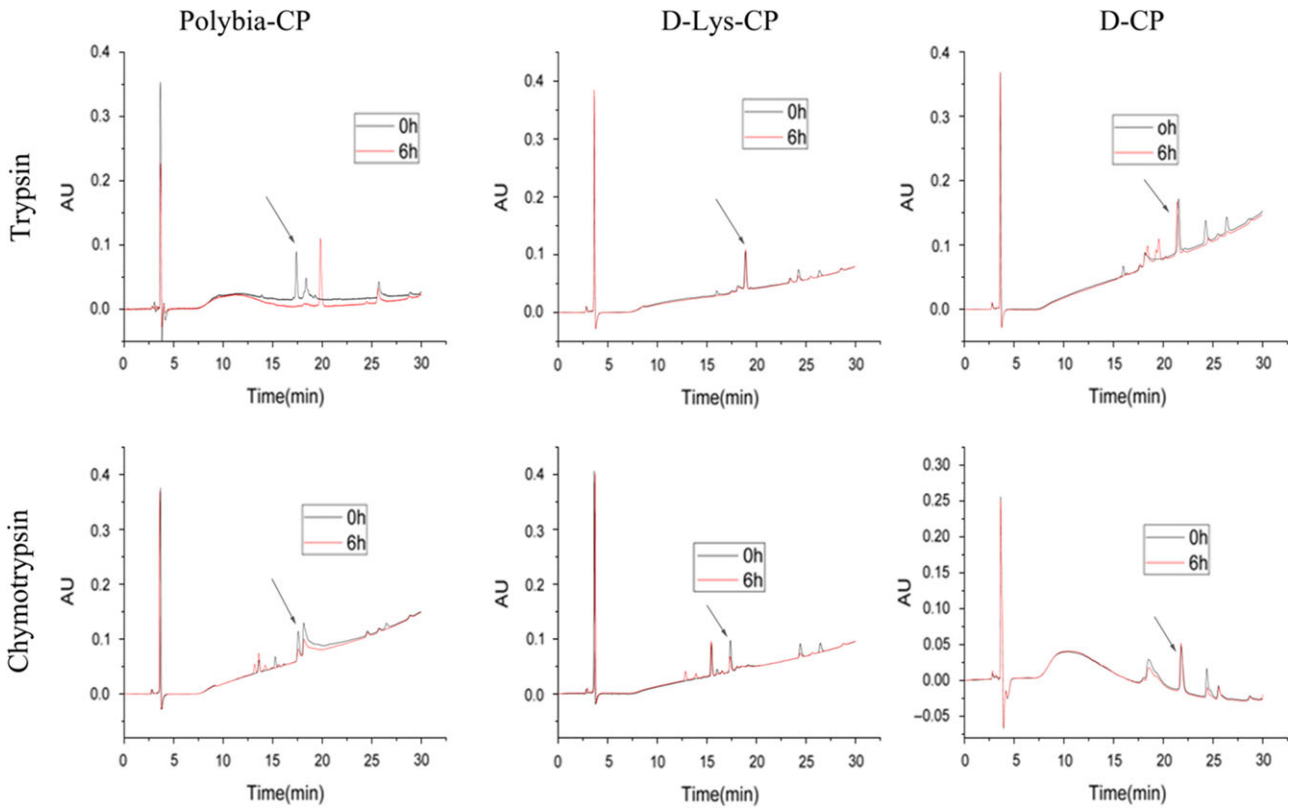

Figure 8. HPLC profiles of polybia-CP, D-Lys-CP, and D-CP treated with trypsin and chymotrypsin The peptides at the concentration of 10 mM were incubated with trypsin or chymotrypsin at 37°C for 0 h (the black line) and 6 h (the red line). The eluted peptides were monitored by a UV monitor at 220 nm. The arrow refers to the main peak.

bio-activity [28,29]. Our results showed that the antimicrobial activity of D-CP was comparable to that of polybia-CP, but the stability of D-CP against trypsin and chymotrypsin was improved greatly (**Fig. 5**). Additionally, D-CP showed improved *in vivo* antimicrobial efficacy in the *E. coli*-infected model (**Supplementary Fig. S1**) because the action target of AMPs has no chiral specificity. Usually, AMPs target at the bio-membrane of pathogens and exert their antimicrobial activity by disrupting the integrity of membrane [30]. D-CP was also proved to target at the cell membrane of bacteria by PI uptake assay. PI is a fluorescent dye that can only pass through the disrupted cell membrane, then bind with DNA and show fluorescence [31]. This may attribute to that the net charge and hydrophobic property of D-CP were not changed after D-amino acid substitution.

High manufacturing cost also limits the use of AMPs. Among all the chemical modification strategies to improve the pharmacokinetics of AMPs, D-amino acid substitution may be one of the most economical and efficient strategies. As we know, polybia-CP is a short cationic AMP, in which lysine is the only one basic amino acid. So lysine is important for the chemical–physical property, secondary structure and bio-activity of AMP. In the present study, the effect of D-lysine substitution of L-lysine on the stability and antibacterial activity of polybia-CP also was evaluated. Our result showed that such kind of partial D-amino acid substitution could improve the stability of polybia-CP against the degradation of trypsin. Although there was some loss in the antibacterial activity, the cytotoxity toward RBCs was lowered.

In summary, our results proved that both the all- and partial D-amino acid substitution strategy could improve the pharmacokinetics of polybia-CP. Although the development of AMPs cannot address all the antibiotic-resistance issue, AMPs with tremendous structural diversity and impressive array of clinically meaningful activities may be an important source of antimicrobial agents in the 'antibiotic-resistance era'. The present study may offer an impetus to the development of new synthetic AMPs.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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