Food Control 20 (2009) 149–156

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Discovery of a novel antimicrobial peptide using membrane binding-based approach

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ARTICLE INFO

Article history:
Received 28 January 2008
Received in revised form 17 March 2008
Accepted 28 March 2008

Keywords:
Antimicrobial peptide
Binding
Membrane disruption
Purification

ABSTRACT

Most known antimicrobial peptides (AMPs) can bind to bacterial cells as the first step to exert their antimicrobial activity. Based on this membrane-binding activity, a novel antimicrobial peptide MDpep9 with the sequence Lys-Ser-Ser-Ser-Pro-Pro-Met-Asn-His was purified from housefly larvae. MDpep9 effectively inhibited the growth of the test bacteria (*Escherichia coli, Salmonella typhimurium, Staphylococcus aureus, Shigella dysenteria, Pseudomonas aeruginosa, Bacillus subtilis,* and *Streptococcus pneumoniae*), with MIC (minimal inhibitory concentration) values ranged from 9 to 72 μg/ml. Among these bacteria, the Gram-positive bacterium, *B. subtilis* 9372 was the most sensitive. Furthermore, the antimicrobial mode study showed that cytoplasmic cell membrane is the target for MDpep9 which can exert its antimicrobial action by disrupting and disintegrating bacterial cell membranes, leading ultimately to loss of cytoplasmic membrane integrity. Moreover, the acidic environment around bacterial cell membrane induced molecular unfolding and increased surface hydrophobicity of MDpep9, promoting the interaction of peptide with bacterial lipid membrane. Our results indicate membrane-binding strategy opens the road to simple and cheap large-scale production of a safe antimicrobial peptide from housefly.

1. Introduction

The microbial contamination problem in food safety is still a major concern not only for developing countries but also for the industrialized world. In particular, microbial contamination of ready to eat products is of concern to human health (Pranoto, Rakshit, & Salokhe, 2005). Meanwhile, since food processors and consumers have expressed a desire to reduce the use of synthetic chemicals in food preservation owing to the potential toxicity of "chemical" food preservatives, the demand for food preservatives from natural sources increases. With several desirable properties such as heat-tolerant, relatively broad antimicrobial spectrum and low toxicity to eukaryotic cells, antimicrobial peptides (AMPs), especially the "food-derived antimicrobial peptides", may serve as a potentially significant group of food preservatives (Osmanafaoflu, 2007; Schneider et al., 2006).

Insects produce a variety of AMPs that play a crucial role in protecting them from invading microorganisms. Since the first discovery of AMPs in insects (Steiner, Hultmark, Engstrom, Bennich, & Boman, 1981), many AMPs have been isolated from insects (Bulet et al., 1993; Fogaca et al., 2004; Konno et al., 2001; Tomie et al., 2003). The housefly (*Musca domestica*) belongs to the Family Diptera. The larva of housefly has been used as food and clinically to cure the serious cutaneous infection caused by bacteria, such as ecthyma and lip boil since the Ming/Qing Dynasty (1368 Anno Domini) up to now in China, and is one ingredient of Chinese traditional famous "Children eight-ingredient precious pastry" for curing bacterial infection of the digestive organs such as vomiting and dysentery, which is potentially an untapped source of bioactive peptides and value-added food production. The potential value of AMPs derived from the edible larvae of housefly could be considered as natural antimicrobial agents for use in food industry as "secondary preservatives".

Demands for highly-purified active AMPs in areas of medicine, food preservatives and emerging technologies are increasing rapidly. To keep up with the growing demand, AMPs purification methods must be developed to be efficient, reliable and cost effective. Although a variety purification schemes have been described for AMPs (Gomes et al., 2005; Halverson, Basir, Knoop, & Michael Conlon, 2000; Lee et al., 1999; Liepke, Zucht, Forssmann, & Ständker, 2001; Ovchinnikova et al., 2004), these isolation methods require multiple steps to produce highly-purified AMPs, resulting in undesirable losses of the already limited material. Thus, the discovery of improved purification methods is a driving force for our research into further application of AMPs.

AMPs cause damage to bacterial membranes, which results in cell death. In an effort to understand how AMPs cause membrane damage and the relation of this interaction to the biological function of AMPs, the peptide–lipid interactions of AMPs have been investigated extensively (Aisenbrey, Bechinger, & Gröbner, 2008; Chen, Lee, & Huang, 2002; Cummings & Vanderlick, 2007; Seto et al., 2007; Sevcsik, Pabst, Jilek, & Lohner, 2007; Van Kan, Demel, Van der Bent, & De Kruijff, 2003; Van Kan, Van der Bent, Demel, & De Kruijff, 2001). However, the mode of action of AMPs is not

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doi:10.1016/j.foodcont.2008.03.006

fully understood and the basis for their selectivity towards specific target cells is not known, but it is undoubted that membrane binding is the initial step involved in membrane disruption process (Lohner & Epand, 1997).

In this paper an innovative method was developed to isolate AMPs from housefly larvae. This approach is based on the membrane-binding activity of AMPs, combining gel filtration chromatography followed by reverse phase high-performance liquid chromatography and MS/MS analysis. By using this method, a novel antimicrobial peptide MDpep9 was successfully isolated from housefly larvae, and the bacterial cell membrane was confirmed to be the target for MDpep9.

2. Materials and methods

2.1. Preparation of dehydrated housefly larvae extracts (HLE)

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 ethylene diamine tetra-acetic 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 12000*g* for 10 min at 4 °C, the water fraction containing peptides was freeze-dried and stored at −20 °C until needed.

2.2. Housefly larvae extract incubated with bacteria (HLE-IB)

Escherichia coli ATCC 25922 was grown from an overnight culture to mid-logarithmic phase in Luria–Bertani (LB, Difco, USA) medium at 37 °C, centrifuged at 2000*g* for 20 min and washed twice in sterile PBS (10 mM, pH 7.0) followed by centrifugation at 2000*g* for 20 min. Pellets were resuspended in PBS to an optical absorbance at 600 nm of 0.8, and then incubated with the same volume of the HLE solution (10 mg/ml in PBS) at 37 °C for 30 min. After incubation, the mixture was centrifuged at 4000*g* for 10 min followed by filtration through 0.22 μm Nalgene syringe filters (Fisher Scientific) to remove the bacterial cells as described by Potter, Hansen, and Gill (2005) and the filtrate was freeze-dried and stored at −20 °C until use.

2.3. Gel filtration on Sephadex G-15 of HLE and HLE-IB

HLE-IB (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 by measuring the absorbance at 220 nm. HLE (20 mg) was also column chromatographed on the Sephadex G-15 column as above.

Compared with the spectrum of HLE, two fractions (fractions B1 and B2) disappeared in the spectrum of HLE-IB, suggesting that these two fractions were removed from the HLE by interaction with the cell membrane material of *E. coli* through centrifugation and filtration. These two filtrations might contain AMPs. The antimicrobial assay demonstrated that fraction B1 and B2 were the most effective among all fractions and the antimicrobial activity of fraction B2 is higher than that of B1. And then fraction B2 was freeze-dried and stored at −20 °C until needed.

2.4. Peptide further purification by reverse phase high- performance liquid chromatography (RP-HPLC)

Fraction B2 was subjected to RP-HPLC YWG C_{18} column (250 × 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 were re-chromatographed on Kromasil C_{18} (250 × 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 was collected, vacuum dried, weighed and used in the subsequent experiments.

2.5. 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). All spectra were measured under the following conditions: MS: Reflector Positive, CID (OFF), mass rang (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 was deposited on the sample slide and left to dry at room temperature. The resulting spectra were analyzed and compared.

2.6. Assay for antimicrobial activity

2.6.1. Microorganisms and growth conditions

All the reference strains were kindly provided by Wuxi disease prevention and control center (Wuxi, China). They are *E. coli* ATCC 25922, *Salmonella typhimurium* 50013, *Staphylococcus aureus* 6538, *Shigella dysenteriae* 51302, *Pseudomonas aeruginosa* ATCC 27553, *Bacillus subtilis* 9372, *Penicillium expansum* ATCC 1117, *Aspergillus niger* ATCC 16404, *Streptococcus pneumoniae* ATCC 49619, and *Saccharomyces cerevisiae* ATCC 40075, respectively. *E. coli* ATCC 25922 was grown in Luria–Bertani (LB, Difco, USA) agar slant at 37 °C. *P. expansum* ATCC 1117 and *A. niger* ATCC 16404 were kept on potato dextrose agar (PDA, Merck, México) slants at 28 °C. *S. cerevisiae* ATCC 40075 was grown in yeast extract peptone dextrose (YEPD, Merck, México) medium at 28 °C. Other microorganisms were grown in nutrient agar (NA, Difco, USA) slants at 37 °C. All media were sterilized by autoclaving using an autoclave at 121 °C for 20 min. All strains were maintained as frozen stocks in appropriate broth plus 20% (v/v) glycerol. Throughout the experiments, strains were subcultured every 2 weeks on agar media to keep the microorganisms viable and kept at 4 °C. Before use in experiments, cultures were propagated twice in liquid media overnight.

2.6.2. Agar diffusion assay

Agar diffusion assay was performed as described by Hou, Shi, Zhai, and Le (2007). Overnight culture of the reference strains were adjusted to 2–5 × 10^8 colony forming units per ml (CFU/ml). One milliliter of such a culture was added to 15 ml respective medium, evenly mixed and poured into Petri dishes (9 cm in diameter). The test samples were dissolved in 10 mM PBS (pH 7.4). The discs (6 cm in diameter) were then applied. Ten microliter 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. PBS was used as negative control, Norfloxacin (10 μg/disk) was used as antibacterial positive control. The experiments were carried out in triplicate.

2.6.3. Minimal inhibitory concentration (MIC)

Minimal inhibitory concentration (MIC) of peptide for each test microorganism was determined with liquid growth antimicrobial assay as described by Yaron, Rydlo, Shachar, and Mor (2003). The overnight culture was washed and resuspended in PBS (10 mM, pH 7.4) by centrifugation at 3000*g* for 10 min to attain the final microbial density 2–4 × 10^7 CFU/ml. Peptide stock solution (576 μg/ml) was prepared with 10 mM PBS (pH 7.4). The peptide solution was sterilized by filtration (Millipore 0.22 filter unit) and diluted two-folds serially in sterile PBS (10 mM, pH 7.4). Fifty microliter of peptide solution was incubated in sterilized 96-well plates with 100 μl media and 100 μl of the test microorganisms disposed as described above, and 50 μl of PBS (10 mM, pH 7.4) tested under the same condition was used as the control. 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. The experiments were carried out in triplicate, and average values are reported.

2.7. Hemolytic activity

Hemolytic activity was evaluated turbidimetrically, as described previously (Park et al., 2004). Erythrocyte suspensions were prepared using fresh rabbit red blood cells (RBCs), washed and resuspended in Tris-buffered saline (TBS, 145 mM NaCl, 10 mM Tris–HCl, pH 7.4). And then RBCs were incubated with peptide (0–100 μg/ml) dissolved in TBS for 60 min at 37 °C. The samples were centrifuged at 3000*g* for 5 min (Centrifuge 5804R, Eppendorf) and the optical density of the supernatant was measured at 414 nm using the microplate reader (Multiskan MK3, Thermo Labsystems Co., USA). No hemolysis (0%) and full hemolysis (100%) were observed in the presence of TBS and 0.1% Triton X-100, respectively. The percent hemolysis was calculated using the following equation:

$$\%\text{hemolysis} = \frac{A - A^0}{A^X - A^0} \times 100$$

where '*A*' is $OD_{414\,nm}$ with peptide solution, 'A^0' is $OD_{414\,nm}$ in TBS, and 'A^X' is $OD_{414\,nm}$ with 0.1% Triton X 100.

2.8. Mode of antimicrobial action of MDpep9

2.8.1. Conformation change of MDpep9 at lower pH around bacterial cells

Changes of molecular surface hydrophobicity of MDpep9 induced by lower pH around bacterial cells were investigated using 8-anilino-1-naphthalenesulfonic acid (ANS) (Sigma Chemical Co., St. Louis, MO). All fluorescent measurements were carried out on an RF-5301PC fluorescence spectrometer (Shimadzu, Kyoto, Japan) equipped with a 1 cm pathlength cell. All pH measurements were made with a glass pH electrode (Cole Parmer) whose measuring range is 0–14 at 25 ± 0.1 °C. The peptide stock solution was diluted with 10 mM KH_2PO_4/Na_2HPO_4 buffers at different pH (2.6, 3, 4, 5, 6, and 7, adjusted with NaOH or HCl) to a target concentration of 9 μg/ml, and then the final pH was checked using a glass pH electrode (Cole Parmer). After a 30 min incubation period, pH was adjusted to neutrality and then fluorescence spectra were measured at 25 ± 0.1 °C in the presence of ANS (20 mM). The excitation was set at 380 nm (slit = 2.5 nm) and the emission spectra were taken in the range of 400–600 nm (slit = 2.5 nm, and 10 nm/s of scanning speed).

2.8.2. Activity on membrane permeability

The experiment was performed according to Chen and Cooper (2002) with some modifications. The overnight culture of *E. coli* ATCC 25922 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 peptide at twice the MIC for different times (15, 30, 60, 120, and 240 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).

2.8.3. Transmission electron microscopy (TEM)

The effect of purified antimicrobial peptide on the ultrastructural morphology of *E. coli* ATCC 25922 was assessed using transmission electron microscopy (TEM). After treatment with purified peptide at twice the MIC for different time (60, 120, and 240 min), *E. coli* ATCC 25922 cells were immediately washed three times with PBS and fixed with 2.5% (v/v) glutaraldehyde. The TEM micrographs were taken using a transmission electron microscope (Hitachi H-7000, Japan).

3. Results and discussion

3.1. Gel filtration on Sephadex G-15 of HLE and HLE-IB

In order to investigate the molecular mechanisms of different biological activities or pharmacological function of AMPs, it is necessary to prepare large amount of AMPs. Isolation of AMPs has been, so far, based on the general purifying steps. However, the separation procedure of these techniques is not efficient to obtain AMPs directly. Many studies have shown that peptide–lipid interactions leading to membrane permeation play a major role in antimicrobial activity (Aisenbrey et al., 2008; Seto et al., 2007; Sevcsik et al., 2007; Van Kan, Demel et al., 2003; Van Kan, Van der Bent, Demel, & De Kruijff, 2001). The association of an antimicrobial peptide with the bacterial membrane's phospholipids is the first process among the overall interactions between the peptide and the living microorganisms. Thus our work has been focused on looking for antimicrobial peptide based on membrane-binding interaction.

Binding assays were used for determining specific membrane-binding activity for antimicrobial peptides in housefly larvae. As shown in Fig. 1, there were five peaks in the gel filtration spectrum of HLE-IB (Fig. 1a) and seven peaks in that of HLE (Fig. 1b). As expected, the disappearance of two peaks (fractions B1 and B2) was observed in the spectrum of HLE-IB compared with the spectrum of HLE. The result suggested that these two fractions (fractions B1 and B2) might contain AMPs, which could interact with the cell membrane material of *E. coli* and were removed by centrifugation and filtration.

In order to confirm the above speculation, all fractions' activities were determined. The results of antimicrobial assay demonstrated that fractions B1 and B2 were the most effective among all fractions and the antimicrobial activity of fraction B2 is higher than that of B1. However, the single separated technique was not enough to obtain preferably purified individual AMP. Thus, in view of our work on the separation of AMP, fraction B2 was subjected to RP-HPLC (Fig. 2) for further purification.

As shown in Fig. 2a and b, the active fraction was both eluted at about 53% acetonitrile by RP-HPLC, suggesting that the molecule is

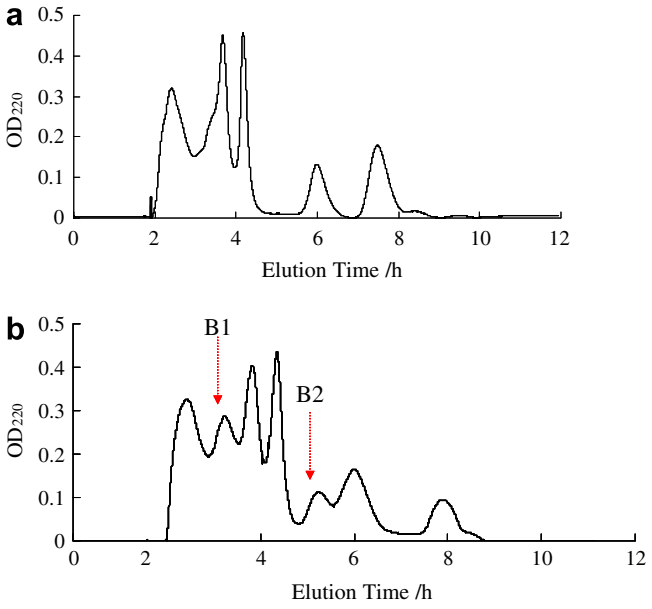

Fig. 1. Elution profile of HLE-IB (a) and HLE (b) on Sephadex G-15 column (45 cm × 2 cm, Amersham Pharmacia Biotech AB, Sweden). Elution buffer: PBS (10 mM, pH 7.0, plus 1 M NaCl); flow-rate: 0.4 ml/min. The elution pattern was monitored by measuring the absorbance at 220 nm.

hydrophobic. 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 (Conlon, Al-Ghaferi, Abraham, & Leprince, 2007a). However, hydrophobicity could result in increases in both antimicrobial potencies and hemolytic activity (Conlon et al., 2007b). Hence a threshold value for effective hydrophobicity was necessary for both hemolytic and antimicrobial activities. In the following toxicity assay, hemolytic activity of the purified peptide was studied, but it did not display hemolytic activity against rabbit red blood cells, suggesting its hydrophobicity is suitable for antimicrobial activity.

Mass spectrometry analysis indicates that the bioactive fraction collected from the RP-HPLC column contained one peptide with molecular masses of 984.4 Da (Fig. 2c). Through analysis and comparison, the purified peptide was found to be a peptide with the sequence Lys-Ser-Ser-Ser-Pro-Pro-Met-Asn-His. 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 "MDpep9".

MDpep9 belongs to Pro-rich peptides and the Pro-Pro and Ser-Ser-Ser are characteristic of this peptide. There are many Pro-rich peptides produced by insects, such as drosocin, pyrrhocoricin, apidaecin, formaecin, lebocin, and abaecin (Otvos, 2002). Those Pro-rich peptides produced by insects are usually shorter than those from mammals and belong to the short Pro-rich AMPs family. The relative abundance of proline residues may lend resistance to common proteases that are present in human tissues. Resistance to proteases is observed for other proline-rich AMPs (Otvos, 2002; Shinnar, Butler, & Park, 2003).

3.2. Antimicrobial activity and hemolytic activity

As shown in Table 1, MDpep9 was active against both Gram-negative (*E. coli, S. typhimurium, P. aeruginosa*, and *S. dysenteria*) and Gram-positive bacteria (*S. aureus, B. subtilis*, and *S. pneumoniae*), with MIC values ranged from 9 to 72 μg/ml, but no antimicrobial activity for fungi (*P. expansum* and *A. niger*) and yeast (*S. cerevisiae*) was detected up to the concentration of 576 μg/ml. The most susceptible bacteria were *B. subtilis* 9372, where a concentration of 9 μg/ml. In food industry, *Bacillus* contamination might originate from soil, water, processing equipment and processing environment. They were encountered in deteriorating of large varieties of food products, such as milk, dairy products, meat products, bakery products, fermented soy beans, mashed potato products, vegetable purees, pasta products, coca herbs, and spices (Kimura, Inatsu, & Itoh, 2002; Nissen, Holo, Axelsson, & Blom, 2001; TeGiffel, Beumer, Leijendekkers, & Rombouts, 1996). Recognition of *Bacillus* species as a potential spoilage problem in ambient-stored foods, resisting heat treatment, and shortening the shelf-life has been documented as challenges for food industries (Mansour, Amri, Bouttefroy, Linder, & Milliere, 1999; Te Giffel, Beumer, Leijendekkers, & Rombouts, 1996). The *subtilis* group has been associated with incidents of foodborne gastroenteritis (Lund, 1990; Rowan, Caldow, Gemmell, & Hunter, 2003). The result presented here suggests a promising strategy to control some strains of *Bacillus* species through the addition of antimicrobial peptide MDpep9.

Table 1
Minimal inhibitory concentrations (MICs) of antimicrobial peptide MDpep9

Microorganisms	MIC (μg/ml)
Gram-negative bacteria	
Salmonella typhimurium 50013	18
Pseudomonas aeruginosa ATCC 27553	36
Shigella dysenteriae 51302	36
Gram-positive bacteria	
Staphylococcus aureus ATCC 6538	36
Bacillus subtilis 9372	9
Streptococcus pneumoniae ATCC 49619	72
Fungi	
Penicillium expansum ATCC 1117	–[a]
Aspergillus niger ATCC 16404	–
Yeast	
Saccharomyces cerevisiae ATCC 40075	–

[a] Antimicrobial activity not detected up to the concentration of 576 μg/ml.

The cytotoxicity of MDpep9 against mammalian cells was examined by measuring the ability to lyse rabbit erythrocytes. In the hemolytic activity assay, it was possible to visually detect hemolysis right after adding 0.1% (v/v) Triton X-100 (positive control), by the observation that a red solution formed, instead of cell sedimentation occurring as in the negative control. The same was observed after adding MDpep9 (0–100 μg/ml), indicating that the peptide cannot disturb erythrocytes membranes. These results demonstrate that MDpep9 may be a good candidate for antimicrobial drug and food preservative, especially as an antibacterial agent against pathogenic bacteria.

The selectivity between prokaryotic cells and eukaryotic cells may result from the electrostatic interactions during the binding process between AMPs and membranes (Ladokhin & White, 2001). The positively charged AMPs were found to bind preferentially to negatively charged membranes. Prokaryotic cell membranes have a much more negative surface potential than eukaryotic membranes (Blondelle, Lohner, & Aguilar, 1999), which may be responsible for the prokaryotic cell membrane-binding of MDpep9 and is a partial explanation for the prokaryotic specificity of antimicrobial peptide MDpep9.

3.3. Mode of action

3.3.1. Change of surface hydrophobicity of MDpep9 induced by lower pH around bacterial cells

For the explanation of membrane-binding strategy and further investigation of the antimicrobial mechanism of MDpep9, confor-

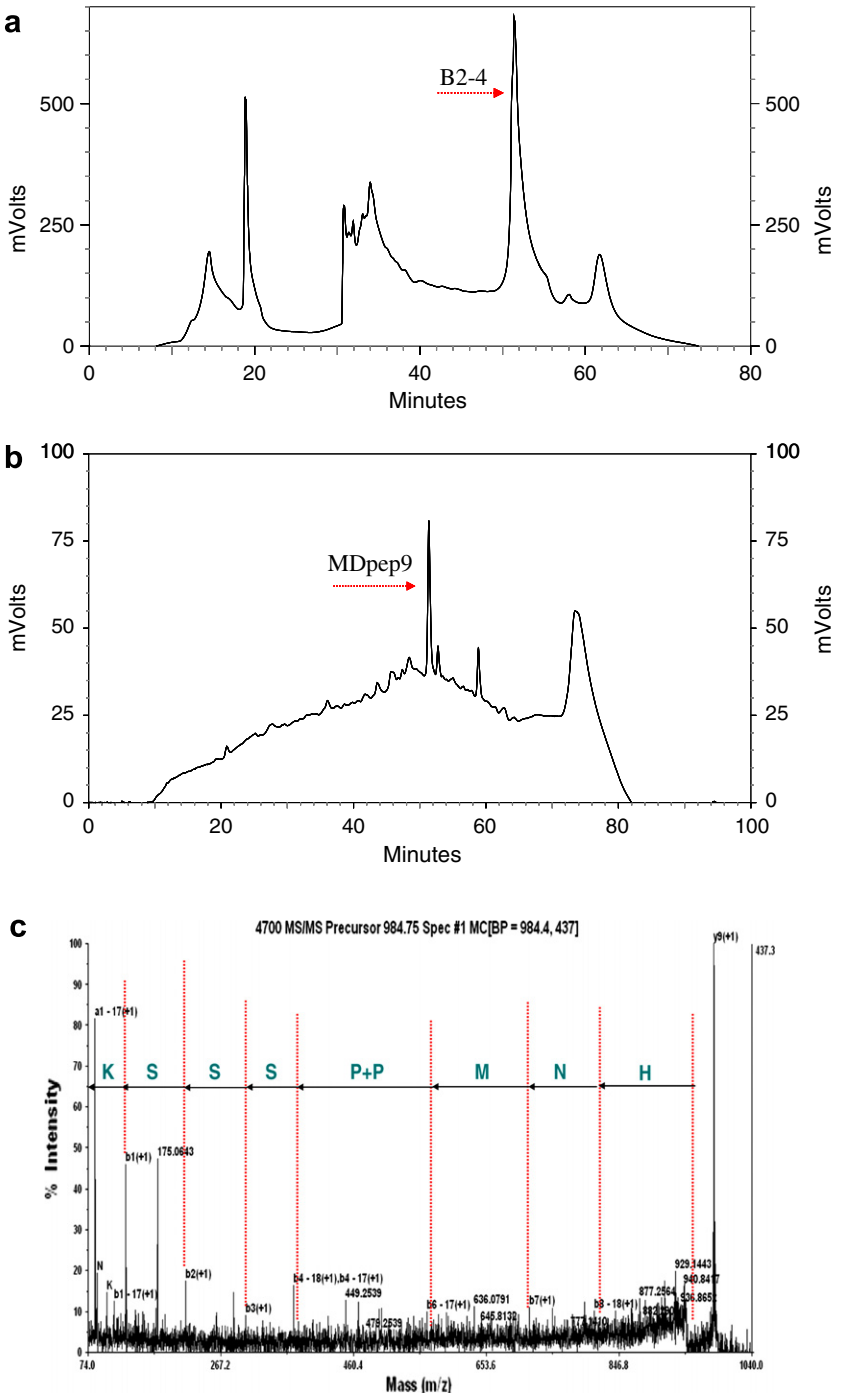

Fig. 2. (a) Chromatographic profile of fraction B2 (YWG C_{18} 250 × 10 mm; flow-rate 1.0 ml/min). (b) Further purification of fraction B2–4 (Kromasil C_{18} 250 × 4.6 mm; flow rate 0.5 ml/min). (c) MS/MS spectrum of MDpep9 (MALDI-TOF MS, reflector positive ion mode).

mational changes of MDpep9 induced by lower pH around bacterial cells were studied. Ean der Goot, Gonzalez-Manas, Lakey, and Pattus (1991) observed that, depending on circumstances, the pH in the vicinity of the membrane might be 1–3 units lower than in the bulk solution. A decrease in pH has been linked to structural changes in many proteins, such as the Bt toxin Cry1C (Butko, Cournoyer, Pusztai-Carey, & Surewicz, 1994), *P. aeruginosa* exotoxin A (Menestrina, Pederzolli, Forti, & Gambale, 1991), tetanus toxin (Calappi, Masserini, Schiavo, Montecucco, & Tettamanti, 1992), and tumor necrosis factor α (Kagan, Baldwin, Munoz, & Wisnieski, 1992). All of these proteins increase their affinity to the membrane at acidic pH (Manceva, Pusztai-Carey, & Butko, 2004). AMPs are membrane-active, but their conformation change at lower pH around bacterial membrane is unknown. The application of the AMPs as food preservative requires comprehensive studies of the interaction of these peptides with a cell membrane (CM).

The stability of proteins is the result of residue–residue and residue–solvent interactions. Antimicrobial peptides, most of which are composed of more hydrophobic amino acids, can self-assemble to form a defined structure. In general, these hydrophobic amino acids are located in the interior region of the protein to avoid exposure to water (Blanco, Ruso, Prieto, & Sarmiento, 2007). Since unfolding of peptides normally involves exposure of buried hydrophobic side chains, surface hydrophobicity of peptides is one of the

structural characteristics to evaluate the change in peptide conformation (Kato & Nakai, 1980; Nakai, 1983). In this study, the changes of molecular surface hydrophobicity of MDpep9 induced by lower pH around bacterial cells were investigated by using of 8-anilino-1-naphthalenesulfonic acid (ANS). ANS is an extensively utilized fluorescent probe for the characterization of protein binding sites. The fluorescence properties of ANS depend on the quantity of hydrophobic binding sites at the protein. The fluorescent probe is virtually non-fluorescent in aqueous solutions, but becomes strongly fluorescent when it is bound to hydrophobic sites in proteins. So the increase in hydrophobicity can induce an increase in the fluorescence intensity of this fluorescence probe.

As shown in Fig. 3, the intensity of fluorescence increased with the decrease of pH values. When pH decreased from 7.0 to 2.6, the quantum yield of the probe doubled. The observed increase in the fluorescence intensity was due to the increased binding of ANS, which is the result of the exposure of previously buried hydrophobic amino acid residues on the protein surface (Gasymov & Glasgow, 2007; Goto & Fink, 1989; Semisotnov & Gilmanshin, 1991; Semisotnov et al., 1987). The fluorescence intensity of bound ANS at pH 2.6 is about two times of that of the native state at pH 7, indicating that there are considerable parts of hydrophobic groups which are wrapped in the hydrophobic core in the native state exposed to the solvent at acidic environment mimicking the vicinity of the lipid membrane.

The results show the conformational change of MDpep9 in the vicinity of the membrane (lower pH). Lower pH increases surface hydrophobicity of MDpep9, which is helpful for the binding interaction with the hydrophobic core of the lipid bilayer (Clackson & Wells, 1995; Manceva et al., 2004; Young, Jernigan, & Covell, 1994). The exposed hydrophobic patches on the surface of AMPs preferentially move into a hydrophobic environment, in this case the lipid tail region of the phospholipid bilayer, anchoring the peptide into the hydrophobic core of the bilayer and promoting the peptide insertion into the bacterial cell membrane. The deep insertion into membrane results in the largest distortion of the lipid packing in the bilayer and significant changes in the lipid phase transition parameters (Van Kan, Ganchev et al., 2003) Maybe once an effective concentration of MDpep9 is reached, the hydrophobic patch on MDpep9 will insert into the lipid bilayer and cause the plasma membrane to break apart leading to cell death.

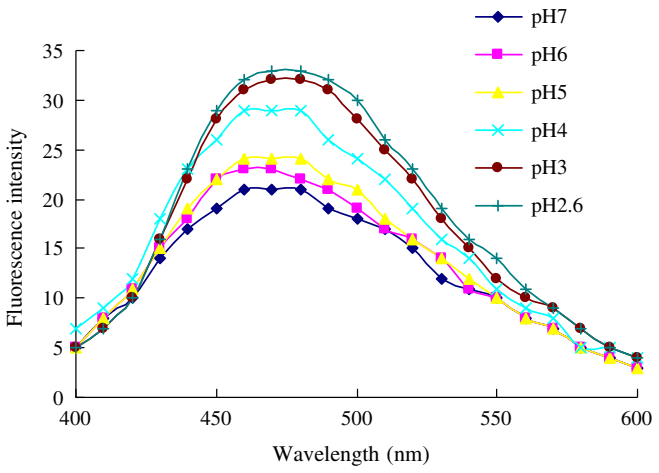

Fig. 3. Fluorescence spectra of MDpep9 in the presence of ANS at different pH. The peptide stock solution was diluted with 10 mM KH_2PO_4/Na_2HPO_4 buffers at different pH (2.6, 3, 4, 5, 6, and 7, adjusted with NaOH or HCl). After a 30 min incubation period, pH was adjusted to neutrality and then fluorescence spectra were measured at 25 ± 0.1 °C in the presence of ANS (20 mM). The excitation was set at 380 nm (slit = 2.5 nm) and the emission spectra were taken in the range of 400–600 nm (slit = 2.5 nm, and 10 nm/s of scanning speed).

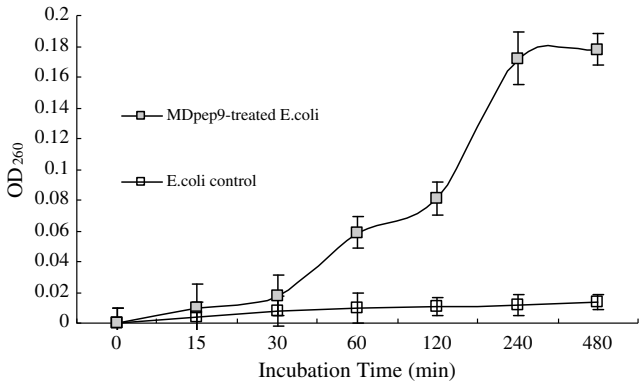

Fig. 4. Total nucleotide leakage from *E. coli* ATCC 25922 cells treated with MDpep9. Strains were incubated with peptide at twice the MICs for different time (15, 30, 60, 120, and 240 mim), and which incubated with 10 mM PBS (pH 7.4) was used as control. The mixture was filtered through 0.22 μm to remove 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).

3.3.2. Activity on the membrane permeability of bacteria

Membrane binding is the initial step involved in peptide-membrane interaction process and the above results demonstrates the lower pH around cell membranes can significantly enhance the membrane binding of MDpep9. 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, a 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.

As shown in Fig. 4, total nucleotide leakage from bacterial cells as a function of incubation time with peptide MDpep9 was plotted. When peptide was interacted with bacterial cells, the induced total leakage content was dependent on the incubation time. For *E. coli* ATCC 25922, there are three stages for membrane permeability. First, upon addition of MDpep9 to *E. coli* ATCC 25922, there was no significant increase in OD_{260} within 30 min. Then a fast increase to 0.185 in OD_{260} was observed (30–240 min). Finally, no increase was showed afterwards. The results suggested that there are at least three steps involved in membrane disruption process. Membrane binding, which is the initial steps, appeared within 30 min.

MDpep9-treated bacterial cells show a significant increase of nucleotide leakage, indicating that MDpep9 can bind to membrane lipid and cause membrane permeabilization to varying extents. The cytoplasmic cell membrane may be the target for MDpep9 which can exert its antimicrobial action by disrupting and disintegrating bacterial cell membranes, leading ultimately to loss of cytoplasmic membrane integrity, which was confirmed by transmission electron microscopy (TEM) (Fig. 5).

To further elucidate the nature of the killing mechanisms of MDpep9, *E. coli* ATCC 25922 was treated with MDpep9 for 240 min, and the bacteria were analyzed by TEM. Compared to the control, MDpep9 treatment resulted in clear morphological changes (Fig. 5). After 60 min treatment, pores were found at the bacterial surface. Further incubation up to 120 min resulted in much more pores that lead to the cytoplasmic lysing and rupturing of the bacterial cell. After 240 min treatment, a ghost-like appearance and an increase in the number of lysed cells was also observed. These data correlate with the rapid and remarkable increase in the nucleotide leakage after peptide-treatment (Fig. 4).

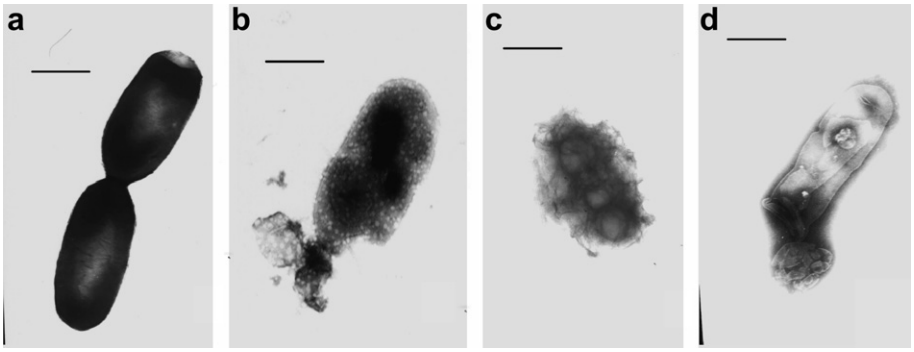

Fig. 5. Morphological changes of *E. coli* ATCC 25922 upon incubation with MDpep9. *E. coli* ATCC 25922 ($2–5 \times 10^7$ CFU/ml) incubated with MDpep9 for 240 min showed time-dependent changes in ultrastructure, not observed in control bacteria. (a) undamaged control cell, (b) 60 min treatment, pore formation at the bacterial surface, (c) 120 min treatment, more pore formation and leakage of cytoplasm, (d) 240 min treatment, 'ghost-like' appearance of *E. coli* cell. Bars represent 0.5 μm.

Sequence analysis shows that MDpep9 is a Pro-rich peptide. Because Pro residues within antimicrobial peptides are known to mediate membrane disruption and/or cell entry (Lu, Marti, & Booth, 2001; Williams & Deber, 1991), it seemed appropriate to explore the mechanism of MDpep9's antimicrobial action in the context of its interactions with the bacterial cytoplasmic membrane. The *cis–trans* isomerization in aqueous buffer provided by Pro (Schibli, Hwang, & Vogel, 1999) appears to facilitate binding to negatively charged membranes and improved membrane translocation of MDpep9, which is part explanation of successful purification of peptide MDpep9 from housefly using membrane-binding strategy.

4. Conclusions

From the present study on the purification and antimicrobial mode study of peptide MDpep9, conclusions can be obtained as the following:

i. The designed membrane binding-based approach to screen AMPs yielded a direct harvest of a novel antimicrobial peptide MDpep9 that preferentially binds to the bacterial cells and promoting in vitro permeation of bacterial cell membrane.
ii. The acidic environment around bacterial cell membrane induced molecular unfolding and increased surface hydrophobicity of MDpep9, promoting the interaction of peptide with bacterial lipid membrane.
iii. The cytoplasmic cell membrane is the target for MDpep9 which can exert its antimicrobial action by disrupting and disintegrating bacterial cell membranes, leading ultimately to loss of cytoplasmic membrane integrity.

To our knowledge, this is the first time that an antimicrobial peptide was discovered using membrane-binding technology. This approach provides a versatile and sensitive method for direct identification of AMPs that might be derive from different sources. Moreover, identification of the AMPs membrane binding and disruption provides new insights into the molecular mechanism underlying antimicrobial activity as well as new targets for purification, but further work identifying the antimicrobial mechanisms is needed for the further development of AMPs.

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