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# Insertion mode of a novel anionic antimicrobial peptide MDpep5 (Val-Glu-Ser-Trp-Val) from Chinese traditional edible larvae of housefly and its effect on surface potential of bacterial membrane

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ABSTRACT

Antimicrobial molecules from insects may serve as a potentially significant group of antibiotics. To identify the effect of antimicrobial peptides (AMPs) on bacterial membrane and obtain further insight in the mechanism of membrane transport of AMPs, the interaction of surface potential and permeation of a novel antimicrobial peptide MDpep5 (Val-Glu-Ser-Trp-Val) from Chinese traditional edible larvae of housefly was examined using liposomes from bacterial lipids extract. Compared with the cationic AMPs, MDpep5 cannot completely disrupt membrane. The uptake of MDpep5 by bacterial liposomes was dependent on the membrane surface potential. The mutual inhibition of the transport of MDpep5 through the cell membrane was caused by the change in surface potential due to the binding of MDpep5 to the membrane. Furthermore, formation of MDpep5-enriched lipid aggregates could lead to the disorder of the bilayer structure. Based on our experimental data, we propose that MDpep5 initiated its antimicrobial activity by profoundly disordering the structure and affecting physical properties of bacterial membrane when binding to the phospholipid which accounts for its bactericidal activity.

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## 1. Introduction

Housefly (*M. domestica*) belongs to Diptera. The larva of housefly has been used in China as food and clinically to cure ecthyma, lip boil and bacterial infection of the digestive organs such as vomiting and dysentery since the Ming/Qing Dynasty (1368 A.D.) up to now. Some researchers have looked at the natural food-derived antimicrobial agents from the larva of housefly, such as antimicrobial peptides (AMPs). As is known, AMPs are ancient host defense molecules in nearly all life forms [1–4]. The AMPs from edible larvae of housefly may serve as a potentially significant group of drug different from synthetic chemicals with potential toxicity and antibiotics causing drug-resistance [5,6].

AMPs can be classified into five groups according to their biochemical properties and chemical structures: cationic peptides, anionic peptides, aromatic dipeptides, peptides derived from oxygen-binding proteins and peptides processed from neuropeptide precursors [7]. For the development of AMPs, it is necessary to unveil the antimicrobial mechanism. Although studies have shown that some AMPs interact with intracellular targets [8,9], these peptides still must traverse the cell membrane to reach their site of action. Consequently, an understanding of peptide–membrane interactions is essential for the improved design and development of AMPs. As the antimicrobial activities of AMPs are generally believed to partly stem from their ability to selectively rupture membranes of various microorganisms, some information has been gained on the mechanism of cationic AMPs transmembrane permeabilisation [10–12], but the mode of action of anionic peptides is still not fully understood.

The presence of the lipid membrane will create high electrostatic fields at the membrane–water interface. This electric potential profile through a membrane includes the surface potential, arising from the net surface charge of the membrane. The net surface charge density and surface potential of cell membrane are related by the Grahame equation [13]:

$$\sigma = \frac{1}{272}\left[\sum_i C_i \exp\left(\frac{-z_i F\psi_s}{RT}\right) - 1\right]^{1/2}$$

where $\sigma$ is the surface charge density in charges per Å$^2$, $C_i$ is the concentration of the $i$th ionic species in the bulk solution, $\psi_s$ is the surface potential and $z_i$ is the valence, $R$ and $T$ are respectively, the gas constant and absolute temperature [14].

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The importance of surface potentials has been recognized since the works of Frankenhäuser and Hodgkin on the squid axon [15]. Sokolov et al. [16] found that surface potentials are relevant to the comprehension of soluble protein interactions with lipid bilayers. The aggregation of AMPs on the surface of bacterial cells was studied by spectroscopy and electron microscopy, and stressed the importance of the electrostatic interactions between the positively charged cationic AMPs and negatively charged cell membrane. It is, however, not yet clear the transmembrane mode of anionic peptides and whether the transmembrane process could influence the electric characteristic of cell membrane, especially the surface potential. Hence, it is important that the surface potential is taken into account when studying the antimicrobial peptide–membrane interactions.

To identify the effect on surface potential and contribute to the elucidation of the mechanism of membrane transport of AMPs, an anionic antimicrobial peptide MDpep5 derived from Chinese traditional edible larvae of housefly was characterized and the electric properties of liposomes made from bacterial phospholipids in presence of MDpep5 were examined.

## 2. Materials and methods

### 2.1. Microorganisms and media

The following bacterial strains were used in antimicrobial assays: *Escherichia coli* ATCC 25922 and *Escherichia coli* JM109 were grown in Luria–Bertani (LB) agar at 37 °C. *Salmonella typhimurium* 50013, *Bacillus subtilis* 9372, *Staphylococcus aureus* ATCC 6538 were grown in nutrient agar slants at 37 °C. All media were sterilized by autoclaving at 121 °C for 15 min and the pH was adjusted before sterilization.

### 2.2. Peptide purification

The powder (100 g) from dehydrated housefly larvae was homogenized in a homogenizer (Ika Labortechnik T 25) in the presence of 1 l of phosphate-buffered saline (PBS, 50 mM, pH 6.0) with 35 μg/ml phenylmethylsulfonyl fluoride (PMSF), 0.2 mg/l ethylenediaminetetraacetic acid (EDTA) and 2‰ 2-mercaptoethonal (5 mM). The homogenate was centrifuged at 4800 × g for 30 min (Eppendorf) followed by heat-treatment at 100 °C for 5 min with continuous agitation and then centrifuged at 12,000 × g for 30 min at 4 °C. For lipid removal from the supernatant, the same volume of *n*-hexane was added, and then the sample was vortexed and centrifuged at 12,000 × g for 10 min at 4 °C. The upper fraction containing lipids was removed and an equal volume of ethyl acetate was added to the water fraction. After vortexing and centrifugation at 12,000 × g for 10 min at 4 °C, the water fraction (20 mg) was subsequently loaded onto Sephadex G-15 column (45 cm × 2 cm, Amersham Pharmacia Biotech AB, Sweden) pre-equilibrated with PBS buffer (10 mM, pH 7.0, plus 1 M NaCl). Sample was eluted with the same buffer at a flow rate of 0.4 ml/min. The elution pattern was monitored at 220 nm, and eluates were collected 5 ml per tube using automatic sample collector and tested for antimicrobial activity.

The active fraction got from gel filtration (**Peak 5**) was subjected to reverse phase high-performance liquid chromatography (RP-HPLC) YWG $C_{18}$ column (250 mm × 10 mm, KromTek Technologies Inc.). The two solvent reservoirs contained the following eluents: (A) 0.1% (v/v) trifluoroactic acid (TFA) (B) 0.1% (v/v) TFA in 80% (v/v) acetonitrile. The elution program consisted of a gradient system (0–100% B in 80 min) with a flow-rate of 1.0 ml/min. Fractions with high activity (**P5-4**) were re-chromatographed on Kromasil $C_{18}$ (250 mm × 4.6 mm, KromTek Technologies Inc.) to obtain chromatographically pure peptide with the same conditions as above at a flow-rate of 0.5 ml/min. The elution pattern was monitored by measuring the absorbance at 214 nm. All solvents were degassed just prior to use. All samples were centrifuged to remove aggregated protein. Each peak was manually collected. The pure antimicrobial peptide (**MDpep5**) was collected, vacuum dried, weighed and used in the subsequent experiments.

### 2.3. Sequence analysis

Mass spectrometric experiment was performed on a matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-TOF MS) (4700 proteomics Analyzer, Applied Biosystems, USA) [17,18]. All spectra were measured under the following conditions: MS: Reflector Positive, CID (OFF), mass range (700–3200 Da), Focus Mass (1200 Da), Fixed laser intensity (6000), Digitizer: Bin Size (1.0 ns). MS/MS: 1 kV Positive, CID (ON), Precursor Mass Windows (Relative 80 resolution (FWHM)), Fixed laser intensity (7000) Digitizer: Bin Size (0.5 ns). α-Cyano-4-hydroxycinnamic acid (Aldrich, Steinheim, Germany) was used as a matrix. The peptide solution was prepared in deionized water at 100 mM concentration. An amount of 0.5 ml of the peptide solution plus 0.5 ml matrix were deposited on the sample slide and left to dry at room temperature. The resulting spectra were analyzed and compared.

### 2.4. Antimicrobial activity: agar diffusion assay and minimal inhibitory concentrations (MICs)

Overnight culture of the respective test microbes were adjusted to 2–5 × $10^8$ colony forming units per ml (CFU/ml), 1 ml of such a culture was added to 15 ml respective medium, evenly mixed and poured into Petri dishes (6 cm in diameter). The lyophilized MDpep5 was dissolved in 10 mM sodium phosphate buffer, pH 7.4 (NaPB) to reach final concentrations. The discs were then applied, 10 μl of the test solutions and control were added to each disc, respectively. Petri dishes were incubated at 37 °C for 16–24 h. The average diameters of the inhibition zone surrounding the discs were measured visually [19]. NaPB was used as negative control. The experiments were carried out in triplicate.

Minimal inhibitory concentration (MIC) of the sample was determined with liquid growth antibacterial assay. The overnight culture at 37 °C of bacterial was washed and resuspended in 10 mM sodium phosphate buffer, pH 7.4 (NaPB) by centrifugation at 3000 × g, 10 min to attain the final microbial density 2–4 × $10^7$ CFU/ml. Stock solution of the peptide was diluted serially in NaPB. Samples (50 μl) were incubated in sterilized 96-well plates with 100 μl medium and 100 μl of the bacteria disposed as described above. The minimal inhibitory concentration (MIC) was considered the lowest peptide concentration that showed no increase in the optical density ($OD_{600}$) read at the microplate reader (Multiskan MK3, Thermo Labsystems Co., USA) after 24 h stationary incubation at 37 °C [20]. NaPB (50 μl) tested under the same condition was used as the negative control. The MICs of Ampicillin was also determined as the positive control to compare with those of MDpep5. The experiments were carried out in triplicate, and average values are reported. When error bars are given in the tables or figures, they refer to the standard deviation.

### 2.5. Bacterial killing assay

*E. coli* ATCC 25922 and *S. aureus* ATCC 6538 were grown at 37 °C in MH broth. Aliquots of exponentially growing bacteria were resuspended in fresh MH broth at approximately $10^7$ cells/ml and separately exposed to MDpep5 at MIC for 0, 5, 10, 15, 20, 25, 30, 40,

50, 60, 70, 80, 90, 100, 110 and 120 min at 37 °C. After these times samples were serially diluted and plated onto MH agar plates to obtain viable colonies. The limit of detection for this method was approximately 10 colony forming units (CFU)/ml. All tubes were incubated overnight at 35 °C and the bacterial growth in each tube was determined by performing consecutive 1:10 (v/v) dilutions of a 0.1 ml aliquot of each tube in MH broth and by plating a 0.1 ml volume of each dilution onto MH agar [21]. The experiments were carried out in triplicate, and average values are reported. When error bars are given in the tables or figures, they refer to the standard deviation.

### 2.6. Total lipids extract of bacterial membranes

Total lipids extract of cell membranes of *E. coli* ATCC 25922 and *S. aureus* ATCC 6538 were obtained using a modified form of the procedure first described by Bligh and Dyer [22]. Essentially, cultures of the microorganism were grown in nutrient broth. When in the exponential phase ($OD_{600}$ = 0.6), 1 ml of culture was extracted, washed twice in Tris buffer (25 mM, pH 7.5), and centrifuged (15,000 × *g*, 5 min) to form a pellet. Pellets were then resuspended in 1 ml Tris buffer (25 mM, pH 7.5) and, to a 0.4 ml aliquot of this cell suspension, 1.5 ml of chloroform–methanol mixture (1:2, v/v) was added. These cell/solvent samples were then vortexed vigorously for 5 min, a further 0.5 ml chloroform added, and the whole again vortexed for 5 min. To each sample, 0.5 ml water was added, the whole vortexed for 5 min and then centrifuged at low speed (1000 × *g*, 5 min) to produce two phases. The lower organic layer was transferred to a fresh centrifuge tube, concentrated using a speed vac (Jouan), and the dried lipid extract stored at −20 °C under $N_2$ [23].

### 2.7. Preparation of bacterial membrane liposomes (large unilamellar vesicles)

Large unilamellar liposomes were prepared by the reverse phase evaporation method, as described previously [24]. Briefly, whole lipid extract of Gram-positive and Gram-negative bacteria were dissolved in a diethyl ether/chloroform mixture (5:1, v/v). 20 mM sodium phosphate buffer was added to the lipids resolved in diethyl ether. The resulting two-phase system was sonicated in a bath-type sonicator for 3 min at 0 °C. The homogeneous lipid emulsion obtained was treated under reduced pressure (about 100 mm Hg) until a stable gel was formed, after which residual ether was further evaporated off at about 700 mm Hg [25]. The liposome dispersion obtained was centrifuged for 20 min at 12,000 × *g* to remove aggregated materials.

### 2.8. Binding of antimicrobial peptide MDpep5 to resting bacterial cells

The assay was essentially performed as described [26] with some modification. Specifically, *E. coli* ATCC 25922 and *S. aureus* ATCC 6538 were grown from an overnight culture to mid-logarithmic phase in Mueller Hinton (MH) at 37 °C and washed twice in sterile phosphate-buffered saline (PBS: 0.01 M $NaH_2PO_4$, 0.137 M NaCl, 2.68 mM KCl, pH 7.0) with centrifugation at 2000 × *g* for 20 min. Pellets were resuspended in PBS to $OD_{600}$ = 0.8, which corresponded to bacterial numbers of about $10^9$ CFU/ml in the final sample. Triplicate bacterial samples were subsequently mixed with equal volumes of PBS containing 1000 μg/ml of peptide to give a final sample with peptide at a concentration of 500 μg/ml. After incubation for 1 h at 24 °C, The suspension was centrifuged for 2 min at 10,000 × *g*. The pellet was washed twice in 200 μl bidistilled water. The concentrations of unbound peptide in each sample's supernatant were subsequently determined according to the method of Walker [27]. All experiments were performed in triplicate.

### 2.9. Intrinsic fluorescence measurement and quenching of Trp emission by acrylamide

The concentration of MDpep5 was adjusted to 2 μM and the Trp residues were excited at 289 nm. The emission spectra were recorded from 300 to 420 nm in the absence and presence of liposomes from bacterial phospholipids.

Acrylamide is a water soluble collisional quencher of Trp fluorescence, and as a polar molecule penetrates the nonpolar regions of neither proteins nor lipid bilayers. Consequently, the extent of quenching by acrylamide depends on protein conformation and association with liposomes. To reduce the absorbance by acrylamide, excitation of Trp at 289 nm was used. Aliquots of a 3.0 M solution of acrylamide were added to peptide-containing solutions in the absence or presence of liposomes at peptide/lipid molar ratio of 1:120. Three scans were averaged, and the values obtained were corrected for dilution and scattering derived from acrylamide titration of a liposome blank. The data were analyzed according to the Stern–Volmer equation [28]:

$$\frac{F_0}{F} = 1 + K_{sv}[Q]$$

where $F_0$ and $F$ are the fluorescence intensities in the absence and the presence of the quencher (Q), respectively, and $K_{sv}$ is the Stern–Volmer quenching constant, providing a measure for the accessibility of Trp to acrylamide.

### 2.10. Activity on membrane permeability

The experiment was performed according to Chen and Cooper [29] with some modifications. The overnight culture of *E. coli* ATCC 25922 and *S. aureus* ATCC 6538 at 37 °C was washed and resuspended in 10 mM PBS (pH 7.4), reaching the final density of 2–5 × $10^7$ CFU/ml. Strains were incubated with target peptide at the MICs for different times (10, 30, 60, 120, 240 and 480 min); strains incubated with 10 mM PBS (pH 7.4) were used as control. The mixture was filtered through 0.22 μm to remove the bacteria cells. The filtrate was then diluted appropriately and the optical density at 260 nm was recorded (UV-2102 PCS, Unico) at room temperature (25 °C).

### 2.11. Measurement of changes in the surface potential of liposomes

Changes in the surface potential of bacterial liposomes were monitored by measuring the changes in the fluorescence intensity of 8-anilino-1-naphthalenesulfonic acid (ANS) which has been widely used to measure the surface potential of the membranes [30] as described previously [31] with some modifications. The measurement was carried out at room temperature in a spectrofluorometer (Hitachi 650-60, Tokyo, Japan) with an excitation wavelength of 365 nm and an emission wavelength of 475 nm. To 300 μl of a suspension of membrane liposomes, 300 μl of ANS solution and 600 μl of apical buffer ($CaCl_2$ 12.6 μM, KCl 2.5 mM, K-gluconate 22.5 mM, $KH_2PO_4$ 4.4 μM, $MgSO_4$ 8.1 μM, NaCl 15 mM, Hepes 10 mM, D-Mannitol 212 mM, $Na_2PO_4$ 3.4 μM, D-Glucose 25 mM) with MDpep5, were added. The final concentration of ANS was 50 μM, and that of liposome was 0.75 μmol phospholipid/ml [32].

Following this, the fluorescence intensity was measured. Corrections for the background fluorescence and light scattering were

made with blanks containing liposome alone or dye alone. Fluorescence intensity, $f$, was defined as

$$f = f_z - (f_t + f_a + f_l) \quad (1)$$

where $f_z$, $f_t$, $f_a$ and $f_l$ are the fluorescence intensity of a membrane liposome-MDpep5-ANS suspension, MDpep5 solution alone, ANS solution alone and a membrane liposome suspension alone, respectively. Relative membrane surface potential, $\psi_{rel}$, was calculated by the following equation,

$$\psi_{rel} = \frac{F_s}{F_c} \quad (2)$$

where $F_s$ and $F_c$ stand for the maximum fluorescence intensity of treated and control bacterial liposomes [30].

### 2.12. Statistical analysis

Analysis of variance (ANOVA) was performed using the General Linear Models procedures GLM) of the Statistical Analysis System (SAS, Version 8.0, 2000, Cary, NC, USA). Error bars are given for a 95% confidence interval. Experiments were triplicated and the means of the three data sets are presented.

## 3. Results and discussion

### 3.1. Purification and antimicrobial activity of peptide MDpep5

In this experiment, the extract of dehydrated housefly larvae was subjected to Sephadex G15 and RP-HPLC. The chromatogram of the sample was shown in Fig. 1. The active fraction got from gel filtration (**Peak 5**) was subjected to RP-HPLC. As shown in Fig. 2b, the active fraction was eluted at about 70% acetonitrile by RP-HPLC, suggesting that the molecule is hydrophobic. As is known, the hydrophobic interactions of AMPs with the cell membrane are important. AMPs can coat the surface of the bacterial membrane with the hydrophobic face towards the lipid components and the polar residues binding to the phospholipid head groups [33].

The bioactive fraction with the purity of 98.7%, as determined by high-performance liquid chromatography (data not shown), was analyzed by mass spectrometry analysis (Fig. 2c). Through analysis and comparison, the purified fraction was found to be a anionic antimicrobial peptide (Val-Glu-Ser-Trp-Val) with total net charge of -1 and total hydrophobic ratio of 60%. According to the above results, search in the NCBI database (http://www.ncbi.nlm.nih.gov/) and Antimicrobial Peptide Database (APD, http://aps.unmc.edu/AP/main.html) found no match, suggesting it is a novel peptide, designated as "MDpep5".

As shown in Table 1, MDpep5 inhibited five tested bacterial pathogens with the MICs ranging from 18 to 32 μg/ml. Further-

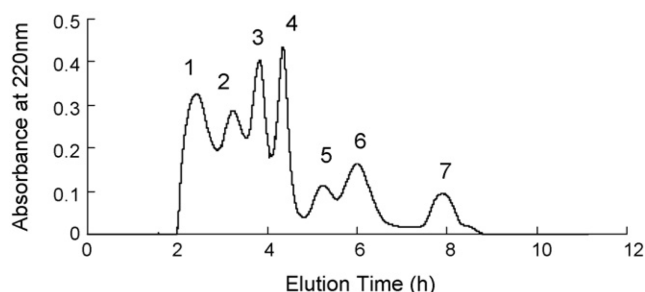


**Fig. 1.** Elution profile of sample on Sephadex G-15 column (45 cm × 2 cm, Amersham Pharmacia Biotech AB, Sweden). Elution buffer: PBS (10 mM, pH 7.0, plus 1 M NaCl); flow rate: 0.4 ml/min. The elution pattern was monitored by measuring the absorbance at 220 nm.

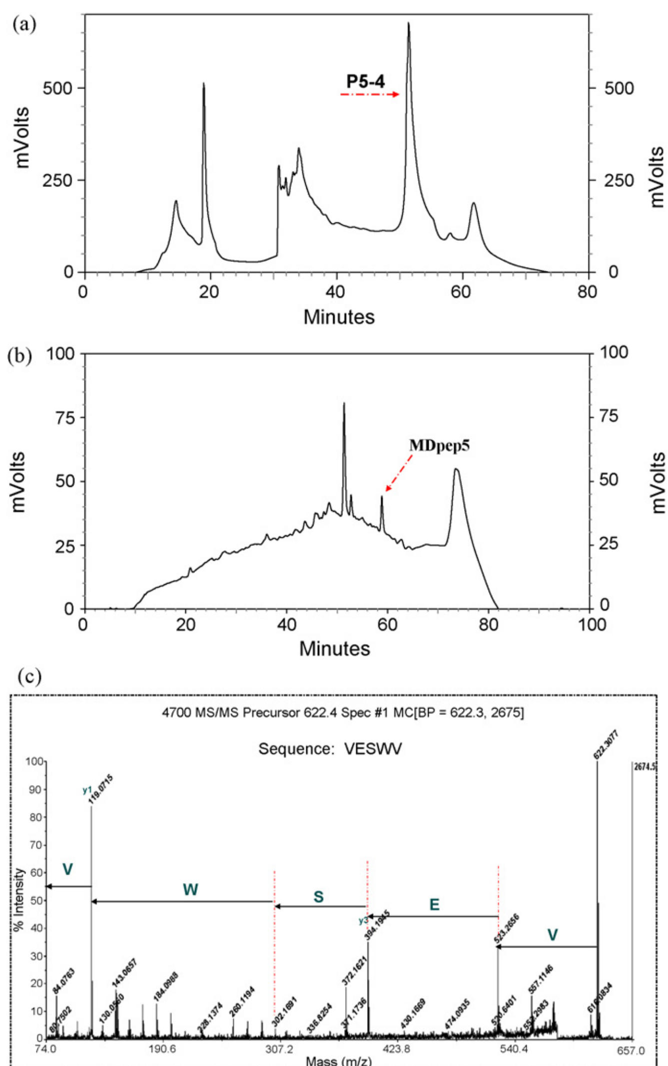


**Fig. 2.** (a) Chromatographic profile of fraction Peak 5 (YWG $C_{18}$ 250 mm × 10 mm; flow rate 1.0 ml/min). (b) Further purification of fraction P5-4 (Kromasil $C_{18}$ 250 mm × 4.6 mm; flow rate 0.5 ml/min). (c) MS/MS spectrum of MDpep5 (MALDI-TOF MS, reflector positive ion mode).

more, MDpep5 showed more inhibitory effect against *E. coli* JM109, a gene-modified strain resistant to ampicillin. While Gram-positive bacteria were more sensitive than Gram-negative bacteria. The result presented here suggests a promising strategy to control some bacteria through the addition of MDpep5.

Kill-curve studies were performed to determine the rate of decrease in viable bacteria upon incubation with MDpep5 for different incubation time (Fig. 3). At incubation time of 10 min a significant decrease was observed, especially for Gram-positive bacteria. No increase in viable-cell counts was shown until 120 min of incubation for both bacteria, suggesting cell growth was completely inhibited. These results show that MDpep5 can limit the increase in viable-cell numbers. Moreover, loss of viability of both Gram-positive and Gram-negative bacteria is incubation time dependent and Gram-positive bacteria are more susceptive to MDpep5.

It is well known that the chemical and physical nature of the cell surface differs widely among different cells. The membrane must play some role in targeting of AMPs to different cells. Gram-positive and Gram-negative bacteria differ fundamentally in the morphology of their surfaces. In Gram-negative bacteria, the outer leaflet of

**Table 1**
Inhibitory zones and minimal inhibitory concentrations (MICs) of MDpep5

| Microorganisms | Diameter of inhibitory zone of MDpep5 (mm) | MICs of MDpep5 (μg/ml) | MICs of ampicillin (μg/ml) |
|---|---|---|---|
| Gram-negative bacteria | | | |
| *Escherichia coli* ATCC 25922 | 24 | 20 | 12.5 |
| *Escherichia coli* JM109 | 17 | 22 | – |
| *Salmonella typhimurium* 50013 | 18 | 32 | 6.25 |
| Gram-positive bacteria | | | |
| *Staphylococcus aureus* ATCC 6538 | 23 | 18 | 1.56 |
| *Bacillus subtilis* 9372 | 25 | 20 | 3.12 |

"–" non-inhibitory within the tested ampicillin concentration.

the outer membrane bilayer is composed mostly of lipopolysaccharide (LPS), a polyanionic molecule. The surface selectivity of cationic AMPs is highly affected by the difference in membrane lipid composition [34]. In contrast, peptidoglycan makes up the cell wall of Gram-positive bacteria and contains teichoic and lipoteichoic acids. The peptidoglycan layer of Gram-positive bacteria is much thicker, 20–80 nm, than in Gram-negative bacteria where it is found to be about ten times smaller [35]. Maybe the subtle differences in the composition of Gram-positive and Gram-negative bacterial cell membranes can result in the differences in their susceptibilities to MDpep5.

### 3.2. Binding of antimicrobial peptide MDpep5 to resting bacterial cells

The interaction of the peptide with membrane surfaces involves several steps, including initial binding to the membrane surface, partial or full insertion into the hydrophobic core of the membrane, and finally induction of translocation across the lipid bilayer [36]. It has been suggested that electrostatic and hydrophobic interactions are predominant forces driving the binding process between AMPs and membranes [37].

Fig. 4 shows that the binding of MDpep5 to Gram-positive and Gram-negative bacteria was a function of the incubation time. The binding isotherm was linear up and membranes were saturated when bacteria were incubated with MDpep5 for 35 min (*E. coli* ATCC 25922) and 55 min (*S. aureus* 6538), respectively. This can be explained as follows: the surface potential of the bacterial membrane originates in the surface negative charge of the membrane. The hydrophobic interactions attract MDpep5 from the solution, which leads through the dissociation equilibrium, to an increase in local concentration of MDpep5 relative to the bulk solution [38]. Since the binding of the first MDpep5 molecules (which possesses a negative charge) to the bacterial membrane creates a more negatively charged outer surface, the negative residues of bound peptide will inhibit the free MDpep5 to adsorb on the membrane and the binding of further molecules becomes more difficult due to the electrostatic repulsion force between the membrane-bound MDpep5 and free MDpep5.

These results suggest that the permeation of MDpep5 through bacterial cell membrane is dependent on the membrane surface potential and the mutual inhibition of the transport of MDpep5 through the cell membrane is caused by the change in surface potential due to the binding of MDpep5 to the membrane.

### 3.3. Insertion mode of MDpep5 with bacterial membrane

The most striking difference between the cytoplasmic membranes of prokaryotic and eukaryotic cells is their lipid compositions. The outer membranes of bacterial cells are comprised largely of negatively charged phospholipids like phosphatidylglycerol, whereas the outer leaflet of the membrane of human erythrocytes is comprised mainly of zwitterionic phospholipids like phosphatidylcholine [39,40]. To study the insertion of MDpep5 into membranes, changes in the fluorescence of the tryptophan (Trp) residues in MDpep5 in aqueous buffer and in the presence of liposomes made from bacterial phospholipids were examined.

Because the fluorescence emission characteristics of Trp are sensitive to its immediate environment, it is often used to monitor the binding of peptides to membranes. As shown in Fig. 5, in Tris–HCl buffer, the wavelength maximum of MDpep5 was around 346 nm, which is the emission region of exposed tryptophan molecule while fluorescence emission of a hidden tryptophan molecule is known to be usually around 320–325 nm, indicating that the Trp residues were fully exposed to the aqueous environment [41]. By contrast, addition of bacterial liposomes to MDpep5 resulted in quenching in emission maximum ($\lambda_{max}$) and blue shift (4 nm) in the $\lambda_{max}$ compared to the peptide alone. Fluorescence quenching can be caused both by collisions (dynamic) and complex formation (static) with

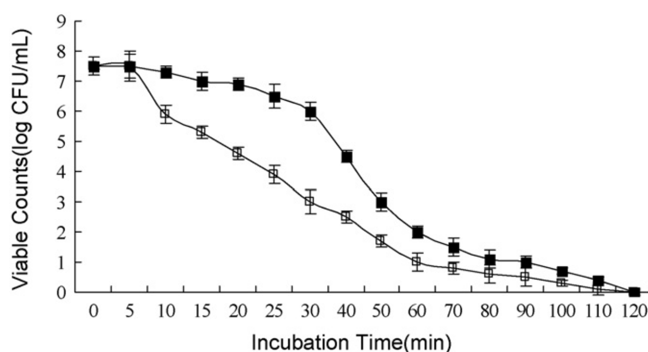


**Fig. 3.** Activity kinetics of MDpep5 against (■) *Escherichia coli* ATCC 25922; (□) *S. aureus* 6538. The experiment was carried out in triplicate, and average values are reported. Error bars refer to the standard deviation.

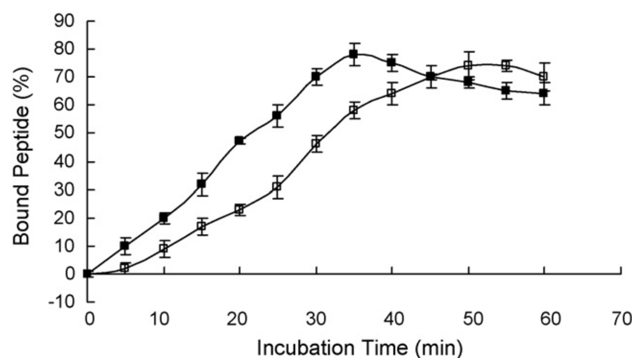


**Fig. 4.** Binding of MDpep5 to bacteria (■) *E. coli* ATCC 25922; (□) *S. aureus* 6538. The bound peptide is plotted as a function of the incubation time. The experiment was carried out in triplicate, and average values are reported. Error bars refer to the standard deviation.

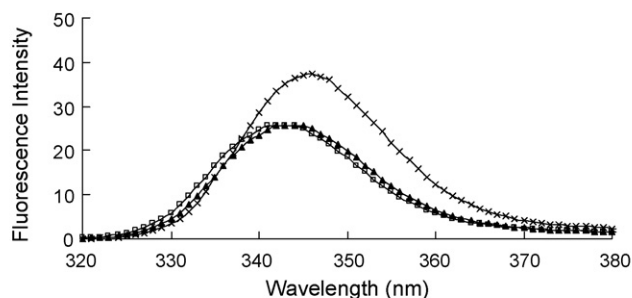


**Fig. 5.** Intrinsic fluorescence of MDpep5 in aqueous buffer and in the presence of bacterial liposomes. (×) Control; (▲) *E. coli* ATCC 25922; (□) *S. aureus* 6538.

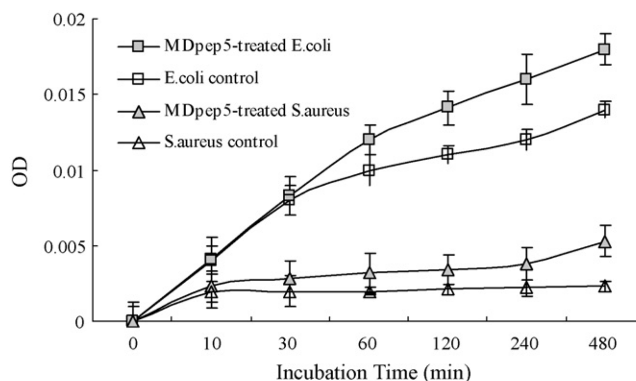


**Fig. 7.** Total nucleotide leakage from *E. coli* ATCC 25922 and *S. aureus* 6538 cells treated with MDpep5. Strains were incubated with peptide at twice the MICs for different time (10, 30, 60, 120, 240 and 480 min), and which incubated with 10 mM PBS (pH 7.4) was used as control. The mixture was filtered through 0.22 μm to remove the bacteria cells. The filtrate was then diluted appropriately and the optical density at 260 nm was recorded (UV-2102 PCS, Unico) at room temperature (25 °C).

the quencher [42]. The blue shift suggests that the peptide strongly binds to the lipid [43,44] and the fluorescence quenching process may be mainly governed by a static quenching mechanism rather a dynamic quenching one [45].

In order to investigate to what extent the Trp residues are exposed to the aqueous phase, and further investigate the conformational change of MDpep5 from solution to membrane, a fluorescence quenching experiment was performed using the neutral quencher acrylamide, a membrane-impermeable fluorescence quencher [46].

Fig. 6 shows that in buffer, increasing the acrylamide concentration caused efficient quenching of the fluorescence of the peptide in a concentration-dependent manner without other effects on the spectra, suggesting that Trp residues are exposed on the molecular surface of MDpep5 facing the buffer. For both *E. coli* ATCC 25922 and *S. aureus* ATCC 6538 liposome, MDpep5 showed a less effective quenching of Trp fluorescence, revealing that the Trp residues had now become less accessible to the quencher. This also supports the blue shifts observed for this peptide in the presence of liposome (Fig. 5), which is consistent with the idea that its Trp residue is buried in the phospholipid membranes.

### 3.4. Effect of MDpep5 on the membrane disruption

Membrane binding is the initial step involved in peptide–membrane interaction process. In order to explore whether the plasma membrane may break apart leading to cell death when an effective concentration of peptide binding to cell membrane is reached, membrane permeability assay was performed. If the bacterial membrane is damaged to a certain extent, release of cytoplasmic constituents of the cell can be monitored. Small ions such as potassium and phosphate tend to leach out first, followed by large molecules such as DNA, RNA, and other materials. Since these nucleotides have strong UV absorption at 260 nm, one can determine membrane integrity through the detection of absorbance at 260 nm.

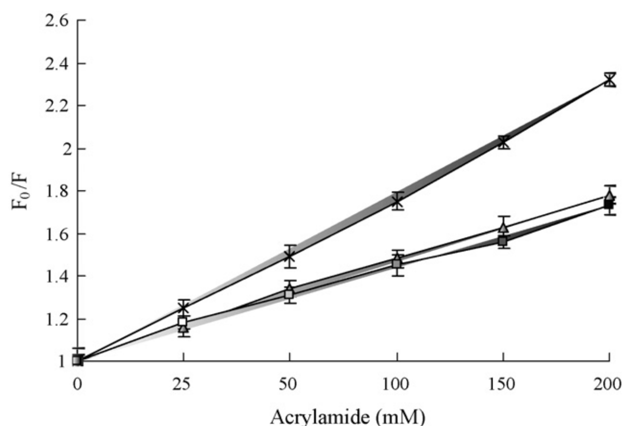


**Fig. 6.** Stern–Volmer plots for the quenching of Trp fluorescence were generated by the sequential addition of acrylamide in the absence (×) or presence of liposomes made from *E. coli* ATCC 25922 phosphorlipids (▲) and *S. aureus* 6538 (□).

As shown in Fig. 7, total nucleotide leakage from bacterial cells as a function of incubation time with peptide MDpep5 was plotted. MDpep5 can induce a little nucleotide leakage, but compared with other pore-forming AMPs [19], it was not significant. The result indicated that disruption of the cytoplasmic membrane was not the lethal event leading to bacterial cell death, which is different from other membrane-insertion AMPs.

As known, most AMPs contain more than five amino acids, which is suitable for forming pores in the bacterial membrane. For MDpep5, there are only five residues in the amino acid chain. Hence, the short chain of MDpep5 may be the primary reason why MDpep5 cannot completely disrupt bacterial membrane and cause nucleotide leakage.

In our above assays, we found that MDpep5 can bind and insert into the cell membrane, in contrast, the leakage result shows it cannot completely disrupt bacterial membrane. It is suggested that the action mode of MDpep5 may be different from the reported cationic peptides which can kill the microorganisms by pore formation in the cell membrane.

We do not know the exact mode of MDpep5, but sequence analysis shows that MDpep5 can easily form peptide–lipid complex with the membrane lipids due to its high total hydrophobic ratio (60%), and formation of such MDpep5-enriched lipid aggregates can lead to the disorder of the bilayer structure, unstabilizing the cell envelope and changing other physical properties of the lipid bilayer, which might be the mechanism by which MDpep5 permeabilizes bacterial membranes, resulting in the subsequent the death of cells.

### 3.5. Effect of MDpep5 on the bacterial electrostatic surface potential

To identify the effect of MDpep5 on the physical properties of the lipid bilayer, we first evaluated the effects of MDpep5 on the surface potential of bacterial membrane using a surface potential-sensitive probe (ANS).

In pure lipid bilayers, the surface potential is due to the negative net charges from charged polar headgroups. The changes in the electrostatic potential may therefore indicate a possible cou-

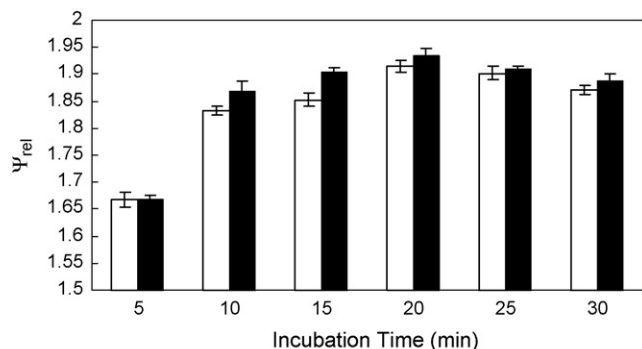


**Fig. 8.** The changes of the intensity of relative membrane surface potential ($\psi_{rel}$) (■) *E. coli* ATCC 25922; (□) *S. aureus* 6538 with respect to the control sample as a function of incubation time with antimicrobial peptide MDpep5.

pling between the charged residues in the AMPs and the molecules of phosphatidylethanolamine and/or phosphatidylglycerol [47]. ANS fluorometric titration, when incorporated in liposomes, facilitates calculation of the variation of the relative electrostatic surface potential ($\psi_{rel}$). Electrical surface potentials are characteristically present at the lipid-solution interface of biological membranes. The surface potential is a measure of the changes of both Gouy–Chapman and dipole potential at the surface of the liposome. The changes of the above values following addition of MDpep5 into the buffer at one liposome side are presented in Fig. 8.

The increase in $\psi_{rel}$ is obviously due to the negative charges of the antimicrobial peptide molecules. The adsorbed peptide molecules are quite crowded, causing the appearance of differences in the surface potential between both liposome sides. This indicates both, that interactions induce increased intermolecular separation and electrostatically unfavorable hyperpolarization of the overall resultant molecular dipoles in the direction perpendicular to the interface. However, with prolonging incubation time, $\psi_{rel}$ decreased, which maybe result from desorption of the membrane-bound MDpep5 from the cell membrane into the cytoplasm. As shown in Fig. 8, although reduction of $\psi_{rel}$ was observed, $\psi_{rel}$ could not decrease to the prime level (compared with control group), which suggests that the translocation of MDpep5 in membrane lead to the change of structural order of lipid membranes. The surface potential of *S. aureus* ATCC 6538 is less susceptible to MDpep5 than those of *E. coli* ATCC 25922, the mechanism of which may be related to the higher surface negative charge of *S. aureus* ATCC 6538 (−1.8) than that of *E. coli* ATCC 25922 (−0.8).

The surface potential (Gouy–Chapman, $\psi_s$) contributes – together with the membrane dipole potential ($\psi_d$) and the transmembrane potentials ($\Delta\psi$) – to the potential difference between the membrane surfaces ($\Delta\varphi$) and determines the electrical potential profile in the membrane [48]. Therefore, the change of the surface potential by MDpep5 would affect the potential profile in the membrane and the interfacial membrane potential difference ($\Delta\varphi$).

Based on our experimental data, we propose that MDpep5 profoundly affects physical properties of bacterial membrane when binding to the phospholipid which accounts for the bactericidal mechanism of MDpep5 and transmembrane behavior of MDpep5 is dependent on the membrane surface potential which originates in the surface negative charge and when an antimicrobial peptide interacts with bacterial cell membrane, it may affect the electrostatic surface potential of the membranes. Therefore, this information could be of considerable interest in establishing a possible relationship between the electrical properties of the phospholipid bilayer surface and AMPs' activity.

## 4. Conclusion

An anionic antimicrobial peptide MDpep5 (Val-Glu-Ser-Trp-Val) derived from housefly was purified and characterized. MDpep5 initiates its antimicrobial activity by binding to the target cytoplasmic membranes of bacterial cells, which is similar to the reported cationic AMPs. However, compared with the cationic AMPs, MDpep5 cannot completely disrupt membrane but affect the normal biochemical properties of cell membrane, thus killing the microorganisms. Our results suggest that the formation of MDpep5-enriched lipid aggregates can lead to the disorder of the bilayer structure and insertion of MDpep5 into bacterial liposomes is dependent on the membrane surface potential. It also suggests that the mutual inhibition of the transport of MDpep5 through the cell membrane is caused by the change in surface potential. Based on our experimental data, we propose that MDpep5 profoundly affects physical properties of bacterial membrane when binding to the phospholipid which accounts for its bactericidal activity. All the results provide the evidence that, together with cationic antimicrobial peptides, anionic antimicrobial peptides may also exist naturally as part of the innate defense system.

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## References


[1] H.G. Boman, Annu. Rev. Immunol. 13 (1995) 61–92.
[2] S. Sambhara, R.I. Lehrer, Expert Rev. Anti. Infect. Ther. 5 (2007) 1–5.
[3] R.E.W. Hancock, Lancet Infect. Dis. 1 (2001) 156–164.
[4] M. Zasloff, Nature 415 (2002) 389–395.
[5] D.I. Andersson, B.R. Levin, Curr. Opin. Microbiol. 2 (1999) 489–493.
[6] D.I. Andersson, Curr. Opin. Microbiol. 6 (2003) 452–456.
[7] M. Salzet, Trends Immunol. 23 (2002) 283–284.
[8] G. Kragol, S. Lovas, G. Varadi, B.A. Condie, R. Hoffmann, L. Otvos Jr., Biochemistry 40 (2001) 3016–3026.
[9] L. Otvos Jr., I.O.M.E. Rogers, P.J. Consolvo, B.A. Condie, S. Lovas, P. Bulet, M.B. Thurin, Biochem. 39 (2000) 14150–14159.
[10] C. Aisenbrey, B. Bechinger, G. Gröbner, J. Mol. Biol. 37 (2008) 376–385.
[11] H.W. Huang, Biochim. Biophys. Acta 1758 (2006) 1292–1302.
[12] Y. Shai, Biochim. Biophys. Acta 1462 (1999) 55–70.
[13] S.G.A. McLaughlin, G. Szabo, G. Eisenman, J. Gen. Physiol. 58 (1971) 667–687.
[14] F. Abdulkader, M.A. Miranda, R. Curi, J. Procopio, J. Biochem. Biophys. Methods 70 (2007) 515–518.
[15] B. Frankenhäuser, A.L. Hodgkin, J. Physiol. 137 (1957) 218–244.
[16] Y. Sokolov, W.S. Mailliard, N. Tranngo, M. Isas, H. Luecke, H.T. Haigler, J. Gen. Physiol. 115 (2000) 571–582.
[17] T. Baczek, J. Pharm. Biomed. Anal. 34 (2004) 851–860.
[18] C.V. Suresh Babu, D.S. Lho, Y.S. Yoo, J. Pharm. Biomed. Anal. 40 (2006) 136–141.
[19] L. Hou, Y. Shi, P. Zhai, G. Le, Food Control 18 (2007) 1350–1357.
[20] S. Yaron, T. Rydlo, D. Shachar, A. Mor, Peptides 24 (2003) 1815–1821.
[21] J. Hindler, Tests to Assess Bactericidal Activity. From: Clinical Microbiology Procedures Handbook, American Society for Microbiology, Washington, DC, 1992, pp. 5.16.14–5.16.24.
[22] E.G. Bligh, W.J. Dyer, Can. J. Med. Sci. 37 (1959) 911–917.
[23] S.R. Dennison, J. Howe, L.H.G. Morton, K. Brandenburg, F. Harris, D.A. Phoenix, Biochem. Biophys. Res. Commun. 347 (2006) 1006–1010.
[24] T. Konishi, M. Murakami, Y. Hatano, K. Nakazato, Biochim. Biophys. Acta 862 (1986) 278–284.
[25] M. Toyomizu, K. Okamoto, Y. Akiba, T. Nakatsu, T. Konishi, Biochim. Biophys. Acta 1558 (2002) 54–62.
[26] H.H. von Horsten, B. Schäfer, C. Kirchhoff, Peptides 25 (2004) 1223–1233.
[27] J.M. Walker, Protein Determination by UV Absorption, From: The Protocols Handbook, Inc. Humana Press Totowa, NJ, 1990, pp. 3–5.
[28] M.R. Eftink, C.A. Ghiron, J. Phys. Chem. 80 (1976) 486–493.
[29] C.Z. Chen, S.L. Cooper, Biomaterials 23 (2002) 3359–3368.
[30] J. Slavik, Biochim. Biophys. Acta 694 (1982) 1–25.
[31] M. Sugawara, H. Oikawa, M. Kobayashi, K. Iseki, K. Miyazaki, Biochim. Biophys. Acta 1234 (1995) 22–28.
[32] M. Sugawara, M. Kurosawa, K. Sakai, M. Kobayashi, K. Iseki, K. Miyazaki, Biochim. Biophys. Acta 1564 (2002) 149–155.
[33] J.M. Conlon, N. Al-Ghaferi, B. Abraham, J. Leprince, Methods 42 (2007) 349–357.

[34] F.Y. Chen, M.T. Lee, H.W. Huang, Biophys. J. 82 (2002) 908–914.
[35] W. Vollmer, J.V.T. Holtje, J. Bacteriol. 186 (2004) 5978–5987.
[36] H. Sato, J.B. Feix, Biochim. Biophys. Acta 1758 (2006) 1245–1256.
[37] V. Frecer, B. Ho, J.L. Ding, Antimicrob. Agents Chemother. 48 (2004) 3349–3357.
[38] K. Nomura, G. Corzo, Biochim. Biophys. Acta 1758 (2006) 1475–1482.
[39] K. Matsuzaki, Biochim. Biophys. Acta 1462 (1999) 1–10.
[40] Z. Oren, Y. Shai, Biopolymers 47 (1998) 451–463.
[41] C.N. N'soukpoé-Kossi, R. Sedaghat-Herati, C. Ragi, S. Hotchandani, H.A. Tajmir-Riahi, Int. J. Biol. Macromol. 40 (2007) 484–490.
[42] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, second ed., Kluwer Academic/Plenum Publishers, New York, 1999, pp. 238–249.
[43] S.T. Yang, S.Y. Shin, J.I. Kim, FEBS Lett. 581 (2007) 157–163.
[44] S. Liu, A. Shibata, S. Ueno, F. Xu, Y. Baba, D. Jiang, Y. Li, Colloids Surf. B: Biointerf. 48 (2006) 148–158.
[45] M. Bogdan, A. Pirnau, C. Floare, C. Bugeac, J. Pharm. Biomed. Anal. 47 (2008) 981–984.
[46] G.R. Thuduppathy, J.W. Craig, V.K.A. Schon, R.B. Hill, J. Mol. Biol. 359 (2006) 1045–1058.
[47] H. van Mil, J.S. van Heukelom, M. Bier, Biophys. Chem. 106 (2003) 15–21.
[48] M.V. Castelli, A.F. Lodeyro, A. Malheiros, S.A.S. Zacchino, O.A. Roveri, Biochem. Pharmacol. 70 (2005) 82–89.