

Study on Effect of Antimicrobial Lipopeptide from *Bacillus subtilis* against *E. coli*

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Antimicrobial lipopeptides (AMLPs) (surfactin, fengycin and their isoforms) produced by *Bacillus subtilis* strain exhibit a broad antimicrobial spectrum against Gram-positive bacteria, Gram-negative bacteria, fungi and virus. In this paper, antimicrobial activity of AMLPs against *Escherichia coli* ATCC 25922 was determined, and the effects of AMLPs on morphological, growth, multiplication, membrane permeability were investigated. Results indicated growth and multiplication of *E. coli* were inhibited efficiently, which was sensitive to AMLPs. The minimal inhibitory concentration (MIC) was 125µM. Studies on bacterial growth curve and microstructure indicated that AMLPs could kill or lyse the *E. coli* cells. Membrane permeability study showed significant increase of permeability of *E. coli* cells. This study indicated the AMLPs could affected the biomembrane.

Key words: Antimicrobial lipopeptides, *B. subtilis*; *E. coli*, Membrane permeability, Minimal inhibitory concentration.

Bacillus subtilis can produce a lot of antimicrobial substance, such as bacillomycin, plipastatin, surfactin, iturin, fengycin, subtilin, bacillopeptins (Besson *et al.*, 1977; Cho *et al.*, 2003; Corvey *et al.*, 2003; Deleu *et al.*, 2005; Shu *et al.*, 2002; Kajimura *et al.*, 1995; Kleerebezem *et al.*, 2004; Kluge *et al.*, 1988; Moyne *et al.*, 2001; Moyne *et al.*, 2004; Peypoux *et al.*, 1976; Tsuge *et al.*, 1996), and the structures of most of which had been identified. We had isolated a novel *Bacillus subtilis* that could produce lipopeptide antimicrobial substance.

In our previous work, culture medium optimization, antibacterial spectra, isolation and purification, structure identity and partial application of AMLPs had been researched, Which indicated AMLPs included surfactin, fengycin and

their congeners. But the effect and process of antibacterial have not be studied yet.

There are many reports about antibacterial mechanism of lipopeptides, however, there is not an agreement about it as yet. Most of them believed functional target organ was cytoplasmic membrane according to molecular hydrophobicity and hydrophilicity. The mechanism was interpreted by barrel-stave (Wieprecht *et al.*, 2000) and carpet (Beven *et al.*, 2003) hypotheses, but reports about hereditary substance, metabolic enzyme, metabolic process, protein synthesis and so on were fewer. This paper studied antibacterial effect of AMLPs against *E. coli* ATCC 25922 from morphostructure of bacterial cell and membrane permeability.

MATERIALS AND METHODS

Microorganism strain and production of the AMLPs

Bacillus subtilis was inoculated into a 250 ml shake flask containing 70 ml of Luria-Bertani

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(LB) medium (5 g beef extract L⁻¹; 10 g peptone L⁻¹; 5 g NaCl L⁻¹; pH 7.2) and cultivated at 37°C and 130 rpm for 24 h. Pre-culture (5 %, v/v) was inoculated into 500 ml shake flask containing 100 ml of Landy medium (Landy *et al.*, 1948) and cultivated at 33! and 180 rpm for 38h for the AMLPs production. At the end of cultivation, the culture was centrifuged at 11000 ×g for 15 min to remove bacterial cell.

Extraction of AMLPs

The supernatant was adjusted to pH 2.0 with 6 M HCl and incubated at 4 °C for 24h, then was centrifuged at 11000 ×g for 15 min to collect sediment, which was dissolved with methanol (pH 7.0) and stored at 4°C for 12 h, then centrifuged again at 11000 ×g for 15 min to gather supernatant including the AMLPs.

Determination and purification of AMLPs

The supernatant was assayed by RS-HPLC (C18 column, ODS-4.6mm×250mm, AGILENT 1100series). The system was operated at a flow rate of 0.5ml min⁻¹ with acetonitrile-trifluoroacetate (3.8 mM, MERK) as mobile phase using gradient elution and monitored at 234 nm (G1314A VWD, JP24020513, AGILENT).

The chromatography spectrum for the separation of AMLPs from supernatant had been obtained. The relation between peak area (y) and concentration of AMLPs (x) was expressed by the following standard curve:

$$y = 7168.5x + 1854.1 \quad (R^2 = 0.9874) \quad (1).$$

The AMLPs was purified by HPLC, and concentrated further. And AMLPs were proved to be a composition of surfactin, fengycin and their isoforms.

Strains and growth conditions

The bacterial strains used were *Escherichia coli* ATCC 25922 and *Bacillus subtilis*. The bacterial strains were grown in a LB medium at 37°C and 150 rpm.

The strain *E. coli* ATCC 25922 was inoculated into a 250 ml shake flask containing 70 ml of beef extract medium and cultivated at 37°C and 150 rpm for 18 h to exponential growth phase as a pre-culture.

Determination of sensitivity of *E. coli*

E. coli cells was gathered at exponent growth phase, and diluted with LB to give a final concentration 1×10⁵ colony-forming unit (CFU) ml⁻¹. 0.1 ml dilution was spread on plate of LB agar and oxford cup was placed on the plate, then 0.2 ml

0.42 mM AMLPs was added into the cup. The plate was incubated at 37°C for 24 h. Then antagonistic discs were determined.

Determination of the minimal inhibitory concentration (MIC)

A modified microdilution technique described by Vorland (Vorland *et al.*, 1998) was used to determine the MIC. Briefly, serial dilutions of AMLPs were made with methanol in 96-well microtitre plates. Each well was inoculated with exponential growing bacteria diluted in LB to give a final concentration of approximate 1×10⁵ CFU ml⁻¹. The MIC was determined as the lowest concentration at which growth was inhibited.

Effect of AMLPs on *E. coli* growth curve

Quantitative assay for *E. coli* growth inhibition was performed following the protocol developed by Broekaert (Broekaert *et al.*, 1990) with some modifications. The pre-culture (5 % v/v) was inoculated into 250 ml shake flask containing 100 ml of LB medium and cultivated at 37°C and 150 rpm for 48h, with different concentrations AMLPs was added at designed time, and *E. coli* growth was monitored by measuring the absorbency of cells culture at 540 nm. *E. coli* cell growth without addition of AMLPs was also determined.

Scanning electron microscopy (SEM)

The pre-cultures were incubated with 1×MIC concentration of the AMLPs at 37°C for 3 h. The cells were then chemically fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer at 4°C for 2 h, collected on a Nuclepore filter (pore size, 0.2 μm) by filtration, and washed three times with the buffer. The cells on the filter were fixed with 1 % osmic acid for 1 h, and then dehydrated with a graded ethanol series. The samples were lyophilized, coated with gold in an ion coater, and examined by scanning electron microscopy on a PHILIPS SEM-505 instrument.

Transmission electron microscopy (TEM)

The pre-cultures were incubated with 1 ×MIC concentrations of AMLPs at 37°C for 3 h. *E. coli* cells were fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer at 4°C for 1 h and embedded in 2% Agar Noble. The agar blocks were then postfixed in 2% osmic acid at 4°C for 1 h and dehydrated in a graded series of alcohols. The cells were embedded in EPON 812 Resin at 60°C. Ultrathin sections (thickness, 50 nm) were stained with uranyl acetate and lead by a modified staining

method (Sato, 1968). Microscopy was performed on a HITACHI H-600 transmission electron microscope.

Effect of AMLPs on membrane permeability of *E. coli* cells

The pre-culture (5 %, v/v) was inoculated into 250 ml shake flask containing 100 ml of LB medium and cultivated at 37°C and 150 rpm, AMLPs with different concentrations were added at designed time, and sampled at different time. The samples were centrifuged at 11000×g for 15 min to remove *E. coli* cells. Absorbency of supernatant was determined at 280 nm and 260 nm respectively. At which protein and nucleic acid have the biggest absorbency peak.

Statistical analysis

The validation data in the verification experiments were analyzed by T-tests using the SPSS 12.0 software.

RESULTS

Determination of sensitivity and minimal inhibitory concentration

Diameter of antagonistic discs of AMLPs was 23.50±2.31mm (Mean±S.D) when its concentration was 0.5mM. The MIC of AMLPs against *E. coli* ATCC 25922 was 125µM. Which showed antibacterial activity of AMLPs against *E. coli* ATCC 25922.

Effect of AMLPs on *E. coli* growth curve

Change of *E. coli* growth curve was observed when AMLPs with different concentration was added at different time (Fig.1). *E. coli* cell density increased gradually with culture time prolonging in control group (Fig.1.a), but

decreased when 1 ×MIC AMLPs was added at 4, 6 h (Fig.1. b, d). Cell density was in a lower level inhibited by adding AMLPs at 4 h, which was markedly difference after culturing for 6h ($P>0.01$). Adding AMLPs at 4 h and 6 h, cell density dramatically decreased from a higher level to a lower lever, which was markedly difference after culturing for 8h and 24h respectively ($P>0.01$). Cell density dropped dramatically when 3 ×MIC AMLPs was added at 4, 6 h (Fig.1. c, e) respectively, which was markedly difference after culturing for 6h and 10h and 24h respectively ($P>0.01$).

Morphological effects

After 3 h of exposure to 1 ×MIC of AMLPs, a profound effect on the cell morphology of *E. coli* was observed using SEM (Fig. 2). Compared to the control with short-pole shape, fringe clear, superficial smooth cells (Fig. 2 a), cells of exposing to the AMLPs became granulation and abnormality with short, crude, round shape (Fig. 2 b), and some of them were similar to coccoidal shape. Cell surface become coarse, fringe irregular, cupped. But lacunose cells or cell fragment weren't observed. Additionally, significant morphological difference between control and treatment groups wasn't observed by light microscopy.

After 3 h of exposure to 1 ×MIC of AMLPs, a profound effect on the cell morphology of *E. coli* was observed using TEM (Fig. 3). Compared to the control with cell figure clear, cytoplasm uniformity, without inanition and obvious cytoplasm leakage (Fig. 3 a), cells treated by 1 ×MIC AMLPs became illegibility of figure and disruption of partial cell wall and vacuole cells due to leakage of cytoplasm (Fig. 3 b).

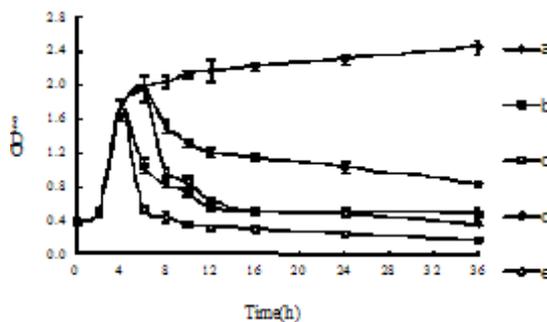


Fig. 1.The effect of AMLPs on *E. coli* growth. *E. coli* growth was monitored by measuring the density of cells culture at 540 nm. a. Control; b,d. 1 ×MIC AMLPs was added at 4, 6 h respectively; c,e. 3 ×MIC AMLPs was added at 4, 6 h respectively; Experiments were performed repeatedly three times and black arrowheads represented time of AMLPs added. The bars represent standard deviation

Morphology of *E. coli* treated with AMLPs by SEM and TEM showed that AMLPs could make cell wall disrupt or pore form. These cells couldn't metabolize normally because cell integrity was destroyed and content leaked, which would lead to death of cell.

Effect of AMLPs on membrane permeability of *E. coli* cells

Different concentrations AMLPs were added at designed time, and sampled at different time. The samples were centrifuged for 15 min at 11000 ×g for removing *E. coli* cells. Absorbency of the supernatant was determined at 260nm (Fig. 4). Absorbency values of medium supernatant at 260 nm decreased gradually with prolonging of culture time in the control (Fig.4.a), which may be because that amino acid, peptide, protein in the medium

was used by bacterial cell, and these substances can absorb ultraviolet light. The result was consistent with *E. coli* growth curve. On the contrary, absorbency of groups treated by AMLPs at 260 nm increased gradually with time prolonging compared to control. The degree of absorbency change of treated groups was increase with AMLPs increasing at the same cell concentration (Fig. 4: b and c, d and e), but was little with increase of cell concentration at the same AMLPs concentration. It maybe the reason that AMLPs concentration to single cell was correspondingly lower with cell concentration increasing at same AMLPs concentration so antibacterial effect become weak. Result showed AMLPs could lead to cell membrane permeability increase or bacterial cell disruption. At normal condition, some big absorbency

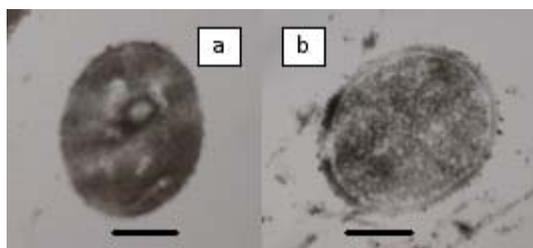


Fig. 2. Scanning electron microscopy of *E. coli* treated with AMLPs. *E. coli* Cells were incubated for 3 h in the absence of AMLPs (a) or in the presence of AMLPs (1 ×MIC) (b) and the specimens were then prepared for scanning electron microscopy. a. Intact cell; b., cell treated with AMLPs; The bars represent 10µm

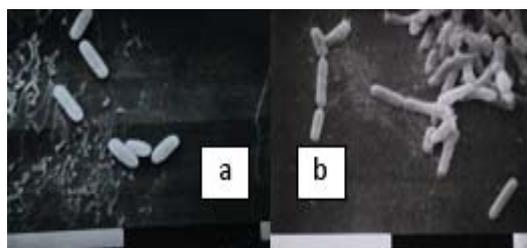


Fig. 3. Transmission electron micrographs of AMLPs-treated *E. coli*. *E. coli* Cells were incubated for 3 h in the absence of AMLPs (a) or in the presence of AMLPs (1 ×MIC) (b) and the specimens were then prepared for transmission electron microscopy. a. Intact cell; b. cell treated with AMLPs. The bars represent 100nm

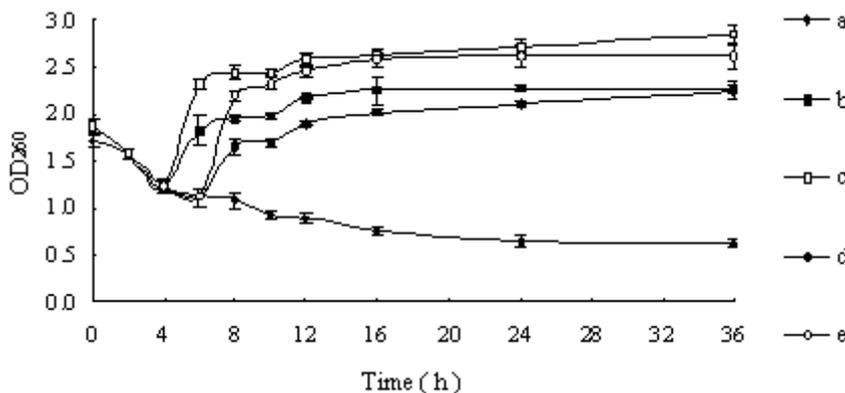


Fig. 4. Effect of AMLPs on membrane permeability of *E. coli* cells. Absorbency of culture supernatant at 260nm. a. Control; b,d. 1×MIC AMLPs was added at 4, 6h respectively; c,e. 3×MIC AMLPs was added at 4, 6h respectively; Experiments were performed three times. The bars represent standard deviation

molecule couldn't traverse cell membrane into culture solution. However, with cell membrane permeability increasing, these molecules would leak into culture solution, resulting in absorbency increase of medium supernatant. This indicated that increase of membrane permeability was a major probable factor which led to the death of *E. coli*.

DISCUSSION

AMLPs MIC was 125 μ M against *E. coli* ATCC 25922, and surfactin and fengycin contained 7-amino acid residues and 10-amino acid residues respectively, but cationic antimicrobial peptides reported generally are peptides containing less than 50 amino acids. The MICs of these peptides for Gram-positive and Gram-negative bacteria, fungi and protozoa were from 0.25 μ g ml⁻¹ to 16 μ g ml⁻¹ (Robinson *et al.* 2005). Additionally, MICs of bovine lactoferricin (LfcinB) homology BLP-2 and human lactoferricin (LfcinH) homology HLP-2 against *E. coli* wild type strain W3110 were 80 μ M and 320 μ M respectively (Farnaud, Patel, Odell, & Evans, 2004). Compared to MICs of most cationic antimicrobial peptides and BLP-2, MIC of AMLPs against *E. coli* ATCC 25922 was higher and similar to HLP-2.

It was reported the antibacterial activity of the amphiphilic α -helical peptides varied with their chain length, and 15 residues was the optimal length in the Beven's study (Beven, Castano, Dufourcq, Wieslander & Wroblewski, 2003), but study of Apponyi et al (Apponyi, 2004) showed the longer peptides were more effective. In the paper, two important compositions of AMLPs were cyclolipopeptide antibacterial substance consisting of 7 and 10 amino acid residues respectively, and it may be because of their shorter peptides chain for their slight bacterial sensitivity. It is assumed that the antimicrobial activity of amphiphilic cationic peptides is effectuated by their action on the cytoplasmic membrane of the target cell, either by pore formation or by membrane thinning or destabilizing effects without forming pores (Gazit, Boman, Boman, & Shai, 1995; Heller *et al.* 2000). In the study, growth curve showed that cell concentration dropped to some extent after AMLPs was added (Fig.1). This AMLPs could make *E. coli* lyse. Further morphologic observation (Fig.2; Fig.3) by SEM and TEM demonstrated

AMLPs could result in cell disruption or pore formation to cell content leak (Fig.4), which made absorbency of medium supernatant at 280nm increase remarkably.

It could be concluded from present study that inhibition, sterilization, lysis of AMLPs to *E. coli* ATCC 25922 was performed by physical action which could result in cell membrane disrupting and pore formation. But further studies need to be done to make sure that whether function AMLPs can result in *E. coli* cell rapid death by comprehensive action of many aspects.

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